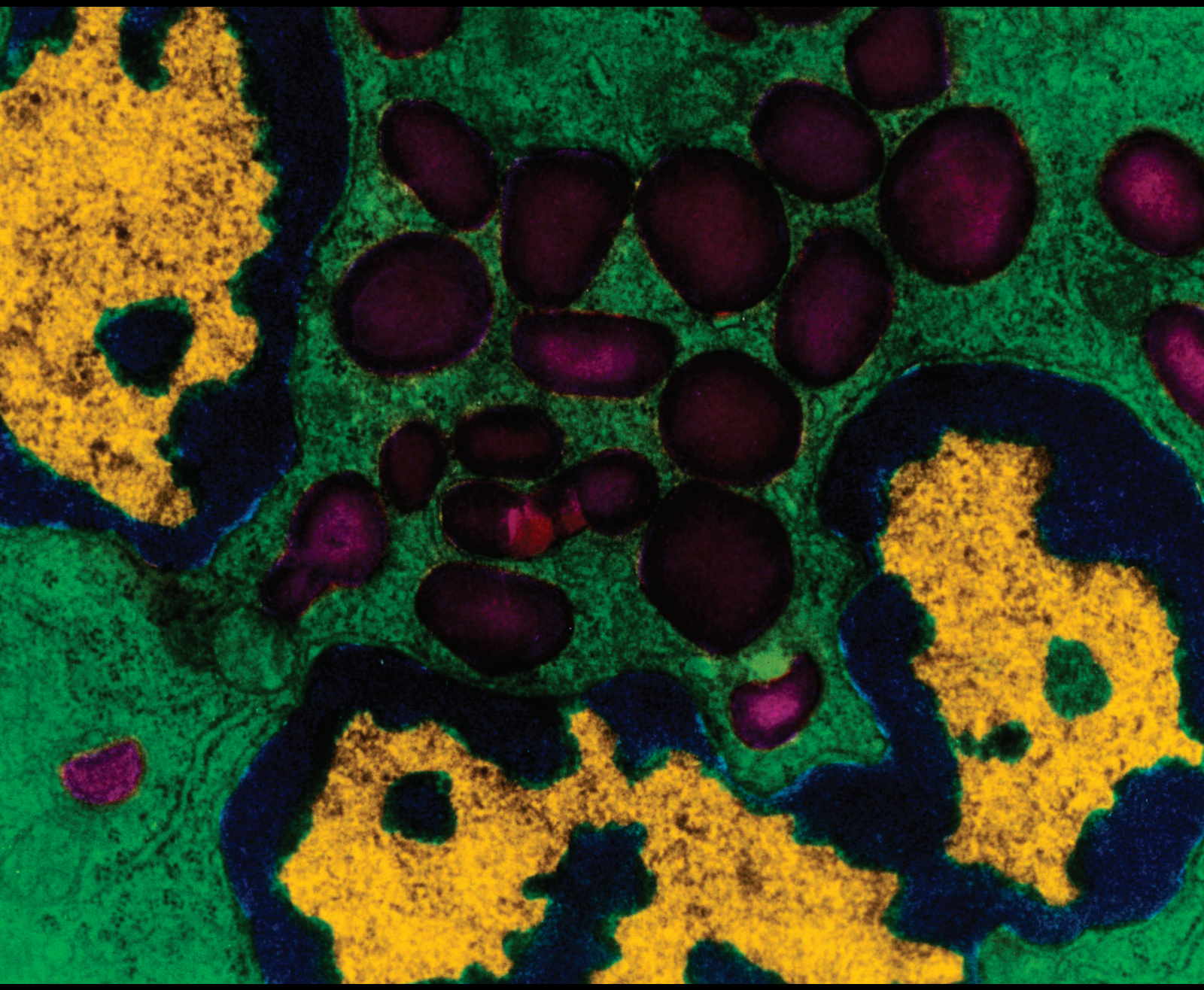
Mediators of Inflammation

Purinergic Signalling in Immune System Regulation in Health and Disease

Guest Editors: Mireia Martín-Satué, Jean Sévigny, and Jesús Pintor



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Editorial

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The concept of a purinergic signalling system was first proposed by Professor Geoffrey Burnstock over 30 years ago. This includes the cellular responses to purine nucleotides, such as ATP, and nucleosides, such as adenosine, that act as extracellular messengers playing a role through specific nucleotide and adenosine receptors in all systems. Indeed, in addition to their role in cellular metabolism, nucleotides as well as nucleosides are extracellular mediators that activate biological responses in all cells. Cells subjected to activation or shear or mechanical stress release nucleotides such as ATP, ADP, UTP, and UDP in large amounts. All cells can release nucleotides in a controlled fashion [1]. The mechanisms of nucleotide release have been the focus of intense research activities but are still not fully understood. While activated platelets and neurons release nucleotides by exocytosis, neutrophils, and T lymphocytes use pannexin-1 hemichannels for nucleotide efflux, some cells also constitutively release nucleotides.

Under normal conditions, blood levels of nucleotides are below 0.1 μM, the level resulting from cell release, degradation by ectonucleotidases and reuptake of nucleotides after dephosphorylation by these enzymes [2]. There are many sources of extracellular nucleotides. In the circulatory system, red blood cells are a major source of nucleotides. The dense granules of the platelets contain a phenomenal amount of ATP and ADP approaching 1 M concentration that are secreted by exocytosis during activation. Nucleotides, primarily ATP, can be released by the endothelial cells and the smooth muscles cells of vessel walls. Nucleotide release can be triggered by shear stress, hypoxia and cell breakdown. As cytosolic ATP concentration is about 5 mM, rupture of the plasma membrane causes an increase in nucleotide concentrations near the injured cells. These mechanisms represent only a few examples of nucleotide release. Cells thus release large amounts of ATP, in a controlled manner but also when a cell is injured or dies which is particularly relevant in situation of inflammation and/or diseases.

Once released, nucleotides activate ubiquitously expressed ligand-gated ionotropic P2X receptors (P2X1–7) and G-protein-coupled metabotropic P2Y receptors ($P2Y_{1,2,4,6,11-14}$) [1]. In addition, the cysteinyl-leukotriene receptor-1 and receptor-2 ($CysLT_1$ and $CysLT_2$) and GPR17 can also respond to nucleotides. P2 receptor subtypes differ with respect to selectivity towards nucleotides. All P2X receptors are activated by ATP, $P2Y_2$ by ATP and UTP, $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ by ADP, $P2Y_4$ by UTP, $P2Y_6$ by UDP (in mouse also by UTP [3]), $P2Y_{11}$ by ATP > ADP, and $P2Y_{14}$ by UDP-glucose. In addition, adenosine, which results from the complete dephosphorylation of ATP and ADP by ectonucleotidases, activates P1-type receptors, which

divide into four types: A_1, A_{2A}, A_{2B}, and A_3. These receptors are widely distributed and their activation has already been demonstrated to modulate a myriad of biological responses.

The responses induced by nucleotides can be modulated by cell surface enzymes generically called ectonucleotidases. Among them, the members of the ectonucleoside triphosphate diphosphohydrolase family (E-NTPDases) [4, 5] which convert tri- and diphosphonucleosides (ATP, ADP) to their monophosphate derivative (AMP). The latter is further hydrolysed to adenosine by ecto-5′-nucleotidase (CD73). Several other enzymes also modulate nucleotide concentrations, namely, nucleotide pyrophosphatases/diphosphodiesterases and alkaline and acid phosphatases. P2 receptor signalling can also be influenced by the ectokinases, that is, ectonucleotide diphosphokinases and adenylate kinases, that interconvert and regenerate nucleotides, respectively [2]. Hydrolysis thus switches off the nucleotide signal and plays a role in adenosine formation. These enzymes thus control relative concentrations of each nucleotide and nucleoside and, accordingly, regulate their effects.

Since its discovery, purinergic signalling has been shown to mediate a wide range of functions in health and disease, important among them being immunomodulation and inflammation. Recent advances have been made in therapies using nucleotide-related drugs in a broad range of pathological conditions such as acute and chronic inflammatory diseases. In this special issue, which is dedicated to purinergic signalling in immune system regulation in health and disease, we present 9 research papers and 4 reviews. The papers deal with the following topics.

By catalyzing the hydrolysis of extracellular AMP to adenosine, ecto-5′-nucleotidase/CD73 was shown to control vascular permeability and immune responses. By using double knockout mice lacking CD73 and another AMP-degrading enzyme, prostatic acid phosphatase (PAP), G. G. Yegutkin et al. show that PAP may have a synergistic role together with CD73 in the immune system by contributing to the lymphoid purine homeostasis and balance of leukocyte subpopulations in the lymph nodes and thymus. However, the absence of PAP alone did not have any significant effects outside the thymus.

S. D. S. Oliveira et al. show that schistosomiasis reduces peritoneal macrophage P2X7 receptor signaling which correlate with increased levels of TGF-β1 in infected mice as well as with reduced cell surface expression of P2X7. The involvement of P2 receptors in chemotactic responses of fibroblasts in the context of regulation of inflammatory responses and tissue damage repair is addressed by M. Pimentel-Santillana et al. in a paper that demonstrates that Prostaglandin E_2 inhibits P2Y-dependent cell migration.

Data presented by L. Oliveira et al. with a rat model suggest that restoration of neuromuscular transmission and immune competence may be possible by targeting common adenosine deficits in patients with autoimmune myasthenia gravis. In another paper, C. Vieira at al. show that postinflammatory ileitis suppresses adenosine neuromodulatory control leading to acceleration of the gastrointestinal transit, which may last more than a week. In an attempt to identify biomarkers for endometriosis, an inflammatory estrogen-dependent complex disorder that is one of the principal causes of infertility in women, L. Texidó et al. demonstrate the presence of ectonucleotidase activities in the contents of endometriomas.

The contribution carried out by K. R. Higgins et al. indicates that the activation of a $P2Y_2$ receptor present in a human monocytic cell line (THP-1) is very efficient inducing the chemokine CCL2. The effect carried out by the purinergic agonists is as good as the one triggered by lipopolysaccharide. Moreover, these researchers have found that polymorphisms of the $P2Y_2$ receptors are important regarding the CCL2 secretion. In this sense, human macrophages expressing 312Ser-$P2Y_2$ displayed significant UTP-induced CCL2 secretion above background compared to 312Arg-$P2Y_2$ expressing macrophages.

Neuroinflammation in the central nervous system occurring during pathologies such as amyotrophic lateral sclerosis and multiple sclerosis is a severe problem associated with those pathologies. S. Amadio et al. studied the role of $P2Y_{12}$ receptors in the neuroinflammatory condition, mainly on ramified microglia and myelinated fibers from primary organotypic cerebellar cultures or tissue slices from rat striatum, cerebellum, and spinal cord from symptomatic animals. These authors suggest that the modulation of $P2Y_{12}$ expression might play a dual role as analytic marker of branched/surveillant microglia and demyelinating lesions and also that potentially could be a predictive value under neuroinflammatory conditions as those found in amyotrophic lateral sclerosis and multiple sclerosis.

Laser therapy, particularly low-level-laser therapy, is being used with success as a complementary treatment for inflammation and wound healing in the dermis. L. Wang et al. present a paper dealing with the effects of red-laser irradiation on extracellular ATP content of mast cells and dorsal root ganglia neurons. In this sense, the authors show that irradiation potentiates extracellular ATP presence in mast cells by promoting ATP synthesis and release, but on the contrary laser attenuates extracellular ATP amounts of dorsal root ganglia neurons by upregulating ecto-ATPase activity.

This issue also presents the following reviews. The review of F. Pedata et al. covers the role of the adenosine receptor A_{2A} in acute injury and neuroinflammation in brain ischemia. A. Guzman-Aranguez et al. review purinergic receptors in ocular inflammation pointing out to the use of purinergic agonists and antagonists as possible therapeutic targets for inflammatory eye disorders. In their review, P. J. Sáez et al. focus on the effects of different cytokines on the intercellular communication mediated by hemichannels and gap junction channels in antigen-presenting cells and their impact on purinergic signaling. Finally, A. R. Santiago et al. reviewed the role of the nucleoside adenosine in microglial proliferation, chemotaxis, and reactivity. The authors focused on the adenosine A_{2A} receptor and discuss its role in Parkinson's and Alzheimer's diseases as well as in glaucoma and diabetic retinopathy.

Although there is still much to learn about the precise role of the adenosine and P2 receptors in the immune system and in inflammation, there is now wider acceptance that

adenosine and nucleotides have a real and important function in this regard. This timely collection of well-informed and insightful articles summarises where we are now and points the future directions we should take. As the purinergic field is growing rapidly and it is getting increasingly demanding to keep up with the literature, we hope that this special issue will not only be useful from this point of view but also will inspire new experiments that reveal further insights regarding adenosine and nucleotides in all the aspects related to inflammation and the immune system.

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References

[1] F. Kukulski, S. A. Lévesque, and J. Sévigny, "Impact of ectoenzymes on P2 and P1 receptor signaling," *Advances in Pharmacology*, vol. 61, pp. 263–299, 2011.

[2] G. G. Yegutkin, "Enzymes involved in metabolism of extracellular nucleotides and nucleosides: functional implications and measurement of activities," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 49, no. 6, pp. 473–497, 2014.

[3] G. Kauffenstein, A. Drouin, N. Thorin-Trescases et al., "NTPDase1 (CD39) controls nucleotide-dependent vasoconstriction in mouse," *Cardiovascular Research*, vol. 85, no. 1, pp. 204–213, 2010.

[4] A. R. Beaudoin, J. Sévigny, and M. Picher, "ATP-diphosphohydrolases, apyrases, and nucleotide phosphohydrolases: biochemical properties and functions," in *Biomembranes: A Multi-Volume Treatise*, A. G. Lee, Ed., vol. 5 of *ATPases*, pp. 369–401, JAI Press, Greenwich, Conn, USA, 1996.

[5] S. C. Robson, J. Sévigny, and H. Zimmermann, "The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance," *Purinergic Signalling*, vol. 2, no. 2, pp. 409–430, 2006.

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Research Article

Modulation of Extracellular ATP Content of Mast Cells and DRG Neurons by Irradiation: Studies on Underlying Mechanism of Low-Level-Laser Therapy

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Low-level-laser therapy (LLLT) is an effective complementary treatment, especially for anti-inflammation and wound healing in which dermis or mucus mast cells (MCs) are involved. In periphery, MCs crosstalk with neurons via purinergic signals and participate in various physiological and pathophysiological processes. Whether extracellular ATP, an important purine in purinergic signaling, of MCs and neurons could be modulated by irradiation remains unknown. In this study, effects of red-laser irradiation on extracellular ATP content of MCs and dorsal root ganglia (DRG) neurons were investigated and underlying mechanisms were explored *in vitro*. Our results show that irradiation led to elevation of extracellular ATP level in the human mast cell line HMC-1 in a dose-dependent manner, which was accompanied by elevation of intracellular ATP content, an indicator for ATP synthesis, together with $[Ca^{2+}]_i$ elevation, a trigger signal for exocytotic ATP release. In contrast to MCs, irradiation attenuated the extracellular ATP content of neurons, which could be abolished by ARL 67156, a nonspecific ecto-ATPases inhibitor. Our results suggest that irradiation potentiates extracellular ATP of MCs by promoting ATP synthesis and release and attenuates extracellular ATP of neurons by upregulating ecto-ATPase activity. The opposite responses of these two cell types indicate complex mechanisms underlying LLLT.

1. Introduction

Low-level-laser therapy (LLLT) is an increasingly used and effective complementary treatment in clinic, especially for wound healing, anti-inflammation, and pain relief. Mast cells (MCs), the vital immune cells, in dermis paly necessary roles in the process of wound healing [1]. MCs have recently been identified to have important anti-inflammatory functions *in vivo* to some extent [2], despite their well-known role as promoter of inflammation. Subcutaneous or mucosal MCs *in vivo* have been suggested to be involved in anti-inflammatory [3] and wound healing [4–7] reactions of LLLT. Recently, subcutaneous MCs have been shown to participate in the initial process of pain relief by laser acupuncture [8], an increasingly and frequently applied version of LLLT. In medicine, red and near-infrared (NIR) lasers, with wavelengths between 600 and 1,000 nm, are frequently applied in LLLT because laser light at these wavelengths can penetrate tissues in the millimeter range as absorption by human skin is low [9]. The approximate skin transmission depth of red light is about 1.5–2 mm [10], mainly reaching MCs residing in cutaneous and subcutaneous skin layers [11]. MCs in skin may, therefore, participate in LLLT mechanisms by being directly activated during red-laser irradiation.

This hypothesis has been supported by some investigations *in vitro*: activation of degranulation [12, 13], $[Ca^{2+}]_i$ elevation [12–14], as well as triggering of whole-cell membrane currents [12] by red-laser irradiation were demonstrated in MCs.

Accumulating evidences suggest that purinergic signaling participates in various physiological and pathophysiological processes. Extracellular nucleotides are important players in regulating inflammatory responses through binding to purinergic P2 receptors present on all inflammatory cells, including MCs [15]. Activation of P1 receptors by adenosine or other agonists can promote the wound healing process [16]. Extracellular ATP is not only the endogenous agonist for several P2 receptors but also the precursor of other related nucleotides. The effect of irradiation on extracellular ATP content of MCs is unknown. In the periphery, MCs morphologically [17] and functionally [18] interact with nerve endings, in which purinergic signaling has a major role [19], and a crosstalk between MCs and peripheral neurons via purinergic signals exists [20]. Whether this crosstalk could be modulated by low-level irradiation remains unknown too.

In the present study, we assessed the responses of extracellular ATP content of MCs and DRG neurons to low-level red-laser irradiation and further explored the underlying mechanisms. The aim of this study is to better understand of the role of ATP purinergic signaling in LLLT effects at the cellular level.

2. Materials and Methods

2.1. Cells Cultivation and Isolation. The human mast cell line HMC-1 was kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA) and cultured as described previously [21]. In brief, the cells were incubated in phenol-red-free IMDM medium, supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% (v/v) fetal bovine serum 1% penicillin, and streptomycin, in a 95% humidity-controlled incubator (Model: 310, Thermo, Thermo electron, Waltham, USA) with 5% CO_2 at 37°C. Cell density was about 3×10^5/mL.

Isolation of rat peritoneal MCs was modified according to Jensen et al. (2006) [22]. Briefly, adult Sprague-Dawley rats (280–320 g) were sacrificed by CO_2 asphyxiation. 30 mL Ca/Mg free hank's balanced salt solution (HBSS) was injected into peritoneal cavity of each rat. After vigorous agitation of the abdominal area for 2 min, injected HBSS was collected and centrifuged at 400 g for 5 min with swing-out rotors. Ammonium-chloride-potassium lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA-Na_2) was used to break erythrocytes. The cell pellet was well resuspended with 8 mL 70% isotonic percoll and gently overlaid with 2 mL RPMI 1640 medium. MCs stayed at the bottom fraction after centrifugation of the cell suspension at 580 g for 15 min. The MC pellet was resuspended in RPMI 1640 medium. The viability and the purity were identified with 0.4% trypan blue and 0.5% toluidine blue (TB). The cell density was adjusted as needed (see Section 2.6) and cells were equilibrated in the incubator for 2 h.

Isolation of rat DRG neurons was modified according to Burkey et al. (2004) [23]. Briefly, adult Sprague-Dawley rats (150–170 g) were sacrificed by CO_2 asphyxiation. Hibateral DRGs were dissected from thoracic to lumbar regions of the vertebral column and placed in oxygenated Ca/Mg free HBSS. The connective tissue sheath around the ganglia was removed and then incubated in 3 mL of F12 medium containing 1.25 mg/mL collagenase IA, 300 U deoxyribonuclease IV (DNase), and 0.05% trypsin IX-S at 37°C for 50 min. At the end of the incubation period, 8 mL F12 medium containing 10% horse serum was added to terminate the enzymatic reaction. Ganglia pellet was collected by centrifugation (200 g, 2 min), resuspended in F12 medium, and mechanically agitated through a fire-polished glass Pasteur pipet until the suspension of dissociated cells was homogeneous. Nerve fragments were discarded by centrifugation at 60 g for 2 min, and isolated individual cells of the bottom pellet were resuspended in F12 medium once more and transferred to a 35 mm petri dish to culture for 2 h. At the end of the incubation, after nonneuronal cells were attached to the bottom, the unattached sensory neurons were collected for equilibration in the incubator for 2 h.

2.2. Skin Tissue Preparation. 4-week-old male Sprague-Dawley rats (Shanghai Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China) weighing 120–150 g were deeply anesthetized with ether. Each animal was placed in an airtight container with several cotton balls soaked with ether (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The rats were not decapitated until they were deeply anesthetized. Hair in the area of the lateral tibia was shaved, and the skin was cut open with scissors to expose connective tissues, which were acutely separated with blunt forceps and scissors before incubation in bath solution (BS) (see Section 2.3) for 1 h. Tissue slices comprised three connective layers, with one loose layer sandwiched between two dense layers. The loose connective tissue was exposed by stretching the two dense layers in opposite direction. Thereafter, the tissue slices were fixed by tiny needles to hot glue support on the chamber bottom. To identify MCs, fixed slices were incubated in 0.5% TB for 10 min and subsequently washed 3 times with 95% ethanol. The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Certificate No. 2013012).

2.3. Reagents and Solutions. The BS for tissue slices contained (in mM) 137 NaCl, 2.7 KCl, 2 $CaCl_2$, 5 $MgCl_2$, 5.6 glucose, and 10 HEPES, pH 7.4 (adjusted with NaOH). The BS for HMC-1 cells was comprised of the following (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 5 $MgCl_2$, 4D-sorbitol, and 10 HEPES, pH 7.4 (adjusted with NaOH). Osmolality of the solutions was 310 mOsm/kg, as verified with osmometer (Model: 3300, Micro Osmometer, Advanced Instruments Inc., Norwood, MA, USA). All experiments were performed at room temperature (23–26°C).

Culture mediums, HBSS, serum, antibiotic, calcium green-1 AM, and pluronic F-127 were bought from Invitrogen Company (USA); all other chemicals were purchased from Sigma Company (USA).

Table 1: Laser system and parameters.

Laser	Parameter
Device	Semiconductor (Model: SB2007047, Shanghai University of TCM, Shanghai, China)
Wavelength (nm)	657
Power output (mW)	35
Mode	Continuous
Irradiated area (cm^2)	0.126
Power density (W/cm^2)	0.28
Exposure time (min)	1–5
Dose (J/cm^2)	17–85

Reagents were prepared in stock solutions and kept at −20°C and diluted in BS or culture medium before use. In some ATP-accessing experiments, HMC-1 cells were pretreated with NEM or BAPTA or ARL 67156 for 20 min before luminescence measurements.

2.4. Irradiation. According to the Kubelka-Munk theory [24], red-light irradiance decreases by >90% at a depth of 1.5–2 mm (see also [10, 25]). Hence, in our present experiments, we started with a dose of 17 J/cm^2, 5% of the dose level used in our previous animal studies [8]. A higher light dose was achieved by prolonging exposure time. The laser device and irradiation parameters used for HMC-1 cells and tissue slice are presented in Table 1. For rat peritoneal MCs and neurons, the laser power density was adjusted to 18 mW/cm^2, and then 20 min exposure generated 21 J/cm^2.

In ATP-related experiments, equilibrated cell suspensions in 1.5 mL Eppendorf tubes (see Section 2.6) were exposed to laser light entering the tube vertically, in order to expose the entire suspension to irradiation. In light and fluorescence imaging experiments, light was introduced at 60-degree angle to tissue slices or HMC-1 cells, and all cells viewed under microscope were irradiated.

To monitor possible heating effects of laser irradiation, temperature was recorded by thermocouple. Temperature changes of perfusion BS induced by red-laser amounted to only 0.2°C during 15 min irradiation.

In order to exclude the possibility that reagents were modified by the laser light, the absorption spectrums of 100 μM NEM, 25 μM BAPTA, and 100 μM ARL 67156 were scanned. None of the drugs showed significant absorbance at the 657 nm laser light.

2.5. Light and Fluorescence Images. To investigate the effects of laser irradiation on rat skin MC degranulation, tissue slices were fixed in a perfusion chamber and micrographs were captured under upright light microscope (Model: NF1, Nikon, Japan).

To estimate the $[Ca^{2+}]_i$ of HMC-1 cells, calcium green-1 fluorescence was measured as described previously [21]. Briefly, HMC-1 cells were grown on glass cover-slips coated with poly-L-lysine before mounting in the perfusion chamber. 5 mM calcium green-1 AM stock solution was dissolved in 20% (w/v) pluronic F-127. HMC-1 cells were loaded in phenol-red-free IMDM containing 4 μM calcium green-1 AM for 45 min; then the loaded cells were superfused with BS. All solutions used in the fluorescence experiments contained 2.5 mM probenecid. Micrographs were captured under inverted light microscope (Model: TE2000-U, Nikon, Japan) by CCD video camera (Orca-ER, Hamamatsu photonics, Hamamatsu, Shizuoka, Japan). Images were digitized and averaged (3 frames), background corrected, and analyzed by an image-processing system (Wasabi software, Hamamatsu photonics, Hamamatsu, Shizuoka, Japan). Fluorescence intensities of individual cells in the viewing field were ascertained by averaging the image intensities collected from regions of interest within each cell. Graphs were colored by Image J software (National Institutes of Health, Bethesda, MD, USA).

All microscopy images were taken with 40x objective magnification (CFI Super Plan Fluor ELWD, 0.6 in N.A.).

2.6. ATP Measurements. ATP content was quantified by bioluminescence assay measuring light output from luciferin-luciferase reactions [26]. To evaluate extracellular ATP content, the cell density was adjusted to about 3.5×10^4/mL. 100 μL aliquots of dispersed cell suspensions were placed in 1.5 mL Eppendorf tubes for HMC-1 cells or into 96-well plate for isolated cells. After equilibration for 2 h in the incubator, all samples were divided into nonirradiated control and irradiated groups exposed to laser light for different irradiation time periods. To measure ATP level in the samples, 100 μL luciferin-luciferase assay mix that was diluted with dilution buffer and was adjusted to isotonicity with mannitol was added to each sample, and light emission was measured immediately by luminometer (GloMax 20/20, Promega, Madison, Wisconsin, USA, for HMC-1 cells or Synergy Mx, BioTek, Winooski, USA for isolated cells).

To determinate the intracellular ATP content, HMC-1 cell density was adjusted to about 7×10^4/mL. 50 μL aliquots of dispersed cell suspensions were transferred into 1.5 mL Eppendorf tubes. 50 μL phenol-red-free IMDM and 100 μL somatic cell ATP-releasing reagents were added to each aliquoted 50 μL sample. After briskly swirling, 100 μL mixtures were transferred to each 1.5 mL Eppendorf tube containing 100 μL luciferin-luciferase assay and light emission was measured immediately by luminometer, as above. Manipulation was gentle to avoid mechanical cell stimulation. Luciferin-luciferase luminescence was calibrated versus ATP standards before and after all sample measurements. Intracellular and extracellular ATP is reported in fmoles/cell.

2.7. Data Analysis. The data were analyzed by ORIGIN software (OriginLab, Northampton, MA, USA) and expressed as averages ± SEM. The n values give the number of measurements obtained from different samples of cells; the N values present the number of independent experiments.

Table 2: The response of extracellular ATP contents to laser irradiation in presence of selected reagents.

Reagents (concentration)	Action	$[ATP]_o$ in Irr/$[ATP]_o$ in control	Conclusion
NEM (100 μM)	Exocytosis blocker	0.96 ± 0.08 (n = 8)	No $[ATP]_o$ increased once exocytosis was blocked
BAPTA-AM (25 μM)	$[Ca^{2+}]_i$ chelating agent	1.01 ± 0.13 (n = 5)	No $[ATP]_o$ increased once $[Ca^{2+}]_i$ was chelated
ARL67156 (100 μM)	Nonspecific ecto-ATPases inhibitor	0.9 ± 0.02 (n = 15)*	Inhibition of $[ATP]_o$ was greatly attenuated when the ecto-ATPases were inhibited
ATP standards (10 pmoles)	—	1.02 ± 0.06 (n = 4)	Extracellular ATP was not affected by laser irradiation

Data are averages ± SEM. Values are relative to control.
$[ATP]_o$ presents extracellular ATP levels.
* $P < 0.05$, compared to the control.

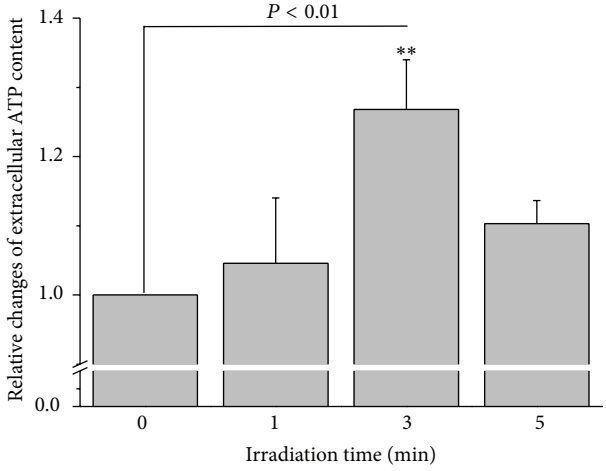

Figure 1: Extracellular ATP content of HMC-1 cells in response to 657 nm and 0.28 W/cm^2 laser irradiation. Relative changes of extracellular ATP level of HMC-1 cells induced by laser irradiation averaged for respective irradiation times of all samples. The data represent averages ± SEM from N = 3-4 independent experiments. ** $P < 0.01$, compared to the preirradiation value.

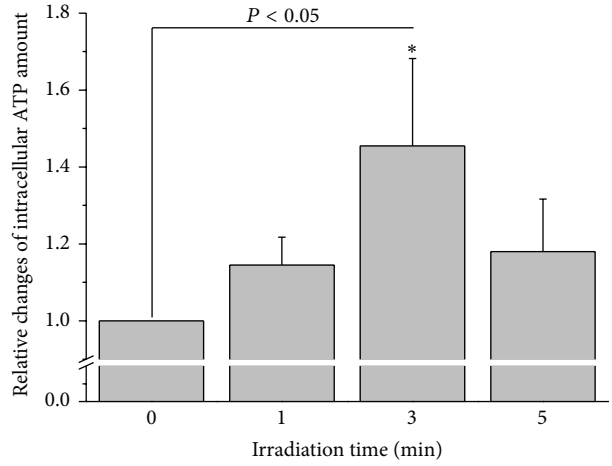

Figure 2: Changes of intracellular ATP content in HMC-1 cells in response to 657 nm and 0.28 W/cm^2 laser irradiation. Relative changes of intracellular ATP content in HMC-1 cells induced by laser irradiation averaged for the respective irradiation times of all samples. The data represent averages ± SEM from N = 3–7 independent experiments. * $P < 0.05$, compared to the preirradiation value.

Differences between sample averages were compared by one-way ANOVA, and $P < 0.05$ was considered to represent statistically significant difference.

3. Results

3.1. Laser Irradiation Enhances the Extracellular ATP Content of HMC-1 Cells. In control HMC-1 cell suspension samples of 3.5×10^4 cells/mL the extracellular ATP content was 0.41 ± 0.06 fmoles/cell (n = 27). ATP content increased progressively with irradiation times of 1 to 3 min. Relative extracellular ATP increase induced by 3 min irradiation was 26.8 ± 7.2% of control ($P < 0.01$, n = 8; Figure 1), and it was blocked by 100 μM NEM, an inhibitor of exocytosis (see Table 2). This indicates that the elevated extracellular ATP originated from a cell regulated release process and not from cell death.

It is interesting that the response tended to decline with longer irradiation time of 5 min (with an increase of only 10.3 ± 3.3% compared to control, n = 9). This phenomenon has been described as the biphasic dose response to low-level light irradiation discovered in 1985 [27]. It was confirmed in numerous subsequent reports that biostimulation can be obtained at weaker doses, while stronger doses result in bioinhibition. This phenomenon is frequently referred to as Arndt-Schultz law [28].

3.2. Laser Irradiation Promotes ATP Synthesis in HMC-1 Cells. Extracellular ATP content depends on three metabolic steps: synthesis, release, and hydrolysis. In various cultured cell types, red and NIR-light irradiations have been reported to promote ATP synthesis [29, 30] that is indexed by intracellular ATP content. Thus in our study, somatic ATP content was assessed. Intracellular ATP in nonirradiated control HMC-1 cells amounted to 14.2 ± 1.8 fmoles/cell (n = 30). It increased slightly during the first minute of irradiation and was significantly enhanced, by 45.5 ± 22.6% compared to control values, after 3 min exposure ($P < 0.05$, n = 15; Figure 2). Similar to the extracellular ATP responses, 5 min

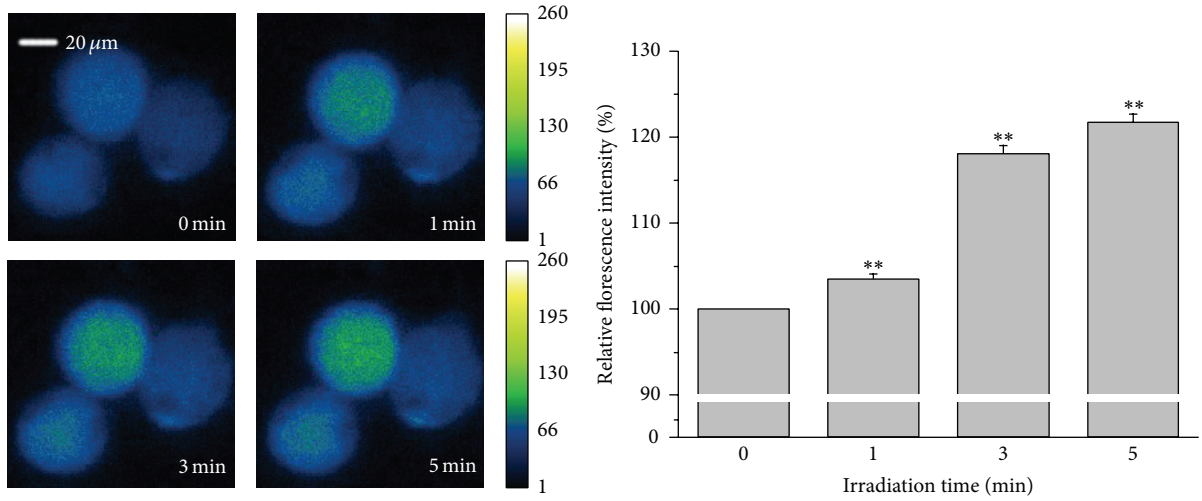

Figure 3: Response of $[Ca^{2+}]_i$ in HMC-1 cells induced by 657 nm and 0.28 W/cm^2 laser irradiation. (a) Representative MCs before and after irradiation. Irradiation times are given in the lower right corners. The pseudocolor calibration bar on the right illustrates $[Ca^{2+}]_i$ (in relative units). (b) Quantitative analysis from several sets of cells normalized to basal $[Ca^{2+}]_i$-dependent fluorescence before irradiation (=100%). Prolongation of laser irradiation enhanced fluorescence intensity in HMC-1 cells. The data represent averages ± SEM from n = 15–17 cells. $^{**}P < 0.01$, compared to control.

irradiation induced a smaller increase of somatic ATP content (by 18.0 ± 13.7% compared to control, n = 9). The coherent response of intracellular with extracellular ATP content may suggest that ATP synthesis partially contributes to elevated external ATP content during irradiation.

3.3. Laser Irradiation Increases $[Ca^{2+}]_i$ in HMC-1 Cells. Usually, $[Ca^{2+}]_i$ elevation is a trigger signal for exocytotic ATP release [31]. Therefore, the response of $[Ca^{2+}]_i$ to irradiation was assessed. We found that laser irradiation significantly elevated $[Ca^{2+}]_i$ in HMC-1 cells (n = 15–17 cells, $P < 0.01$). An example experiment is shown in Figure 3(a), which illustrates the increase in Ca^{2+} fluorescence at different irradiation times. Increase of relative fluorescence intensity became apparent after 1 min irradiation and amounted to 3.5 ± 0.6% (n = 17 cells, $P < 0.01$) of the controls. It rose to 18.0 ± 1.0% (n = 15 cells, $P < 0.01$) and 21.5 ± 1.1% (n = 15 cells, $P < 0.01$) with irradiation times of 3 and 5 min, respectively (Figure 3(b)). In order to judge whether irradiation-induced $[Ca^{2+}]_i$ elevation correlates with the ATP release, intracellular free Ca^{2+} in HMC-1 cells was chelated by BAPTA. The results showed that pretreatment of HMC-1 cells with 25 μM BAPTA blocked the extracellular ATP rise induced by 3 min irradiation (Table 2). This suggests that the extracellular ATP level depends on $[Ca^{2+}]_i$-dependent ATP release.

3.4. Laser Irradiation Activates Wild-Type MCs. In order to confirm the effects of red-laser irradiation on wild-type MCs, we measured the response of skin MCs to irradiation *in situ*. Connective tissue slices isolated from the lateral side of the rat tibia contained MCs at high density [32]. Figure 4(a) shows two MCs in a slice section; resting cells exhibited smooth and complete membranes. Identification of MCs was confirmed by staining with 0.5% TB. Once the slices were exposed to red-laser irradiation for 5 min, some MCs in these slices started to degranulate, displaying rough membrane and releasing granules that scattered around the cells (Figure 4(b)). Since peritoneal MCs are of the connective tissue phenotype as skin MCs and the available number of isolated peritoneal MCs is much higher than skin MCs, rat peritoneal MCs were taken to determinate the effects of red-laser irradiation on ATP mobilization. Similar to the responses observed with HMC-1 cells, 21 J/cm^2 irradiation of rat peritoneal MCs also showed tendency of enhanced extracellular ATP content, although the increase did not reach statistical significance (Figure 5).

3.5. Laser Irradiation Attenuates Extracellular ATP Content of Rat DRG Neurons by Modulation of Ecto-ATPase Activity. For nonirradiated neurons, extracellular ATP content was 0.8±0.1 fmoles/cell (n = 28). In contrast to MCs, extracellular ATP level was attenuated by 26.0 ± 2.6% in DRG neurons at 21 J/cm^2 irradiation (n = 27, $P < 0.01$, Figure 6). To eliminate the possibility that ATP outside neuronal cells could be degraded by the laser irradiation, the effect of light on ATP was evaluated. The results showed that ATP was not affected by laser irradiation (Table 2). Considering that multiple ecto-ATPases are expressed on DRG neurons [33], we wondered whether the suppressive effect resulted from modulation of ecto-ATPase activity by irradiation. Figure 6 illustrates the response of extracellular ATP content of DRG neurons to irradiation in the presence of the nonspecific ecto-ATPases inhibitor ARL 67156. 100 μM ARL 67156 partially attenuated the irradiation-induced inhibition of extracellular ATP (n = 15, $P < 0.05$, Figure 6). This suggests that at least

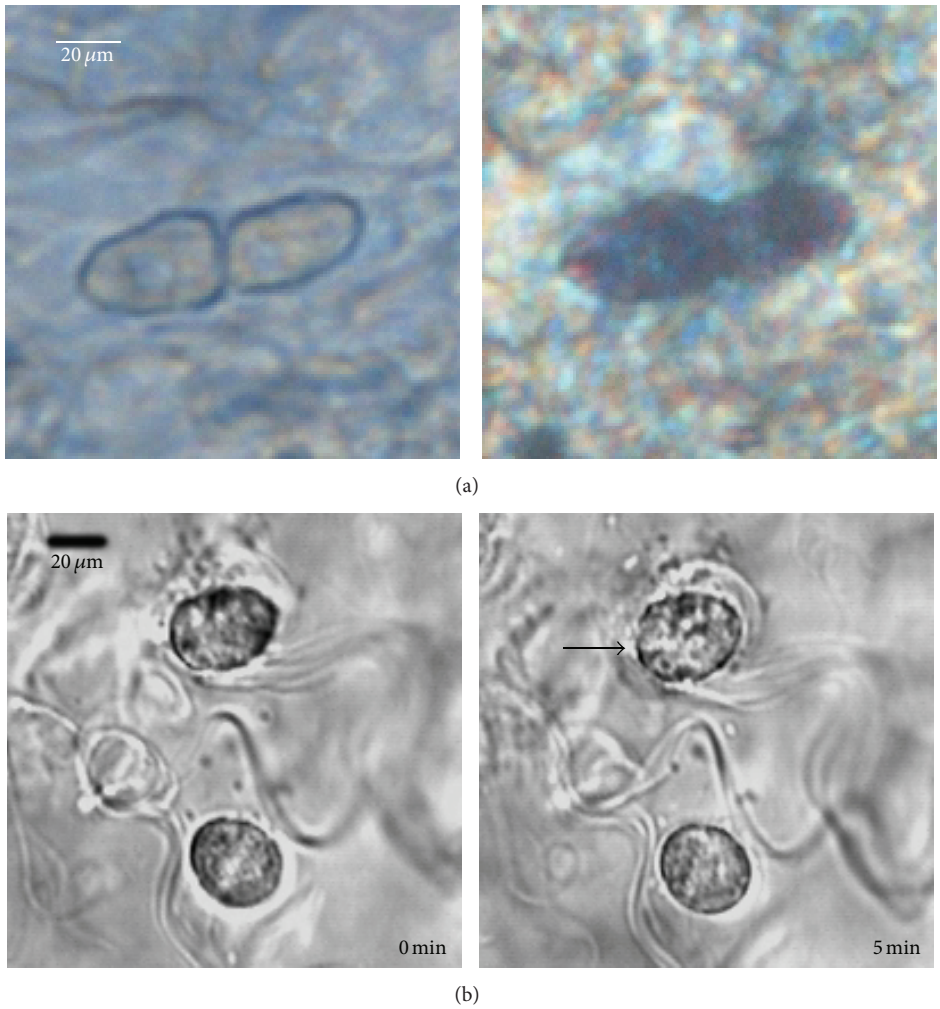

Figure 4: Response of MCs in rat tissue slices to 657 nm and 0.28 W/cm^2 laser irradiation. (a) MCs in connective tissue slices isolated from rat skin. The left graph shows their smooth and complete plasma membrane. The right graph presents the same MCs stained by 0.5% TB. (b) MCs in tissue slices showed smooth cell membranes before irradiation (left graph). Some MCs started to degranulate once they were exposed to irradiation. The cell membrane became rough, and some black granules were seen scattered around the cell (right graph). The arrow points to degranulating cell. Irradiation times are given in the lower right corners.

to some extent the irradiation reduced external ATP level of DRG neurons by stimulating the ecto-ATPases activity. The mechanisms of the remaining suppressive effects in the presence of ARL 67156 need further exploration.

4. Discussion

In vivo MCs have been demonstrated to play a role in LLLT for anti-inflammatory [3], wound healing [7], as well as pain relief [8]. Not only the total number of MCs but also degranulation ratios were increased by red-laser irradiation [6]. In *in vivo* tests, increasing degranulation [12, 13], histamine release [34, 35], and $[Ca^{2+}]_i$ [12–14, 34, 35] as well as whole-cell membrane currents [12] were demonstrated in MCs irradiated by green-, blue-, or red-laser. Extracelluar ATP is an important autocrine/paracrine mediator to modulate system functions in various tissues by binding to P2 receptors. Red-laser irradiation-induced elevation of extracellular ATP level of MCs has been illustrated in our previous work [14]. In the present study, we confirmed such potentiated effects and demonstrated dose dependency. Extracellular ATP content is influenced by several processes, including synthesis, release, and hydrolysis. In our study, the irradiation-induced coherent response of intracellular and extracellular ATP content suggests that the stimulating effects of irradiation on extracellular ATP can partially be attributed to enhance ATP synthesis. Promoting ATP synthesis by red and NIR-light irradiation has been reported in various cultured cell types [29, 30]. The ATP synthesis depends on the state of the cellular respiratory chain. The absorption of monochromatic light by components of the cellular respiratory chain, such as cytochrome c oxidase, has been reported [35, 36]. Besides providing more ATP to be loaded into the secretory vesicles, increased ATP synthesis also will facilitate ATP-driven transporters such as

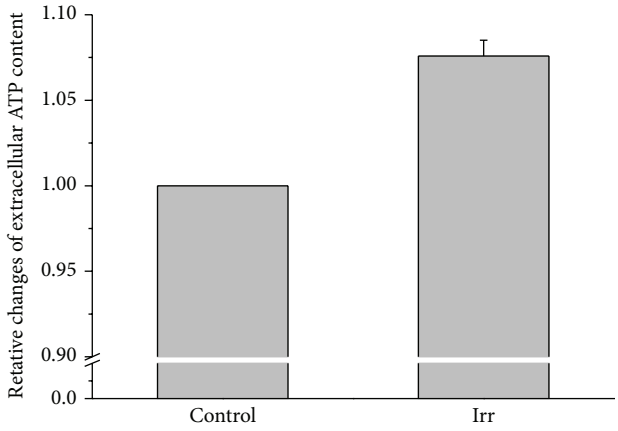

Figure 5: Extracellular ATP content of rat peritoneal MCs in response to 657 nm, 18 mW/cm^2, and 21 J/cm^2 laser irradiation. Changes of extracellular ATP content of rat peritoneal MCs induced by laser irradiation averaged for respective irradiation times of all samples. The data represent averages ± SEM from n = 12-13.

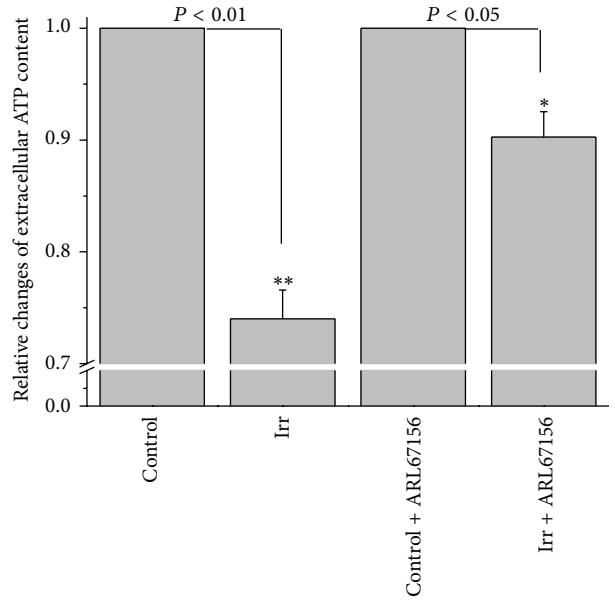

Figure 6: Extracellular ATP content of rat DRG neurons in response to 657 nm, 18 mW/cm^2, and 21 J/cm^2 laser irradiation. Relative changes of extracellular ATP level of rat DRG neurons induced by laser irradiation averaged for respective irradiation times of all samples. The data represent averages ± SEM from N = 3–7 independent experiments. $^{**}P < 0.01$ compared to the preirradiation value; $^{*}P < 0.05$ compared to the preirradiation in the presence of ARL 67156 value.

Ca^{2+} and Na^+/K^+ ATPase. Whether the respiratory chain is involved in the processes described in our work needs further investigation. The idea of "photo-switched" ligands that modulate the membrane channels gating has been put forward [37, 38]. This might be another mechanism underling in our researches.

Our results also revealed that irradiation could elevate $[Ca^{2+}]_i$ in MCs, which is required as the trigger signal for ATP release [31], leading to increased extracellular ATP level. Our experiments with BAPTA-loaded cells provided further evidence that the extracellular ATP level depends on the $[Ca^{2+}]_i$ rise. Since extracellular ATP is the endogenous agonist of P2X receptors which allow extracellular Ca^{2+} to enter the cells [19], and MCs themselves express P2X receptors [39], we hypothesize that the ATP secreted from MCs could produce positive feedback by autocrine activation of the P2 receptors. Such a mechanism could amplify irradiation effects in local areas to treat local disorders, for example, to act in anti-inflammatory reactions [3] in wound-healing [7], or in local pain relief [8].

Like the subcutaneous MCs, peripheral neuritis in skin is also in a depth that can be reached by irradiation during LLLT [40]. In our work, irradiation attenuated extracellular ATP content of DRG neurons, which is opposite to the response of MCs. The depressive effects of irradiation on peripheral nerve system have been reviewed [41]. Since soluble and membrane-attached ecto-ATPases, that hydrolyze extracellular ATP, exist in peripheral nervous system, including DRG peripheral endings [33], modulation of ecto-ATPases of DRG neurons by irradiation could explain the effect on extracellular ATP levels. In our work, 100 μM ARL 67156 abolished the irradiation-induced depression extracellular ATP of DRG neurons suggesting an upregulation of ecto-ATPases on DRG neurons. The ecto-ATPases hydrolyze extracellular ATP into AMP, the precursor of adenosine which is a vital mediator involved in pain relief by binding to A1 receptors [42, 43]. The peripheral nervous system is the main domain of purinergic receptors, including A1 receptors [19]. Thus modulation of ecto-ATPases by irradiation might be one of the mechanisms underlying analgesic effects of LLLT.

MCs interact with nerve endings morphologically [17] and functionally [18]. There exists crosstalk via purinergic signals between them [20]. Concerning the opposite responses to irradiation in MCs and neurons, we hypothesize that stimulated ecto-ATPase on DRG neurons could hydrolyze part of the extracellular ATP released by the MCs during LLLT.

5. Conclusion and Summary

Low-level red-laser irradiation of MCs and DRG neurons has opposite effects on extracellular ATP content. Irradiation of MCs enhanced extracellular ATP level in a dose-dependent manner, which is brought about by increased intracellular ATP synthesis and $[Ca^{2+}]_i$. For DRG neurons, irradiation depressed the extracellular ATP content due to upregulation of ecto-ATPases. The opposite responses of these two cell types indicate complex mechanisms underlying LLLT that need further investigations.

Abbreviations

ARL67156: 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5′-triphosphate trisodium salt hydrate FPL 67156

BS: Bath solution
BAPTA-AM: 1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
$[Ca^{2+}]_i$: Intracellular calcium activity
DRG: Dorsal root ganglia
HBSS: Hank's balanced salt solution
HMC-1: Human mast cell line-1
LLLT: Low-level-laser therapy
NEM: N-Ethylmaleimide
MCs: Mast cells
NIR: Near-infrared
TB: Toluidine blue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] C. Noli and A. Miolo, "The mast cell in wound healing," *Veterinary Dermatology*, vol. 12, no. 6, pp. 303–313, 2001.

[2] J. Kalesnikoff and S. J. Galli, "New developments in mast cell biology," *Nature Immunology*, vol. 9, no. 11, pp. 1215–1223, 2008.

[3] R. R. Marinho, R. M. Matos, J. S. Santos et al., "Potentiated anti-inflammatory effect of combined 780 nm and 660 nm low level laser therapy on the experimental laryngitis," *Journal of Photochemistry and Photobiology B: Biology*, vol. 121, pp. 86–93, 2013.

[4] M. Bayat, M. M. Vasheghani, N. Razavie, and M. R. Jalili, "Effects of low-level laser therapy on mast cell number and degranulation in third-degree burns of rats," *Journal of Rehabilitation Research and Development*, vol. 45, no. 6, pp. 931–938, 2008.

[5] C. E. Pinfildi, R. E. Liebano, B. S. Hochman et al., "Effect of low-level laser therapy on mast cells in viability of the transverse rectus abdominis musculocutaneous flap," *Photomedicine and Laser Surgery*, vol. 27, no. 2, pp. 337–343, 2009.

[6] M. C. M. C. Pereira, C. B. D. Pinho, A. R. P. Medrado, Z. D. A. Andrade, and S. R. D. A. Reis, "Influence of 670 nm low-level laser therapy on mast cells and vascular response of cutaneous injuries," *Journal of Photochemistry and Photobiology B: Biology*, vol. 98, no. 3, pp. 188–192, 2010.

[7] F. L. C. V. Berbert, G. Sivieri-Araújo, L. T. O. Ramalho, S. A. L. Pereira, D. B. R. Rodrigues, and M. S. De Araújo, "Quantification of fibrosis and mast cells in the tissue response of endodontic sealer irradiated by low-level laser therapy," *Lasers in Medical Science*, vol. 26, no. 6, pp. 741–747, 2011.

[8] K. Cheng, X.-Y. Shen, G.-H. Ding, and F. Wu, "Relationship between laser acupuncture analgesia and the function of mast cells," *Zhongguo Zhen Jiu*, vol. 29, no. 6, pp. 478–483, 2009.

[9] Q. Peng, A. Juzeniene, J. Chen et al., "Lasers in medicine," *Reports on Progress in Physics*, vol. 71, no. 5, Article ID 056701, 2008.

[10] P. Whittaker, "Laser acupuncture: past, present, and future," *Lasers in Medical Science*, vol. 19, no. 2, pp. 69–80, 2004.

[11] L. C. J. Yong, "The mast cell: origin, morphology, distribution, and function," *Experimental and Toxicologic Pathology*, vol. 49, no. 6, pp. 409–424, 1997.

[12] D. Zhang, A. Spielmann, L. Wang et al., "Mast-cell degranulation induced by physical stimuli involves the activation of transient-receptor-potential channel TRPV2," *Physiological Research*, vol. 61, no. 1, pp. 113–124, 2012.

[13] L. Wang and W. Schwarz, "Activation of mast cells by acupuncture stimuli," *Forum on Immunopathological Diseases and Therapeutics*, vol. 3, no. 1, pp. 41–50, 2012.

[14] L. Wang, J. Sikora, L. Hu, X. Shen, R. Grygorczyk, and W. Schwarz, "ATP release from mast cells by physical stimulation: a putative early step in activation of acupuncture points," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 350949, 7 pages, 2013.

[15] F. Jacob, C. P. Novo, C. Bachert, and K. Van Crombruggen, "Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses," *Purinergic Signalling*, vol. 9, no. 3, pp. 285–306, 2013.

[16] M. D. Valls, B. N. Cronstein, and M. C. Montesinos, "Adenosine receptor agonists for promotion of dermal wound healing," *Biochemical Pharmacology*, vol. 77, no. 7, pp. 1117–1124, 2009.

[17] M. G. Blennerhassett, M. Tomioka, and J. Bienenstock, "Formation of contacts between mast cells and sympatetic neurons in vitro," *Cell and Tissue Research*, vol. 265, no. 1, pp. 121–128, 1991.

[18] O. Bauer and E. Razin, "Mast cell-nerve interactions," *News in Physiological Sciences*, vol. 15, no. 5, pp. 213–218, 2000.

[19] M. P. Abbracchio, G. Burnstock, A. Verkhratsky, and H. Zimmermann, "Purinergic signalling in the nervous system: an overview," *Trends in Neurosciences*, vol. 32, no. 1, pp. 19–29, 2009.

[20] G. Burnstock, "Acupuncture: a novel hypothesis for the involvement of purinergic signalling," *Medical Hypotheses*, vol. 73, no. 4, pp. 470–472, 2009.

[21] L. Wang, G. Ding, Q. Gu, and W. Schwarz, "Single-channel properties of a stretch-sensitive chloride channel in the human mast cell line HMC-1," *European Biophysics Journal*, vol. 39, no. 5, pp. 757–767, 2010.

[22] B. M. Jensen, E. J. Swindle, S. Iwaki, and A. M. Gilfillan, "Generation, isolation, and maintenance of rodent mast cells and mast cell lines," *Current Protocols in Immunology*, vol. 3, 2006.

[23] T. H. Burkey, C. M. Hingtgen, and M. R. Vasko, "Isolation and culture of sensory neurons from the dorsal-root ganglia of embryonic or adult rats," *Methods in Molecular Medicine*, vol. 99, pp. 189–202, 2004.

[24] R. R. Anderson and J. A. Parrish, "The optics of human skin," *Journal of Investigative Dermatology*, vol. 77, no. 1, pp. 13–19, 1981.

[25] P. Avci, A. Gupta, M. Sadasivam et al., "Low-level laser (light) therapy (LLLT) in skin: stimulating, healing, restoring," *Seminars in Cutaneous Medicine and Surgery*, vol. 32, no. 1, pp. 41–52, 2013.

[26] G. A. Kimmich, J. Randles, and J. S. Brand, "Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system," *Analytical Biochemistry*, vol. 69, no. 1, pp. 187–206, 1975.

[27] E. Mester, A. F. Mester, and A. Mester, "The biomedical effects of laser application," *Lasers in Surgery and Medicine*, vol. 5, no. 1, pp. 31–39, 1985.

[28] H. Chung, T. Dai, S. K. Sharma, Y.-Y. Huang, J. D. Carroll, and M. R. Hamblin, "The nuts and bolts of low-level laser (Light) therapy," *Annals of Biomedical Engineering*, vol. 40, no. 2, pp. 516–533, 2012.

[29] T. Karu, L. Pyatibrat, and G. Kalendo, "Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro," *Journal of Photochemistry and Photobiology B: Biology*, vol. 27, no. 3, pp. 219–223, 1995.

[30] S. Benedicenti, I. M. Pepe, F. Angiero, and A. Benedicenti, "Intracellular ATP level increases in lymphocytes irradiated with infrared laser light of wavelength 904 nm," *Photomedicine and Laser Surgery*, vol. 26, no. 5, pp. 451–453, 2008.

[31] F. Boudreault and R. Grygorczyk, "Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations," *Journal of Physiology*, vol. 561, no. 2, pp. 499–513, 2004.

[32] L.-N. Wang, W. Schwarz, Q.-B. Gu, and G.-H. Ding, "Skin slice used as a model for investigating acupuncture effects," *Zhen Ci Yan Jiu*, vol. 34, no. 5, pp. 291–296, 2009.

[33] H. O. Vongtau, E. G. Lavoie, J. Sévigny, and D. C. Molliver, "Distribution of ecto-nucleotidases in mouse sensory circuits suggests roles for nucleoside triphosphate diphosphohydrolase-3 in nociception and mechanoreception," *Neuroscience*, vol. 193, pp. 387–398, 2011.

[34] W.-Z. Yang, J.-Y. Chen, J.-T. Yu, and L.-W. Zhou, "Effects of low power laser irradiation on intracellular calcium and histamine release in RBL-2H3 mast cells," *Photochemistry and Photobiology*, vol. 83, no. 4, pp. 979–984, 2007.

[35] Z.-H. Wu, Y. Zhou, J.-Y. Chen, and L.-W. Zhou, "Mitochondrial signaling for histamine releases in laser-irradiated RBL-2H3 mast cells," *Lasers in Surgery and Medicine*, vol. 42, no. 6, pp. 503–509, 2010.

[36] M. T. T. Wong-Riley, H. L. Liang, J. T. Eells et al., "Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: role of cytochrome c oxidase," *The Journal of Biological Chemistry*, vol. 280, no. 6, pp. 4761–4771, 2005.

[37] K. Hill and M. Schaefer, "Ultraviolet light and photosensitising agents activate TRPA1 via generation of oxidative stress," *Cell Calcium*, vol. 45, no. 2, pp. 155–164, 2009.

[38] L. Wang, D. Zhang, and W. Schwarz, "TRPV channels in mast cells as a target for low-level-laser therapy," *Cells*, vol. 3, no. 3, pp. 662–673, 2014.

[39] E. Bulanova and S. Bulfone-Paus, "P2 receptor-mediated signaling in mast cell biology," *Purinergic Signalling*, vol. 6, no. 1, pp. 3–17, 2010.

[40] T. Karu, "Primary and secondary mechanisms of action of visible to near-IR radiation on cells," *Journal of Photochemistry and Photobiology B: Biology*, vol. 49, no. 1, pp. 1–17, 1999.

[41] R. Chow, P. Armati, E.-L. Laakso, J. M. Bjordal, and G. D. Baxter, "Inhibitory effects of laser irradiation on peripheral mammalian nerves and relevance to analgesic effects: a systematic review," *Photomedicine and Laser Surgery*, vol. 29, no. 6, pp. 365–381, 2011.

[42] M. J. Zylka, "Pain-relieving prospects for adenosine receptors and ectonucleotidases," *Trends in Molecular Medicine*, vol. 17, no. 4, pp. 188–196, 2011.

[43] N. Goldman, M. Chen, T. Fujita et al., "Adenosine A1 receptors mediate local anti-nociceptive effects of acupuncture," *Nature Neuroscience*, vol. 13, no. 7, pp. 883–888, 2010.

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Research Article

Deficits in Endogenous Adenosine Formation by Ecto-5′-Nucleotidase/CD73 Impair Neuromuscular Transmission and Immune Competence in Experimental Autoimmune Myasthenia Gravis

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AMP dephosphorylation via ecto-5′-nucleotidase/CD73 is the rate limiting step to generate extracellular adenosine (ADO) from released adenine nucleotides. ADO, via A_{2A} receptors (A_{2A}Rs), is a potent modulator of neuromuscular and immunological responses. The pivotal role of ecto-5′-nucleotidase/CD73, in controlling extracellular ADO formation, prompted us to investigate its role in a rat model of experimental autoimmune myasthenia gravis (EAMG). Results show that $CD4^+CD25^+FoxP3^+$ regulatory T cells express lower amounts of ecto-5′-nucleotidase/CD73 as compared to controls. Reduction of endogenous ADO formation might explain why proliferation of $CD4^+$ T cells failed upon blocking A_{2A} receptors activation with ZM241385 or adenosine deaminase in EAMG animals. Deficits in ADO also contribute to neuromuscular transmission failure in EAMG rats. Rehabilitation of A_{2A}R-mediated immune suppression and facilitation of transmitter release were observed by incubating the cells with the nucleoside precursor, AMP. These findings, together with the characteristic increase in serum adenosine deaminase activity of MG patients, strengthen our hypothesis that the adenosinergic pathway may be dysfunctional in EAMG. Given that endogenous ADO formation is balanced by ecto-5′-nucleotidase/CD73 activity and that A_{2A}Rs exert a dual role to restore use-dependent neurocompetence and immune suppression in myasthenics, we hypothesize that stimulation of the two mechanisms may have therapeutic potential in MG.

1. Introduction

Autoimmune *Myasthenia Gravis* (MG) is the most common T-cell dependent acquired neuromuscular disorder, which in practical terms is characterized by skeletal muscle weakness and fatigability on repetitive use due to autoantibodies directed towards muscle-type nicotinic ACh receptors (nAChR). These antibodies reduce the number of effective receptors to nearly one-third of the normal (reviewed in [1]), leading to a decrease in the safety margin of the neuromuscular transmission, which is particularly relevant during high-frequency nerve activity. By decreasing the generation of postsynaptic action potentials, this condition leads to muscle weakness [2, 3]. Although the therapeutic approach must be individualized according to patients complains, treatment of MG can be divided into symptomatic (acetylcholinesterase inhibitors) and long-term immunosuppression (e.g., corticosteroids, azathioprine,

monoclonal antibodies, and removal of the thymus). The overall goal is to restore normal clinical neuromuscular function dealing with the exaggerated immune reaction, while minimizing side effects. Thus, understanding common features that regulate both the safety factor of neuromuscular transmission and the T-cell drive for specific autoantibodies production may be clinically relevant for devising novel and unifying therapeutic strategies to manage MG.

Adenosine (ADO) is an ubiquitous molecule acting as a potent modulator of both neuronal and immunological responses through the activation of adenosine A_{2A} receptors ($A_{2A}R$) [4, 5]. A seminal work from our group at the rat motor endplate demonstrated for the first time that ADO could facilitate the release of neurotransmitters via prejunctional $A_{2A}R$ activation, besides the classical neuroinhibitory action mediated by adenosine A_1 receptors (A_1R) [4]. Later on, we showed that tonic activation of facilitatory $A_{2A}R$ on motor nerve terminals contributes to overcome tetanic depression during high-frequency neuronal firing through the increase in Ca^{2+} influx via Ca_v1 (L-type) channels [6]. This led us to propose that manipulation of $A_{2A}R$ activation could be of clinical interest to preserve neuromuscular transmission in compromised (low safety factor) myasthenic motor endplates. In fact, we proved that impairment of Ca^{2+} influx via Ca_v1 (L-type) channels due to deficits in $A_{2A}R$ tonus contributes to tetanic failure in rats with toxin-induced *Myasthenia gravis* (TIMG) [7]. Moreover, the $A_{2A}R$ is now considered an important negative modulator of T-cell function and is also being recognized as a relevant player in the immunopathogenesis of MG [8]. The $A_{2A}R$ activation plays a dual role on T cells. This receptor inhibits T-cell receptor (TCR)-mediated signaling, which consequently leads to a decrease in IL-2 production and CD25 expression, and, consequently, to a decline in T-cells proliferation [5, 9]. In addition, activation of the $A_{2A}R$ has been shown to increase the expression of FoxP3 in cognate antigen-activated T cells, thus promoting the differentiation of inducible regulatory T cells (T_{reg}) [10].

Taking this into consideration, disorders like *Myasthenia gravis* (MG) may benefit from therapeutic strategies targeting common molecular elements involved in both neuromuscular and immunological impairment. Impairment of the $A_{2A}R$ neuromodulatory tonus was recently demonstrated in the TIMG rat model [7]. In parallel, Li and collaborators reported decreases in the $A_{2A}R$ expression on both $CD4^+$ T cells and B cells residing in spleen and lymph nodes following experimental autoimmune *Myasthenia gravis* (EAMG) induction [8]. Thus, one may predict that the adenosinergic $A_{2A}R$-mediated pathway might be a common deficient feature underlying both neuronal and immunological dysfunctions occurring in MG.

AMP dephosphorylation via ecto-5′-nucleotidase/CD73 is the rate limiting step to generate extracellular ADO from released adenine nucleotides. At the rat motor endplate, ADO originating from the catabolism of released adenine nucleotides together with ACh preferentially activates facilitatory $A_{2A}R$ on nerve terminals [11–13]. While at the immunological system, formation of ADO by ecto-5′-nucleotidase/CD73 is essential for the immunosuppressant activity of $CD4^+CD25^+FoxP3^+$ T_{reg} [14]. The pivotal role of ecto-5′-nucleotidase/CD73 in controlling the extracellular ADO levels prompted us to investigate its contribution in the pathogenesis of autoimmune *Myasthenia Gravis* (MG) in order to conceive novel therapeutic strategies to manage this relatively frequent, yet highly incapacitating, disease. In this study, we used a rat model of EAMG that best reproduces the features of human MG, in both clinical and histopathological terms.

2. Materials and Methods

2.1. Induction and Clinical Assessment of EAMG. Female Wistar rats, weighting approximately 100 g (Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06.30–19.30 h)—dark (19.30–06.30 h) cycle, with food and water *ad libitum* and randomly divided into three groups (naïve, control, and EAMG). Under general anesthesia, with ketamine (75 mg/kg) and medetomidine (100 mg/kg) by intraperitoneal administration, rats in the EAMG group were immunized by subcutaneous injection at four sites (two hind footpads and shoulders) with 50 μg of R97-116 peptide (DGDFAIVKFTKVLLDYTGHI, JPT Peptide Technologies GmbH), a synthetic peptide corresponding to a specific region on the α subunit of the rat nicotinic AChR, made up in complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO, USA). Injections were performed on day 0 and were boosted on day 30 with the same peptide in incomplete Freund's adjuvant (IFA) [15]. The control group was immunized with CFA and IFA emulsions, respectively, containing phosphate-buffered saline (PBS) instead of the nAChR R97-116 peptide at the respective time points. Animals in the naïve group were left untreated. Evaluation of disease manifestations in immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor and hunched posture and muscle strength by grip strength test (BIOSEB, France), and fatigability was assessed after exercise (repetitive paw grips on the cage grid). Disease severity was graded as follows: grade 0, normal strength and no fatigability; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present at rest; grade 3, severe clinical signs at rest, no grip, and moribund; and grade 4, death [15]. Each animal was weighted and evaluated for disease manifestation twice weekly until sacrifice by decapitation on day 42 [15]. Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All the animals included in this study were submitted to the same experimental procedure.

2.2. Serum Adenosine Deaminase Activity. The whole blood was collected from the three different groups of rats after decapitation and total serum adenosine deaminase (ADA)

activity was determined at 37°C by an enzymatic spectrophotometric method on a Cobas Mira S autoanalyser (Roche Diagnostics, Switzerland), according to the method of Giusti [16].

2.3. Muscle Contraction Recordings. The experiments were performed using either left or right phrenic nerve-hemidiaphragm preparations (4–6 mm width). Each muscle was superfused with gassed (95% O_2-5% CO_2) Tyrode solution (pH 7.4) containing (mM) NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2, and choline 0.001, at 37°C. Tension responses were recorded isometrically at a resting tension of 50 mN with a force transducer and displayed on a Hugo-Sachs (Germany) recorder. Supramaximal intensity rectangular pulses of 40 μs duration and a current strength of 8 mA were applied to phrenic nerve. This was done to achieve synchronization of phrenic motoneuron firing in order to reduce the number of silent units. Pulses were generated by a Grass S48 (USA) stimulator coupled to a stimulus isolation unit (Grass SIU5) operating in a constant current mode. Tetanic failure (fatigue) of hemidiaphragm muscle contractions was achieved using high frequency (50 Hz) intermittent (17 pulses per sec, during 3 minutes) nerve stimulation [17]. The percentage of contractile reduction force was calculated by assessing the percentage of variation of the peak force at the last train (applied at 180 seconds of stimulation) comparatively to the peak force observed at the beginning of the intermittent stimulation.

2.4. [^{3}H]-ACh Release Experiments. The procedures used for labeling the preparations and measuring evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release have been previously described [4, 12]. Briefly, phrenic nerve-hemidiaphragm preparations were mounted in 3 mL capacity Perspex chambers heated to 37°C. Nerve terminals were labeled for 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi/nmol) under electrical stimulation at a frequency of 1 Hz (0.04 ms duration, 8 mA). The phrenic nerve was stimulated with a glass-platinum suction electrode placed near the first division branch of the nerve trunk to avoid direct contact with muscle fibers. Washout of the preparations was performed for 60 min, by superfusion (15 mL/min) with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Release of [^{3}H]-ACh was evoked by electrical stimulation of the phrenic nerve with 750 pulses applied at 5 Hz frequency (see stimulation conditions on myographic recordings section). Two stimulation periods were used: at 12 min (S_1) and at 39 min (S_2) after the end of washout (time zero). Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Tritium outflow was evaluated by liquid scintillation spectrometry (% counting efficiency: 40 ± 2%) after appropriate background subtraction using 2 mL bath samples collected automatically every 3 min. After the loading and washout periods, the preparation contained (5542 ± 248) × 10^3 disintegrations per minute per gram (DPM/g) wet weight of tissue and the resting release was (132 ± 12) × 10^3 DPM/g (n = 8). The fractional release was calculated to be 2.38 ± 0.14% of the radioactivity present in the tissue at the first collected sample. The evoked release of [^{3}H]-ACh was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period [12]. The change in the ratio between the evoked [^{3}H]-ACh released during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs.

2.5. Kinetics of the Extracellular AMP Catabolism by HPLC (UV Detection). The extracellular AMP catabolism was evaluated, at 37°C, on phrenic-nerve hemidiaphragm preparations from naïve, control, and EAMG rats. After a 30 min equilibration period, the organ bath was emptied and 2 mL of a 30 μM AMP in gassed Tyrode's solution was added to the preparations at time zero. Samples of 75 μL were collected from the bath at different times up to 45 min for HPLC with UV detection (HPLC-UV, LaChrome Elite, Hitachi, Merck, Germany) analysis of the variation of substrate disappearance and product formation [13, 18]. In all experiments, the concentration of products at the different times of sample collection was corrected by subtracting the concentration of products in samples collected from the same preparation incubated without adding substrate. Only IMP, inosine (INO), and hypoxanthine (HX) were spontaneously released from the preparations in concentrations that did not exceed 1 μM [13]. There was no spontaneous degradation of AMP at 37°C in the absence of the preparation. Concentration of the substrate and products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the different concentrations of the products, and concentration of the substrate. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase activity. The negligible activity of this enzyme at the end of the experiments was an indication of the integrity of the cells during experimental period.

2.6. Release of Endogenous Adenosine by HPLC (Diode Array Detection). Experiments were performed using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection) analysis. After a 30 min equilibration period, the preparations were incubated with 1.5 mL gassed Tyrode's solution, which was automatically changed every 3 min by emptying and refilling the organ bath with the solution in use. The preparations were electrically stimulated once, 15 min after starting sample collection (zero time), using 750 pulses delivered at a 5 Hz frequency. In these experiments, only the sample collected before stimulus application and the two samples collected after stimulation, were retained for analysis. Bath aliquots (50–250 μL) were frozen in liquid nitrogen immediately after collection, stored at −20°C (the enzymes are stable for at least 4 weeks), and analyzed within 1 week of collection by HPLC with diode array detection (Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode

array detector, and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 μM, 2.1 mm × 150 mm) equipped with a guard column (5 μm, 2.1 mm × 1 mm) using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was 200 μL/min and the column temperature was maintained at 20°C. The autosampler was set at 4°C and 50 μL of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine. Stimulation-evoked release of adenosine was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenosine determined after stimulus application.

2.7. Immunofluorescence Staining and Confocal Microscopy Observation. One of the major constrains regarding immunolabelling of the mammalian neuromuscular junction is the presence of abundant intramuscular connective tissue, which conceals the synaptic region leading to poor penetration of the labeling antibodies into the tissue. To circumvent this problem, we pretreated muscle fragments with Tyrode's solution continuously gassed with 95% O_2-5% CO_2, containing 0.1% collagenase (type I; Sigma Aldrich) for 30 min, in order to increase the access of the antibodies to the neuromuscular junction. Then, the muscle sections were stretched to all directions and pinned onto petri dishes coated with Sylgard. The tissues were, finally, fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, and sodium periodate 0.01 M) overnight at 4°C and stored in a cryoprotector solution at −20°C. Using a cryostat (Leica CM1950; Leica Microsystems, Nussloch, Germany) kept at −25°C, serial cross-sections of the muscle strips (45 μm) were cut.

After sectioning, tissue fragments were incubated overnight at 4°C with a blocking buffer solution, consisting in foetal bovine serum 10%, bovine serum albumin 1%, and Triton X-100 1% in PBS. Afterwards the samples were incubated with primary antibodies diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 0.5%, and Triton X-100 0.5% in PBS), at 4°C, for 48 h. For immunofluorescent staining of $A_{2A}R$, the rabbit anti-canine $A_{2A}R$ polyclonal antibody (1 : 75; A2aR21-A, Alpha Diagnostics International Inc.) and the mouse anti-human $A_{2A}R$ monoclonal (1 : 50; 05-717, Clone 7F6-G5-A2, Chemicon) with cross-reactivity with rat $A_{2A}R$, were incubated overnight. After incubation, the sections were washed in PBS supplemented Triton X-100 0.3% (3 cycles of 10 min). Then, species-specific secondary antibodies were applied to tissues samples overnight, at 4°C, in the dark upon which the samples were incubated for 15 min at room temperature with α-bungarotoxin (α-BTX) (1 : 1500) conjugated with tetramethylrhodamine (TMR-BTX) (Molecular Probes) to provide nAChR detection. Finally, tissue samples were mounted on optical-quality glass slides using the anti-fading agent VectaShield (VectorLabs) and stored at 4°C. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

The $A_{2A}R$ monoclonal antibody (05-717, Clone 7F6-G5-A2, Chemicon) recognizes an epitope in the third intracellular loop. It detected $A_{2A}R$ in paraformaldehyde-fixed mouse brain striatal sections; no signal was observed in $A_{2A}R$ knockout mice (see http://www.emdmillipore.com/PT/en/product/Anti-Adenosine-Receptor-A2a-Antibody%2C-clone-7F6-G5-A2,MM_NF-05-717). To test the specificity of the antibody for the $A_{2A}R$ (AlphaDiagnostics, A2aR21-A), some sections were processed with the primary antibody preadsorbed with a control antigen corresponding to a 30-amino-acid sequence of the intracellular C-terminus of the canine A2aR/ADORA2A (Gene Accession # P11617) (A2aR21-P, Alpha Diagnostics International Inc., San Antonio, TX, USA, http://www.4adi.com/objects/catalog/product/extras/A2aR21-S-A-P.pdf). Preadsorption was performed by incubating the $A_{2A}R$ primary antibody overnight at 4°C with 10-fold molar excess of the antigen peptide sequence. Sections were then processed as described earlier with the preabsorbed antiserum and with the normal antiserum, in parallel. During documentation of $A_{2A}R$ preabsorption controls, settings on the confocal microscope were adjusted appropriately to show $A_{2A}R$-immunoreactivity for sections that were processed normally (no preabsorption) and these settings were maintained when documenting pre-absorption controls to minimize bias, during capture and printing of digital images.

2.8. $CD4^+$ T Cell Enrichment. Popliteal and inguinal lymph nodes were removed from naïve, control, and EAMG animals and homogenized to single-cell suspensions. $CD4^+$ T lymphocytes-enriched suspensions were prepared by incubation of total lymph node cells with anti-CD4 magnetic microbeads (Miltenyi Biotech) that were further separated on LS columns (Miltenyi Biotech) according to the manufacturer's recommendations. The proportions of $CD4^+$ cells in the enriched suspensions typically ranged from 80 to 90%.

2.9. T-Cells Analysis by Flow Cytometry. Different combinations of antibodies were used to characterize cells derived from the different groups of animals. 1×10^6 cells/mL of $CD4^+$ T cell-enriched suspensions were blocked with 10% (V/V) mouse and rabbit sera before incubation with the following primary antibodies: FITC-conjugated anti-rat CD4 (1 : 100, eBioscience, clone OX35), PE-conjugated anti-rat CD25 (1 : 100; eBioscience, clone OX39), and rabbit anti-rat CD73 antibody (1 : 750; kindly provided by Professor Jean Sévigny, Univ. Laval, Québec, QC, Canada; can be obtained at http://www.ectonucleotidases-ab.com) during 30 min at 4°C in the dark. The anti-CD73 antibody was revealed with biotin-conjugated anti-rabbit IgG (Fc specific) (1 : 750, Sigma Aldrich) followed by incubation with Streptavidin PE-Cy7 (1 : 100; eBioscience). The specificity of the anti-rat CD73

antibody was confirmed by immunoblotting, flow cytometry, and immunohistochemistry [19].

For analysis of FoxP3 expression, cells were fixated and permeabilized using the fixation/permeabilization FoxP3 Kit (eBioscience) and labeled with PE-Cy5-conjugated anti-mouse/rat FoxP3 (1 : 100, eBioscience, clone FJK-16s). Isotype matched fluorochrome conjugated mAbs of irrelevant specificity were used as negative controls. The samples were analyzed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, Miami, FL). The collected data files (100 000 events per sample) were converted for analysis, with the CELLQUEST software, v3.2.1f1 by using FACS CONVERT, v1.0 (both from Becton Dickinson, San Jose, CA).

2.10. Proliferation Assays. $CD4^+$ T cells were isolated from the popliteal and inguinal lymph nodes of control and EAMG animals by using magnetic cell sorting rat $CD4^+$ microbeads (Miltenyi Biotech, Inc., Auburn, CA, USA) following the manufacturer's instructions. The CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen, Eugene, OR, USA) was used for cell labelling. A CFSE (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester) stock solution (10 mM in DMSO) stored at −20°C was thawed and diluted in PBS with 0.1% BSA to a final concentration of 10 μM. $CD4^+$ T cells were resuspended at 2×10^6/mL in PBS with 0.1% BSA and further incubated with an equal volume of the diluted CFSE solution, for 7 min at 37°C. Cells were washed three times with complete RPMI medium. $CD4^+$ T cells were plated at 5×10^4/well in U-shape 96-well plates without stimulus or stimulated with 1 μg/mL plate-bound anti-CD3 mAb (clone G4.18) and 1 μg/mL soluble anti-CD28 mAb (clone JJ319) (both from eBioscience). Additionally, cells were supplemented with 30 μM or 100 μM AMP every 12 h, in the absence or presence of either ADA (0.5 U/mL) or ZM241385 (50 nM). Unlabelled stimulated cells were used to define cell autofluorescence. Each condition was set in triplicate and cultures were maintained for 72 h at 37°C and 5% CO_2. Proliferation was determined based on CFSE fluorescence by flow cytometry analysis. Representative CFSE histograms are presented. The percentage of effect of AMP on $CD4^+$ T cells proliferation was calculated by using the mean proliferation index of $CD4^+$ T cells in the absence of the drug compared with the proliferation index of $CD4^+$ T cells incubated with AMP. When using the modifiers ADA (0.5 U/mL) or ZM 241285 (50 nM), the effect percentage of AMP on $CD4^+$ T cells proliferation was calculated by using the mean proliferation index of $CD4^+$ T cells in the presence of ADA (0,5 U/mL) or ZM 241285 (50 nM) compared with the proliferation index of $CD4^+$ T cells incubated with AMP alone.

2.11. Drugs and Solutions. Adenosine deaminase (ADA, type VI, 1803 U/mL, EC 3.3.3.4), adenosine monophosphate (AMP), choline chloride, hemicholinium-3, CFA, and IFA was from Sigma (Sigma, St. Louis, MO, USA), 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol(ZM 241385) was from Tocris Bioscience (Tocris Bioscience, Bristol, UK). [Methyl-^{3}H]choline chloride (ethanol solution, 80 Ci mmol^{-1}) was obtained from Amersham International (Amersham, UK). ZM 241385 was made up in dimethyl sulphoxide (DMSO). All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used, were observed.

2.12. Statistics. Results are expressed as mean ± SEM, with n indicating the number of animals used for a particular set of experiments. Statistical analysis of data was carried out using paired or unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test. Values of $P < 0.05$ were considered to represent significant differences.

3. Results and Discussion

3.1. Neurophysiological and Immunological Features of EAMG in the Wistar Rat. Most commonly, EAMG as a model of MG in humans can be induced in rats by immunization with nAChR purified from Torpedo electric organ in CFA and by passive transfer of serum from EAMG rats or from patients with MG [20]. Both in rat EAMG and in human MG, the production of autoantibodies to AChR is dependent on T-cell help [1]. In this study, we used a synthetic peptide corresponding to the R97-116 sequence found in α subunit of the rat nAChR in CFA to induce EAMG in Wistar rats [15]. These animals were screened for markers of neuromuscular and immunological imbalance. Rat EAMG is characterized clinically by two distinct phases: a transient acute phase with weakness (affecting predominantly the forelimbs, head, neck, laryngeal, and respiratory muscles) that begins approximately 7 days post immunization (p.i.) and with recovery after 3-4 days and a progressive chronic phase with onset approximately 28 days p.i., where weakness is progressive, often ending in death.

Previous studies have demonstrated a positive correlation between increase in serum adenosine deaminase (ADA) activity, the enzyme that inactivates ADO into INO, and the clinical score of myasthenic patients [21]. Moreover, symptoms and T cell mediated reactivity have been inversely related to the percentage of $CD4^+CD25^+FoxP3^+$ T_{reg} cell population in human MG [22]. In this context, we thought it will be interesting to evaluate the total serum ADA activity and the relative proportion of $CD4^+CD25^+FoxP3^+$ T_{reg} in the cell suspensions obtained from draining popliteal and inguinal lymph nodes in the rat model of EAMG to see if they compare to the human disease. Figure 1(a) shows that serum ADA activity was significantly ($P < 0.05$) higher (47 ± 8 U/L, $n = 12$) in EAMG animals as compared to both control (20 ± 2 U/L, $n = 13$) and naïve (25 ± 6 U/L, $n = 9$) littermates. The increase in serum ADA activity that we show here for the first time has been considered a hallmark of MG pathophysiology [21], since ADA influences proliferation and differentiation of lymphocytes, especially of T cells [23]. This

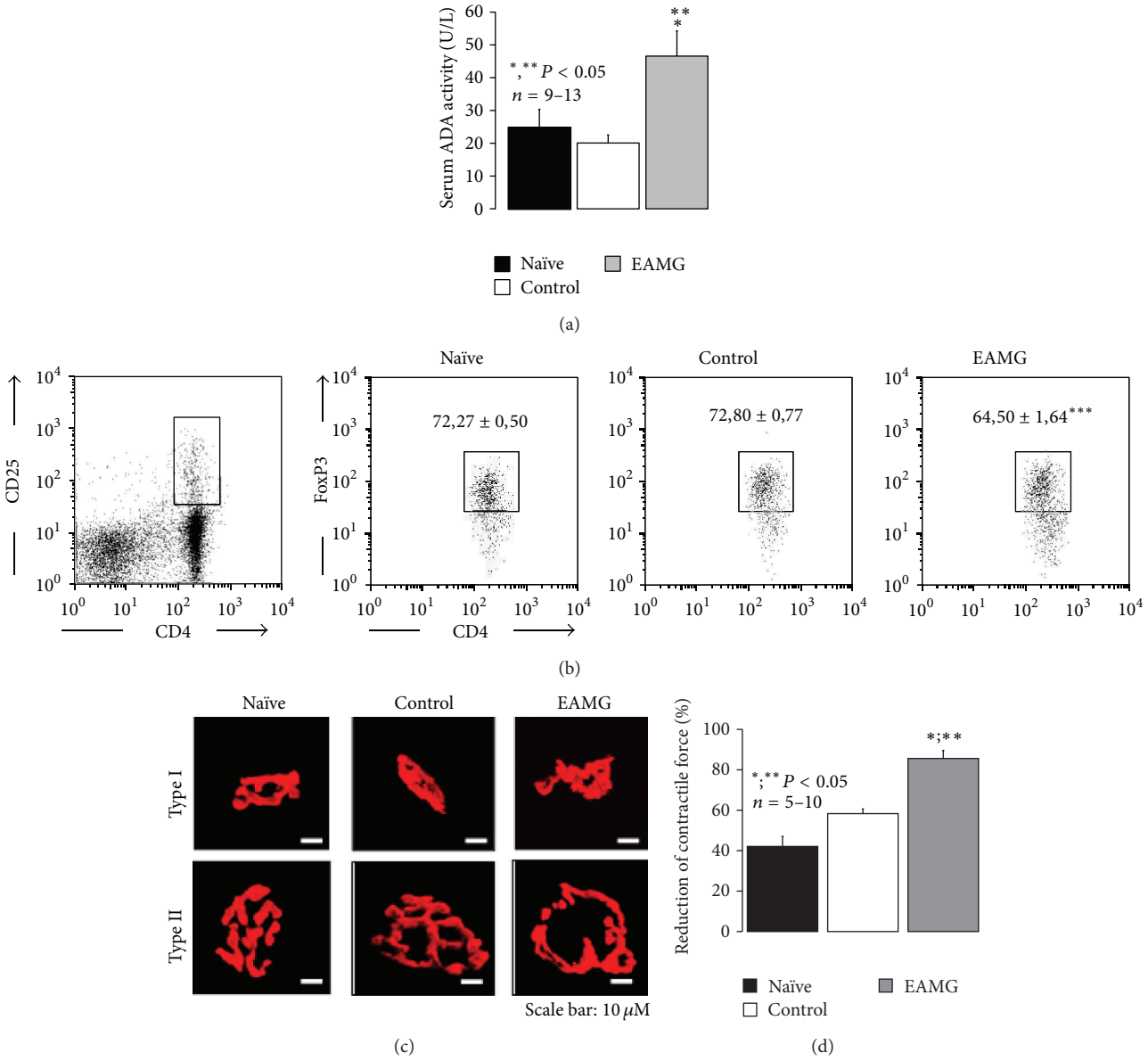


Figure 1: Neuromuscular and immunological deficits of rats with EAMG. Experiments were performed six weeks after immunization with the peptide R97-116 corresponding to the α-subunit of nAChR in CFA (EAMG), as compared to age-matched naïve and control littermates. (a) Measurement of serum adenosine deaminase (ADA) activity. Results are mean ± SEM of 9 naïve, 13 control, and 12 EAMG rats. $^{*,**}P < 0.05$ (one-way ANOVA following Dunnett's modified t-test) compared to naïve and control animals, respectively. (b) Flow cytometry analysis of intracellular FoxP3 expression in $CD4^+CD25^+$ T cells collected from popliteal and inguinal lymph nodes from naïve, control, and EAMG rats. Gating strategy to delimit $CD4^+CD25^+$ cells is shown on the left dot plot. Dot plots on the right show FoxP3 expression within gated $CD4^+CD25^+$ cells. Gate inside these dot plots correspond to $FoxP3^+$ cells. Dot plots are a representative example of each indicated group. Numbers inside dot plots correspond to mean percentage ± SEM of $FoxP3^+$ cells within $CD4^+CD25^+$ population. Statistically significant difference between EAMG ($n = 13$) and both naïve ($n = 9$) and control ($n = 13$) groups is indicated ($^{***}P < 0.001$; one-way ANOVA and Bonferroni's post hoc test). (c) Confocal microscopy micrographs showing type I and type II motor endplates from hemidiaphragm sections labeled with TMR-α-BTX *(red)* from naïve, control, and EAMG animals. Scale bar: 10 μm. (d) Reduction of contractile strength of isolated hemidiaphragm preparations from naïve, control, and EAMG animals during a 3 min period of intermittent phrenic nerve stimulation (17 pulses per second delivered at 50 Hz frequency). Results are mean ± SEM of 10 naïve, 6 control, and 5 EAMG rats. $^{*,**}P < 0.05$ (one-way ANOVA following Dunnett's modified t-test) compared to naïve and control animals, respectively.

effect seems to be specific, as the exacerbation of total serum ADA activity was observed only in EAMG, but not in control and naïve animals. In addition, EAMG animals exhibit a significant ($P < 0.001$) reduction in the relative proportion of $CD4^+CD25^+FoxP3^+$ T_{reg} cells among the $CD4^+CD25^+$ T cell population obtained by draining popliteal and inguinal lymph nodes ($64.50 \pm 1.64\%$, $n = 13$) as compared to both control ($72.80 \pm 0.77\%$, $n = 13$) and naïve ($72.27 \pm 0.50\%$, $n = 9$) animals (Figure 1(b)). These findings obtained by immunizing Wistar rats with the R97-116 peptide sequence of the nAChR α subunit are in agreement with those verified in the EAMG model induced in Lewis rats either with the Torpedo nAChR [24] or with the same rat R97-116 peptide [25], as well as in the human MG [22, 26]. No significant differences ($P > 0.05$) were found among the three animal groups in the percentage of total $CD25^+$ cells within the $CD4^+$ T cell population ($7.29 \pm 0.40\%$, $8.17 \pm 0.28\%$ and $8.56 \pm 0.46\%$ for naïve, control, and EAMG groups, resp.). Interestingly, long term administration of enzyme replacement therapy with pegylated bovine ADA (PEG-ADA) has been associated to manifestations of immune dysregulation including autoimmunity [27]. The increased turnover of ADO by ADA seems to interfere with the $CD4^+CD25^+FoxP3^+$ T_{reg} mediated control of immune responses since T_{reg} cells isolated from PEG-ADA-treated patients are reduced in number and show decreased activity [27].

Neuromuscular transmission failure in autoimmune MG results from an antibody attack to postsynaptic muscle nAChRs decreasing their number and causing a disorganization of receptor clusters at the motor endplate (reviewed in [1]). Therefore, we decided to evaluate the occurrence of similar morphological changes at diaphragm motor endplates of EAMG rats by immunofluorescence confocal microscopy. Previous studies from our and many other laboratories demonstrated that immunofluorescence labeling of postsynaptic α1-subunits of nAChR with α-bungarotoxin conjugated with tetramethylrhodamine was instrumental to evaluate histological modifications of the neuromuscular junction from myasthenic animals (see e.g., [7]). The diaphragm was chosen because it is a highly active skeletal muscle (duty cycle ~25–40%) of mixed fiber composition. Figure 1(c) shows typical motor endplates of slow (type I) and fast (type II) muscle fibers from naïve, control, and EAMG rats labeled with tetramethylrhodamine conjugated with α-bungarotoxin. Planar (two-dimensional) area measurements showed expected size differences. Those at type I diaphragm muscle fibers were smaller and with less exuberant postsynaptic folding compared to type II fibers. Motor endplates of EAMG animals exhibit significant morphological alterations as compared to naïve and control rats. These changes were similar to the ones observed in samples from MG patients [28]. Changes, which include significant ($P < 0.05$) reductions in the total area of nAChR labeling per endplate, were observed predominantly on type II fibers (Figure 1(c)). Data are in agreement with previous findings showing a reduction in the number of effective nAChR receptors. Most probably, autoantibodies present in EAMG animals bind to the nAChR to cause receptor internalization and degradation. The antibody-nAChR complex also binds to complement resulting in damage of the postsynaptic membrane, which typically has fewer secondary synaptic folds and a widened synaptic cleft that leads to loss of functional receptors (Figure 1(c); see e.g., [29]).

These morphological changes reduce the safety margin of neuromuscular transmission. Considering that the reduced skeletal muscle strength during repetitive nerve stimulation reflects the neuromuscular/immunological imbalance operating in EAMG, we performed myographic recordings using diaphragm preparations stimulated indirectly via the phrenic nerve trunk under fatigue conditions. These were produced by high-frequency (50 Hz) intermittent (17 pulses per sec, during 3 minutes) phrenic nerve stimulation [17]. Figure 1(d) shows that muscle fatigue was significantly ($P < 0.05$) more intense in EAMG animals than in both naïve and control littermates.

Overall these data suggest that immunization of rats with a single peptide fragment homologous to a region of the α-subunit of the nAChR leads to pathophysiological and clinical features observed in human MG. Thus, we are confident that the EAMG rat model might be extensively used to unravel the pathogenesis of MG and to explore novel therapeutic strategies to manage this disease [30].

3.2. Altered Expression of Ecto-5′-nucleotidase/CD73 in $CD4^+$ T Cell Subsets from Lymph Nodes of EAMG Rats. Adenosine formation from released adenine nucleotides via ecto-5′-nucleotidase/CD73 expressed on T_{reg} cells regulates the function of stimulated T cells through $A_{2A}R$ activation [14, 31]. In a recent study, Li et al. [8] reported a reduction in the expression of $A_{2A}R$ on both T and B cells residing in lymph nodes of EAMG animals. Here, we focus our attention on the distribution of ecto-5′-nucleotidase/CD73 in $CD25^-$, $CD25^+Foxp3^+$ and $CD25^+Foxp3^-$ T cells among the total $CD4^+$ T cells population obtained from inguinal and popliteal lymph nodes of EAMG animals as compared to their naïve and control littermates. Figure 2 shows a significant ($P < 0.05$) reduction in the proportion of $CD4^+CD25^+FoxP3^+$ T_{reg} cells expressing CD73 in popliteal and inguinal lymph nodes of EAMG animals (13.98 ± 1.44, $n = 5$) as compared to naïve (22.21 ± 0.81, $n = 5$) and control (20.23 ± 2.70, $n = 5$) groups. A decrease was also observed in the mean fluorescence intensity (MFI) due to CD73 staining on $CD4^+CD25^+FoxP3^+$ T_{reg} cells of the EAMG group (11.84 ± 0.63) as compared to the other assessed groups, both naïve (17.31 ± 0.51) and control ($13{,}29 \pm 1.47$) (Figure 2). In contrast, no significant differences were found in the MFI and in the proportion of CD73-expressing effector ($CD4^+CD25^+FoxP3^-$) T cells and nonactivated ($CD4^+CD25^-$) T cells, among all groups analyzed.

The decrease in the proportion of cells expressing ecto-5′-nucleotidase/CD73, within the $CD4^+CD25^+FoxP3^+$ T cell population isolated from the lymph nodes of myasthenic rats, might have functional repercussions given that the ecto-5′-nucleotidase/CD73 pathway is responsible for increasing the production of ADO by T_{reg} cells, which exerts an immunosuppressive action on activated T cells via $A_{2A}R$ activation [14, 31]. The reduction in ecto-5′-nucleotidase/CD73 expression in T_{reg} cells from EAMG rats suggests that the regulatory

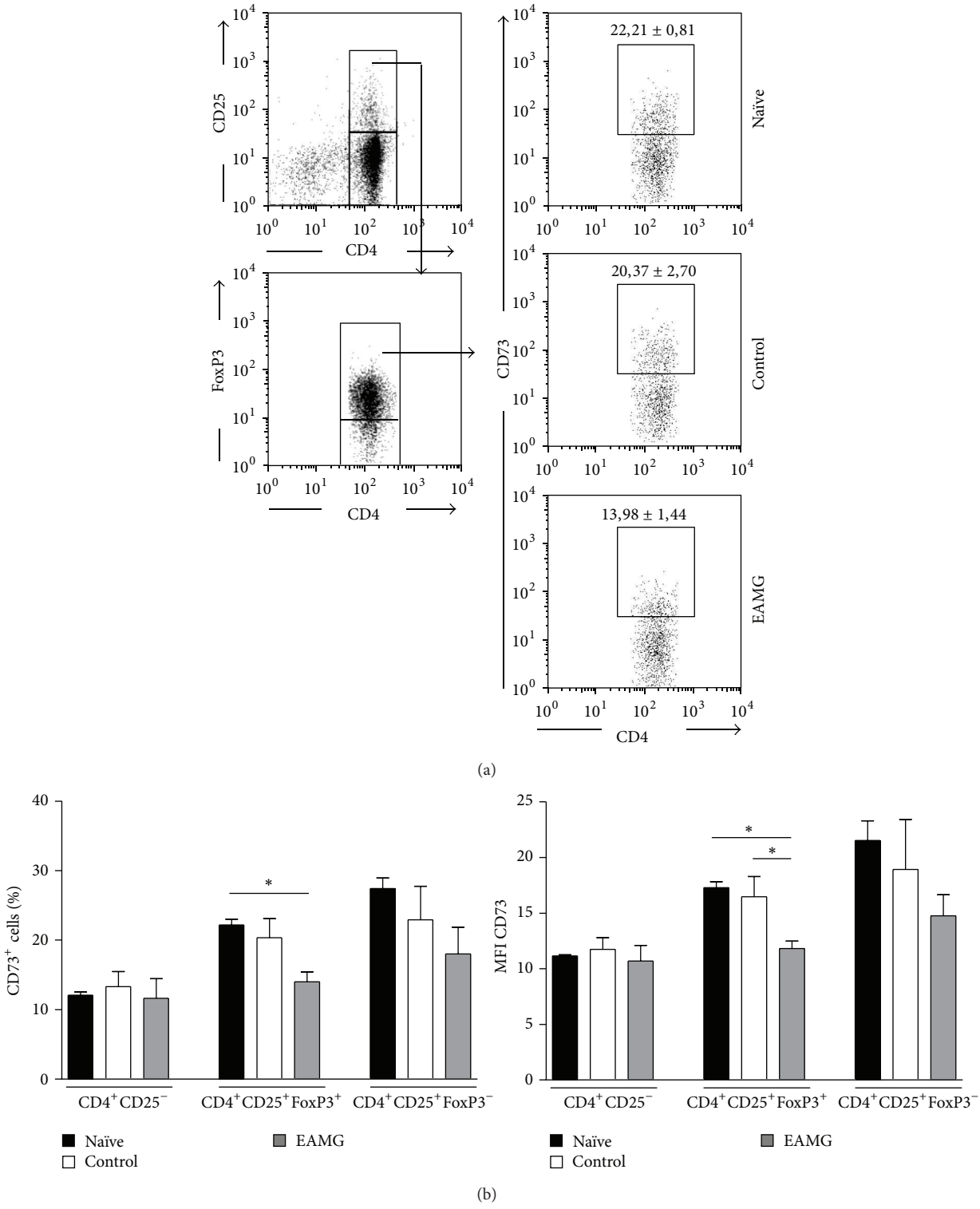


Figure 2: (a) Flow cytometry analysis of surface CD73 expression on $CD4^+CD25^+FoxP3^+$ T cells of popliteal and inguinal lymph nodes from naïve, control, and EAMG animals. Gating strategies are indicated on dot plots on the left. Dot plots on the right show CD73 expression within $CD4^+CD25^+FoxP3^+$ T cells and are a representative example of each indicated group. Numbers inside dot plots correspond to mean percentage ± SEM of $CD73^+$ cells. (b) Percentage of $CD73^+$ cells (left) and mean fluorescence intensity (MFI) due to CD73 (right) staining within the indicated cell populations. Bars represent means ± SEM of 5 experiments for each animal group. $^*P < 0.05$, $^{**}P < 0.01$ (one-way ANOVA and Bonferroni's post hoc test) compared to naïve and control animals, respectively.

loop of ADO accumulation in close proximity of these cells might be impaired. This was hypothesized because deficits in tonic $A_{2A}R$ activation of T_{reg} have been shown to decrease FoxP3 mRNA production [10] leading to insufficient expression of ecto-5′-nucleotidase/CD73 [32]. Indeed, Nessi et al. [33] put forward the hypothesis that the inability of $CD4^+CD25^+FoxP3^+$ T_{reg} to revert ongoing EAMG may be due to inadequate control of activated T cells leading to B-cell activation and differentiation into nAChR antibody-secreting plasma cells. In agreement with this hypothesis, we report here that incubation of $CD4^+$ T cells with ZM241385 (50 nM, a selective $A_{2A}R$ antagonist) or with ADA (0.5 U/mL, the enzyme that inactivates ADO into inosine) significantly ($P < 0.05$) enhanced cells proliferation, respectively, by 21 ± 3% ($n = 4$) and 18 ± 4% ($n = 4$) in control animals, but removal of the $A_{2A}R$ tonus by endogenous ADO failed to cause similar effects in cells from EAMG rats (data not shown). Moreover, T_{reg} cells inability to sustain relatively high concentration of ADO can be further aggravated by increased serum ADA activity [27].

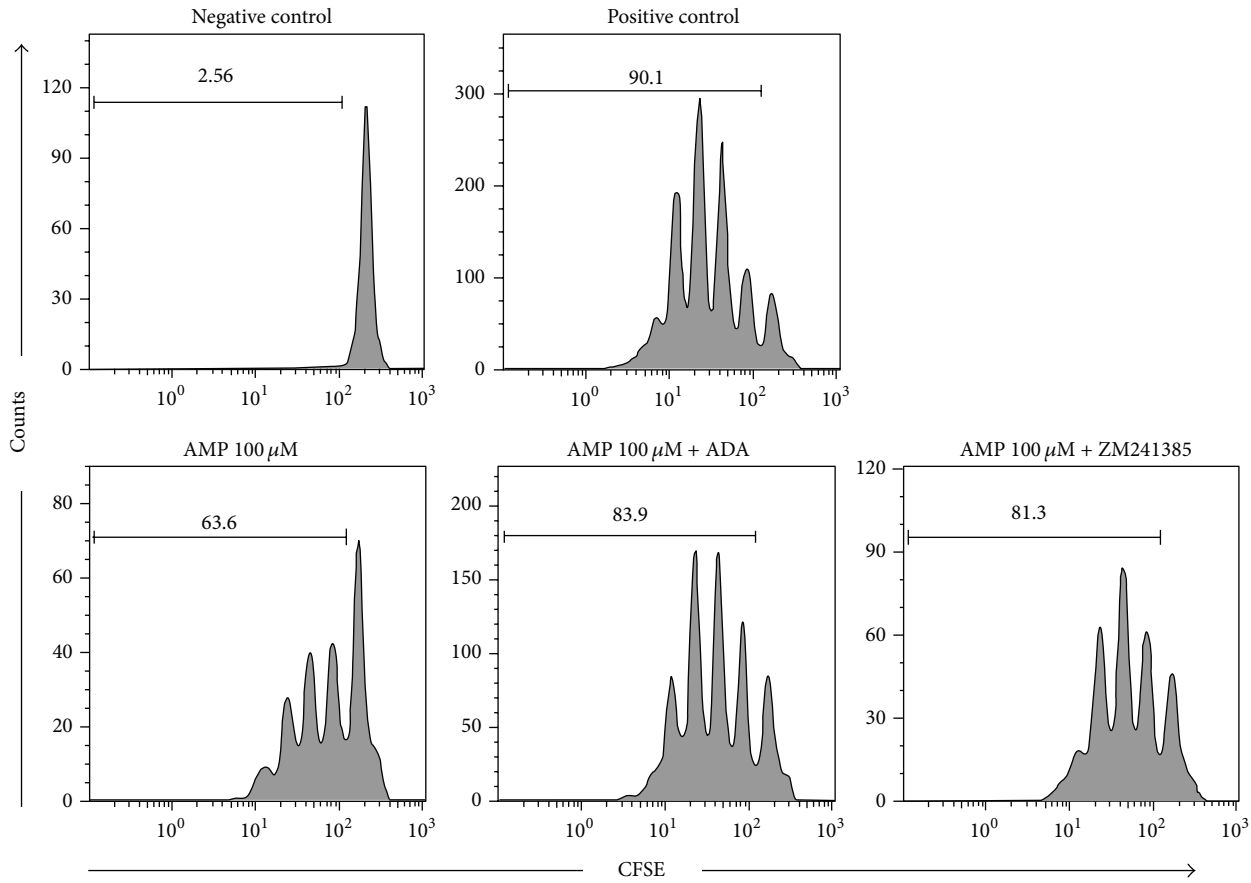
The recovery of extracellular ADO levels may be an attractive pharmacological therapy to restore the immunological competence in myasthenic animals. Data shown in Figure 3 demonstrate that $A_{2A}R$-mediated suppression of $CD4^+$ T cells proliferation can be rehabilitated almost to control levels when cells isolated from EAMG rats were supplemented with the nucleoside precursor, AMP (30–100 μM). That is, AMP (30–100 μM) concentration dependently suppressed proliferation of $CD4^+$ T cells isolated from both control and EAMG animals. The immune suppressive effect of AMP (30–100 μM) was significantly ($P < 0.05$) attenuated when it was applied together with ZM241385 (50 nM) or ADA (0.5 U/mL) (Figure 3), suggesting that the nucleotide has to be hydrolyzed into ADO, which acts via $A_{2A}R$ to suppress $CD4^+$ T-cells proliferation. Results show that activation of $A_{2A}R$ rehabilitated by AMP dephosphorylation into ADO, via ecto-5′-nucleotidase/CD73, can restore immune competence in EAMG rats, strengthening the hypothesis that immune suppression deficits reside on low ADO generation from released adenine nucleotides to levels below those required to tonically activate $A_{2A}R$.

3.3. Recovery of the Facilitatory $A_{2A}R$-Mediated Tonus by ADO Generated via Ecto-5′-nucleotidase/CD73 at the Motor Endplate of EAMG Rat. The presence of $A_{2A}R$ at the motor endplate of naive rats was demonstrated by immunofluorescence confocal microscopy using two distinct commercially available antibodies (Figure 4, for details see Materials and Methods). The antibody from Alpha Diagnostics International Inc. (A2aR21-A) is directed against a 30-amino-acid peptide (A2aR21-P) in the intracellular C-terminus of the canine $A_{2A}R$, which is only 43% conserved in the rat $A_{2A}R$ (see http://www.4adi.com/objects/catalog/product/extras/A2aR21-S-A-P.pdf). Although it has been shown to cross-react with the rat $A_{2A}R$, we are aware that specificity has to be proven in transfected cells or in knock-out animals. For this reason, we used an alternative antibody from Chemicon (05-717), which was designed to recognize an epitope in the third intracellular loop of the human recombinant $A_{2A}R$ (Clone 7F6-G5-A2) while cross-reacting significantly with the rat receptor (see e.g., [34]); immunostaining with this antibody was abrogated in the striatum of the $A_{2A}R$ knock-out mouse (see http://www.emdmillipore.com/PT/en/product/Anti-Adenosine-Receptor-A2a-Antibody%2C-clone-7F6-G5-A2,MM_NF-05-717). Data show that the immunostaining pattern, with both antibodies, A2aR21-A and 05-717, was quite similar. Preadsorption with the peptide A2aR21-P abrogated staining with the A2aR21-A antibody while keeping the same acquisition settings on the confocal microscope. Taking this into account and the requirements for colocalization tests conducted in parallel, whose results are beyond the scope of this study, we continued the experiments with the antibody from Alpha Diagnostics International Inc. (A2aR21-A).

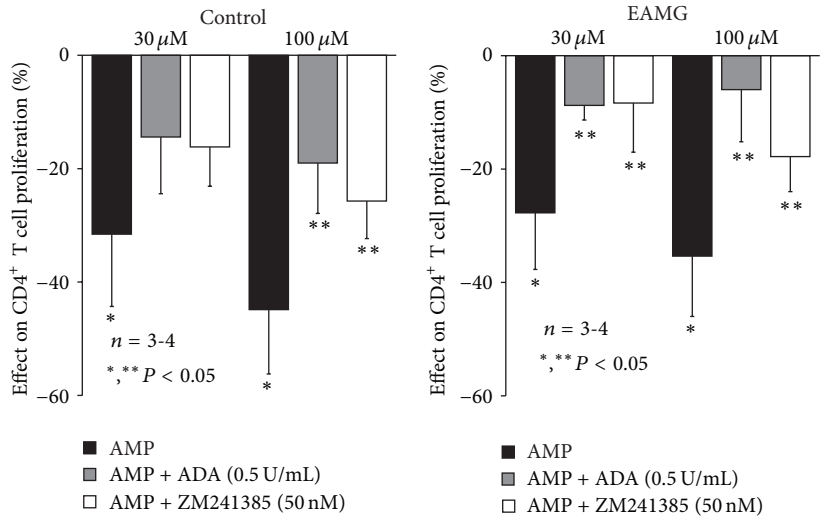
Figure 4(a) shows that immunoreactivity against $A_{2A}R$ is located predominantly on nerve axons and presynaptic buttons in close opposition to the motor endplate region, which is highly enriched in nAChRs labeled with α-bungarotoxin conjugated with tetramethylrhodamine. We found no evidence to suggest localization of $A_{2A}R$ in skeletal muscle fibers. No clear distinction was observed on the $A_{2A}R$ immunoreactivity pattern between the three animal groups, naïve, control, and EAMG (Figure 4(a)). Despite this, discrete changes in the expression of $A_{2A}R$ in EAMG rats cannot be ruled out and deserve further attention.

Using a neurochemical approach, we evaluated the $A_{2A}R$-mediated tonus on [^{3}H]-ACh release from hemidiaphragm preparations indirectly stimulated via the phrenic nerve trunk with 750 supramaximal repetitive pulses delivered with frequency of 5 Hz (Figures 4(b) and 4(c)). Selective blockade of $A_{2A}R$ with ZM241385 (50 nM) decreased nerve-evoked [^{3}H]-ACh release roughly by 30% in both naïve and control rats (Figures 4(b) and 4(c)), indicating that endogenous ADO exerts a predominant facilitatory tonus, via the activation of $A_{2A}R$, on neuromuscular transmission (see e.g., [4, 12]). Conversely, ZM241385 (50 nM) failed to decrease the evoked [^{3}H]-ACh release in EAMG animals (Figure 4(c)). Data from confocal microscopy and neurochemical studies suggest that despite the fact that $A_{2A}R$s are present on motor nerve terminals of EAMG animals (Figure 4(a)), activation of these receptors by endogenously generated ADO is significantly impaired (Figures 4(b) and 4(c)).

Previous studies from our group demonstrated that amplification of transmitter release caused by $A_{2A}R$ becomes evident at high levels of synaptic ADO accumulation [12, 35]. Therefore, one may hypothesize that deficits in ADO accumulation at the synaptic cleft may contribute to the loss of ADO neurofacilitation in myasthenic animals. Figure 5(a) shows that EAMG animals accumulate smaller amounts of ADO (1.25 ± 0.18 nM/mg of tissue, $n = 13$) following phrenic nerve stimulation compared to control animals (2.45 ± 0.45 nM/mg of tissue, $n = 13$). No changes were observed between groups regarding the baseline levels of ADO (Figure 5(b)).



(a)



(b)

Figure 3: (a) Flow cytometric evaluation of plated anti-CD3 and soluble anti-CD28 mAbs (1 μg/mL) induced proliferative response of 5×10^4 CFSE-labelled $CD4^+$T cells, sorted from popliteal and inguinal lymph nodes of a control animal, cultured for 3 days in the absence (positive control) or presence of AMP (100 μM), AMP (100 μM) plus ADA (0,5 U/mL), or AMP (100 μM) plus ZM241385 (50 nM), as indicated. Negative control corresponds to unstimulated cells (no mAbs added). Numbers within histograms correspond to the percentage of cells that divided at least once. Results shown are a representative example of 3 to 4 independent experiments performed in different animals. (b) AMP induced inhibition of $CD4^+$ T cell proliferation obtained from popliteal and inguinal lymph nodes from control and EAMG animals. Bars represent means ± SEM of 3 control and 4 EAMG animals. $^*P < 0.05$ compared to the absence of AMP and $^{**}P < 0.05$ compared to the AMP effect (one-way ANOVA and Bonferroni's post hoc test).

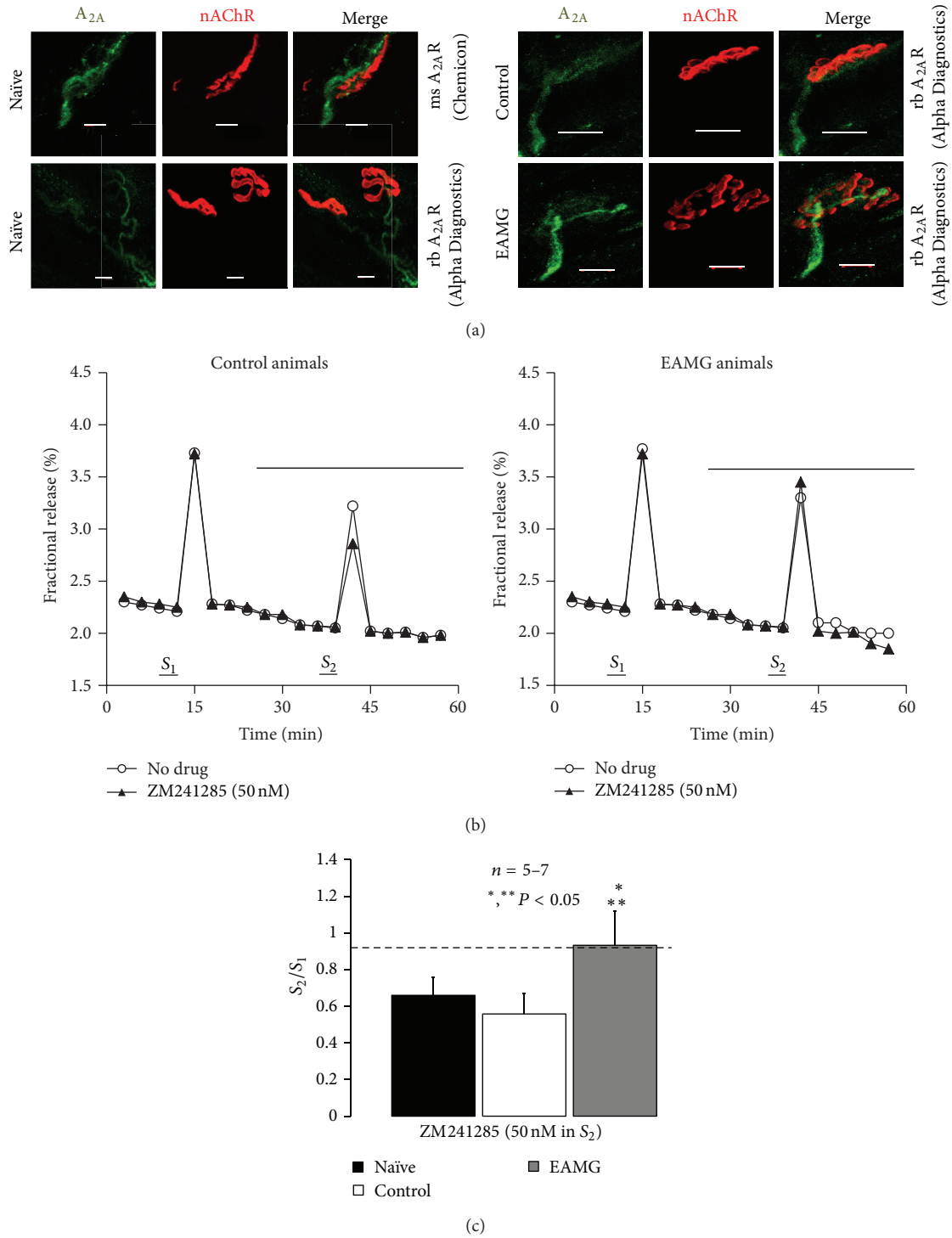


Figure 4: Tonic activation of $A_{2A}R$ is significantly impaired at motor endplates of myasthenic rats. (a) Confocal micrographs showing immunoreactivity against $A_{2A}R$ (*green*) on motor endplates from rat hemidiaphragms labeled with TMR-α-BTX (*red*) from naïve, control, and EAMG rats. Scale bar: 10 μm. Two distinct $A_{2A}R$ antibodies, AlphaDiagnostics (A2aR21-P) and Chemicon (05-717, Clone 7F6-G5-A2), were used was indicated. (b) Time course of tritium outflow from phrenic nerve terminals from control and EAMG animals taken from typical experiments in the absence (no drug, open circles) and in the presence of the selective $A_{2A}R$ antagonist, ZM241285 (50 nM) (filled triangles). [^{3}H]-ACh release was elicited by stimulating the phrenic nerve trunk with 750 pulses delivered with a frequency of 5 Hz at the indicated times (S_1 and S_2). ZM241285 (50 nM) was applied 15 min before S_2. (c) Modification of the S_2/S_1 ratio caused by ZM241285 (50 nM) in naïve, control, and EAMG rats. Each column represents pooled data from five (naïve and control) and seven (EAMG) animals. The vertical bars represent mean ± SEM. $^{*,**}P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared to naïve and control (CFA) rats.

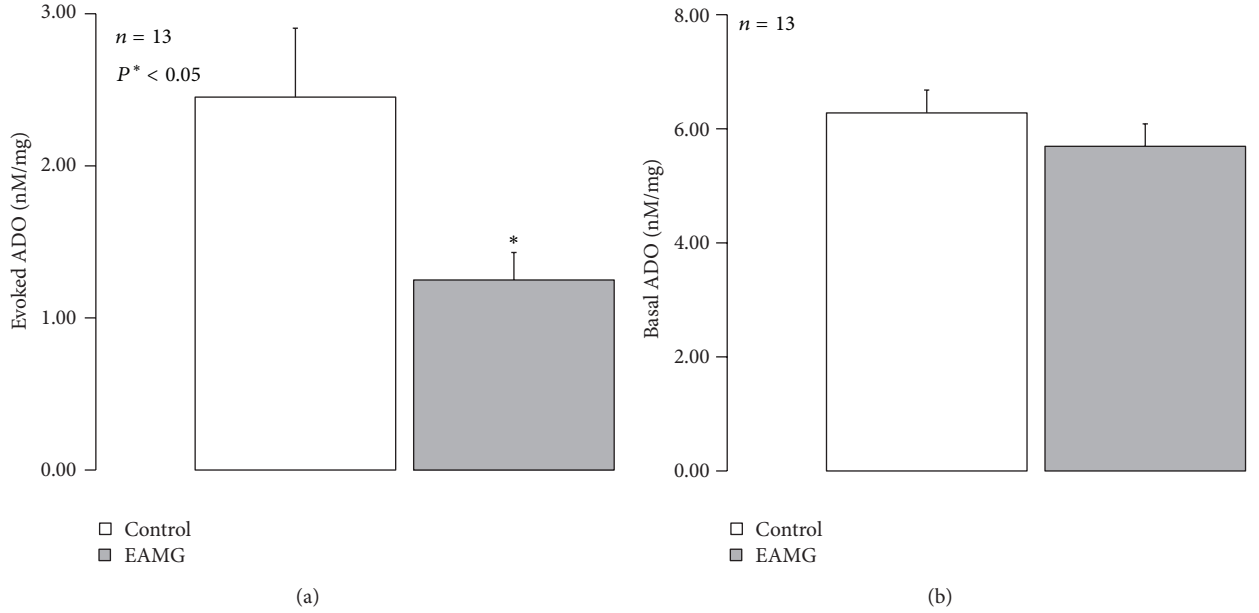


Figure 5: The amount ADO released upon phrenic nerve stimulation is lower in EAMG animals. The ordinates represent the (a) evoked release of ADO upon phrenic nerve trunk electrical stimulation (750 pulses applied at 5 Hz frequency) and (b) basal ADO quantified by HPLC(diode array detection). Nerve-evoked release of ADO was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenosine determined after stimulus application. The data are means ± S.E.M. of 13 animals of each group (naïve, control, and EAMG).

Considering that ADO originating from the catabolism of released adenine nucleotides together with ACh preferentially activates facilitatory $A_{2A}R$ on nerve terminals [11, 12] and knowing the regulatory potential of ecto-5′-nucleotidase/CD73 for adenosine formation, we thought it was important to compare the activity of this enzyme at the skeletal neuromuscular junction of naïve, control, and EAMG animals by assessing the time course of the extracellular catabolism of AMP (30 μM) (Figure 6(a)). The kinetics of the extracellular catabolism of AMP (30 μM) and ADO formation in EAMG animals was very similar to that observed in control rats (Figures 6(a) and 6(b), resp.). No significant changes ($P > 0.05$) were detected when comparing the half degradation time of extracellular AMP (30 μM) in EAMG (22 ± 3 min, $n = 6$), control (26 ± 1 min, $n = 6$), and naïve (26 ± 3 min, $n = 4$) rats (cf. [13]).

Given the similarity of extracellular adenosine formation via ecto-5′-nucleotidase/CD73 among the three animal groups, we tested whether AMP could restore the ADO tonus required to sustain transmitter release from stimulated motor nerve terminals of EAMG rats, as we observed in the toxicological MG rat model [7]. Figure 6(c) shows that exogenously added AMP (100 μM) consistently enhanced nerve-evoked [^{3}H]-ACh release from hemidiaphragm preparations of EAMG animals (28 ± 7%, $n = 7$) by a similar amount to that obtained in naïve (31 ± 5%, $n = 4$) and control (29 ± 8%, $n = 4$) muscles. Pretreatment with ADA (0.5 U/mL), the enzyme, that inactivates ADO into INO, prevented the facilitatory effect of AMP (100 μM) on both control (−4 ± 6%, $n = 4$) and EAMG (10±9%, $n = 4$) animals. Likewise, selective blockade of $A_{2A}R$ with ZM241285 (50 nM) also attenuated AMP-induced facilitation of evoked [^{3}H]-ACh release in control (9 ± 5%, $n = 4$) and EAMG (15 ± 3%, $n = 4$) animals (data not shown). These results indicate that the facilitatory effect of AMP (100 μM) on evoked transmitter release requires its conversion, into ADO and, subsequent, activation of $A_{2A}R$.

4. Conclusion

ADO is an extracellular signaling nucleoside that has a unique dynamic role in the regulation of synaptic neurotransmission [4, 6] and immunosuppressive [5, 9, 10] responses, via the activation of $A_{2A}R$. Ecto-5′-nucleotidase/CD73 is the rate limiting enzyme for ADO production from released adenine nucleotides, which plays a strategic role in calibrating the duration and magnitude of the purinergic signal delivered to immune cells [32] and to the motor endplate [11]. The study of adenosinergic-based therapies acting on A2AR and CD73 urges in order to provide novel strategies oriented simultaenously to suppress immune responses and to promote neuromuscular transmission. In this work we gathered information of the immunological and neuronal imbalance related to the adenosinergic pathway in a rat model of EAMG (see Figure 7), which compares to the human MG in both pathophysiological and clinical features.

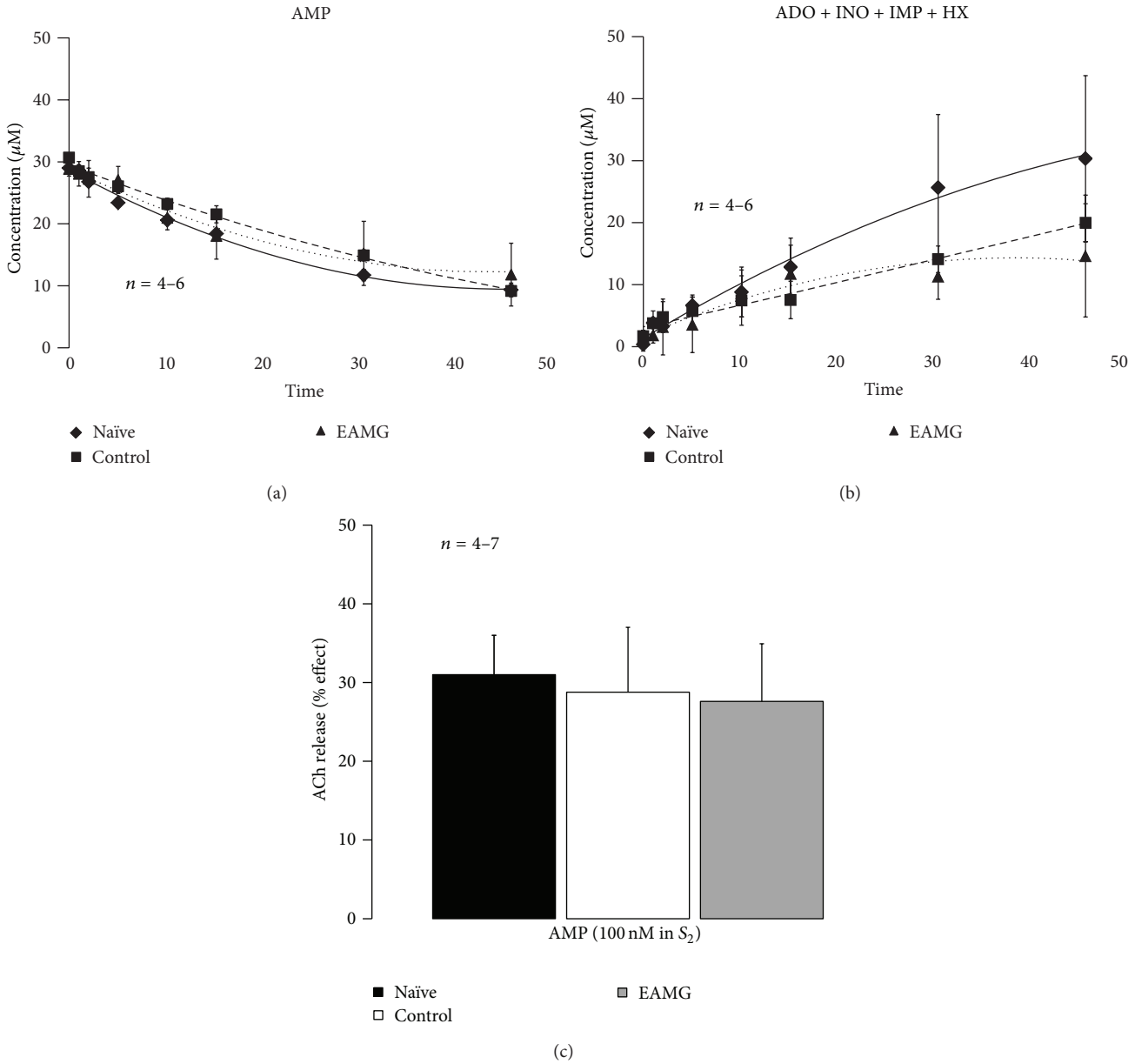


Figure 6: Dephosphorylation of AMP, to ADO via ecto-5′-nucleotidase/CD73, facilitates the release of [^{3}H]-ACh from stimulated phrenic motor nerve terminals of naïve, control, and EAMG rats. (a) and (b) show the kinetics of the extracellular AMP (30 μM) catabolism (a) and formation of ADO plus nucleoside derivatives (inosine (INO) and hypoxanthine (HX)) (b) in hemidiaphragm preparations from naïve, control, and EAMG rats. AMP (30 μM) was added to the preparation at zero time; samples were collected from the bath at indicated times on the abscissa and retained for HPLC analysis. Data shown are averages pooled from 4 naïve, 6 control, and 6 EAMG animals. The vertical bars represent SEM and are shown when they exceed the symbols in size. (c) Facilitatory effects of AMP (100 μM) on evoked [^{3}H]-ACh release (5 Hz, 750 pulses, S_1 and S_2) from hemidiaphragm preparations from naïve, control, and EAMG rats. AMP (100 μM) was applied 15 min before S_2. Each column represents pooled data from 4 (naïve and control) and 7 (EAMG) animals. The vertical bars represent mean ± SEM.

To our knowledge, this is the first attempt to tackle common deficits affecting unbalanced neuromuscular transmission and autoimmune responses in a EAMG model. Insufficient amounts of adenosine to promote immune cells communication and neuromuscular transmission via $A_{2A}R$ activation seem to be operating in EAMG animals, yet the underlying mechanisms responsible for these findings seem to be diverse in the two systems. Deficits of ADO generation on immune cells are mainly dictated by a decreased expression of the ADO generating enzyme, ecto-5′-nucleotidase/CD73, on T_{reg} cells, which may be further aggravated by a decreased expression of $A_{2A}R$ on B and T cells [8]. The coordination, between increased serum ADA activities (Figure 1(a)) with the inability of T_{reg} cells to maintain high extracellular levels of ADO coming from adenine nucleotides (Figure 2), relieves suppression of specific nAChR-T lymphocytes leading to

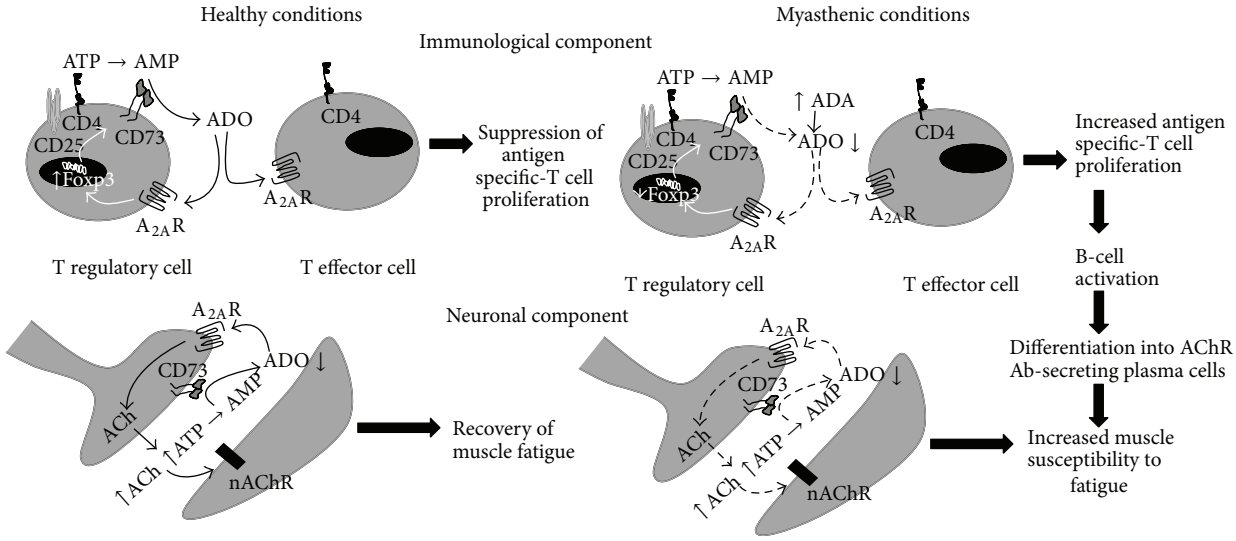


Figure 7: Participation of the adenosinergic system on neuroimmunological deficits present in EAMG rats. In healthy animals, $A_{2A}R$ activation by ADO generated from the catabolism of released nucleotides via ecto-5′-nucleotidase/CD73 downmodulates T effector ($CD4^+CD25^+FoxP3^-$) cells proliferation in response to specific antigens by increasing the activity of T_{reg} ($CD4^+CD25^+FoxP3^+$) cells expressing FoxP3-dependent gene products, like ecto-5′-nucleotidase/CD73. At the neuromuscular junction, ecto-5′-nucleotidase/CD73 activity leads to the formation of ADO from released ATP (from both nerve and muscle), which facilitates acetylcholine release via prejunctional $A_{2A}R$ activation that is necessary to resist tetanic depression. In EAMG rats, increases in serum adenosine deaminase (ADA) together with ecto-5′-nucleotidase/CD73 in T_{reg} ($CD4^+CD25^+FoxP3^+$) cells lead to insufficient amounts of extracellular ADO. The lack of the $A_{2A}R$ immunosuppressive tonus contributes to the loss of peripheral tolerance to nAChR. Thus, increases in the proliferation of antigen-specific T effector cells triggers B cells differentiation into plasma cells and secretion of antibodies directed towards motor endplates nAChR clusters. This antibody attack leads to nAChR internalization/degradation and to complement-mediated morphological changes of the myasthenic postsynaptic membrane (e.g., fewer secondary synaptic folds, widening of the synaptic cleft). These changes contribute to neuromuscular transmission failure, which is further aggravated by deficits in the production of extracellular ADO, probably from released adenine nucleotides, namely, ATP. Impairment of tonic $A_{2A}R$-mediated facilitation of transmitter Impairment of tonic $A_{2A}R$-mediated facilitation of transmitter release turns myasthenic skeletal muscles unable to resist fatigue.

cell proliferation and to antigen-specific B cells activation in myasthenic animals (Figure 7). Regarding the motor endplate the lack of $A_{2A}R$ activity does not seem to be related to changes in the ability of ecto-5′-nucleotidase/CD73 to metabolize adenine nucleotides leading to ADO formation (Figure 6(a)) or to deficits in the $A_{2A}R$ receptor expression on motor nerve terminals (Figure 4(a)). The autoantibody attack on postsynaptic nAChR may compromise predominantly the retrograde release of ADO (Figure 5(a)) and/or its precursor, ATP, from affected skeletal muscle fibers, leading to a functional loss of $A_{2A}R$-mediated facilitation of ACh release and to neuromuscular transmission failure in EAMG rats.

Highly incapacitating neuromuscular transmission deficits associated with MG, namely, muscle weakness and fatigability, may result from an attack of the complement system on antibody-nAChR complexes bound to the motor endplate leading to its disorganization and nAChR loss [30]. In this work, we extended the mechanisms associated to neuromuscular transmission failure in EAMG by implicating deficits in the ADO pathway. Like that observed with the toxin-induced MG animal model [7], data presented in this study indicates that endogenous adenosine generated in myasthenic motor endplates during repetitive nerve firing (Figure 5(a)) may be insufficient to preserve transmitter release during repetitive neuronal firing via tonic activation of presynaptic facilitatory $A_{2A}R$ (Figure 4(c)). The conjunction of these findings with the data already presented in the bungarotoxin-induced MG rat model, where no evidence of significant damage of muscle integrity have been reported [7, 36] suggests that a decreased accumulation of adenosine leading to insufficient tonic activity of $A_{2A}R$ on motor endplates of myasthenic animals may be linked to muscle paralysis caused by the loss of nAChR, instead of the immune mediated disruption of endplate morphology. Muscle paralysis with μ-conotoxin GIIIB, a toxin that blocks muscle-specific voltage-gated Na^+ channels without affecting neuronal function [37], decreased nerve-evoked ATP (~15%) and ADO (>90%) outflow [7]. Moderate-to-severe MG patients have impaired oxidative metabolism and a noticeable shift to glycolytic metabolism during exercise, which yields to higher-end Pi/ATP ratio and reduced levels of synaptic ADO levels [38]. Besides the fact that exocytosis of ATP may occur synchronously with ACh in a frequency-dependent manner [13, 39], the nucleotide may also be released by tetanic stimulation of skeletal myotubes from healthy animals, through pannexin-1 hemichannels, within a 15 s to 3 min time scale [40]. Taken together, these findings imply that ATP and the end product of the ectonucleotidase cascade, ADO, might be considered

essential retrograde mediators between muscle activity and synaptic adaptations, a situation that may be deregulated under pathological conditions, like the EAMG. At this point, one cannot exclude deficiencies in the conversion of released adenine nucleotides into ADO (by ecto-NTPDases) upstream the ecto-5′-nucleotidase/CD73, which may concur to explain the lack of ADO tone regulating neurotransmitter release at the motor endplate. This hypothesis certainly deserves further studies in the near future.

Our results show for the first time that immune suppression and neuromuscular transmission deficits in EAMG animals may be rehabilitated by $A_{2A}R$ activation, which can be achieved by shortcutting ecto-NTPDases with exogenous AMP serving as an ADO precursor (see Figures 3 and 6). Thus, maintenance of ecto-5′-nucleotidase/CD73 activity may be crucial to define the pattern of extracellular ATP-derived ADO formation favoring $A_{2A}R$ activation in order to rehabilitate cell communication deficits in myasthenics. Li and collaborators have already showed that administration of the $A_{2A}R$ agonist, CGS21680C, 29 days post EAMG induction (therapeutic treatment) ameliorated disease severity and decreased the number of Th1 and Th2 cells while increasing the number of Treg cells [8], thus suggesting that targeting $A_{2A}R$ may have putative therapeutic applications in T cell-based autoimmune diseases. The potential significant side effects of chronic systemic use of drugs acting on $A_{2A}R$, as well as the residual action of the $A_{2A}R$ ligands on other ADO receptor subtypes, pushed forward a new era of drugs designed for local augmentation of the nucleoside function dictated by the proximity of ecto-5′-nucleotidase/CD73 [41, 42]. In this context, ecto-5′-nucleotidase/CD73 may assume a pivotal role as a pharmacological target to fine tune $A_{2A}R$ activity. The time-space coincidence expression of both ecto-5′-nucleotidase/CD73 and $A_{2A}R$ receptors offers new appealing therapeutic targets for immunotherapy and neuromuscular transmission reinforcement of myasthenia with minimal side effects.

Abbreviations

EAMG:	Experimental autoimmune *Myasthenia Gravis*
MG:	*Myasthenia Gravis*
nAChR:	Nicotinic acetylcholine receptor
$A_{2A}R$:	Adenosine A_{2A} receptor
AMP:	Adenosine monophosphate
ADO:	Adenosine
ADA:	Adenosine Deaminase
CFA:	Complete Freund adjuvant
IFA:	Incomplete Freund adjuvant
FoxP3:	Forkhead transcription factor 3
T_{reg}:	Regulatory T cells
LN:	Lymph nodes
TMR-α-BTX:	Tetramethylrhodamine conjugated with α-bungarotoxin
PEG-ADA:	Pegylated bovine ADA, rb, rabbit, and ms, mouse.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] J. M. Lindstrom, "Acetylcholine receptors and myasthenia," *Muscle & Nerve*, vol. 23, no. 4, pp. 453–477, 2000.

[2] B. M. Conti-Fine, M. Milani, and H. J. Kaminski, "Myasthenia gravis: past, present, and future," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 2843–2854, 2006.

[3] N. P. Hirsch, "Neuromuscular junction in health and disease," *British Journal of Anaesthesia*, vol. 99, no. 1, pp. 132–138, 2007.

[4] P. Correia-de-Sá, A. M. Sebastião, and J. A. Ribeiro, "Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat," *British Journal of Pharmacology*, vol. 103, no. 2, pp. 1614–1620, 1991.

[5] B. Csóka, L. Himer, Z. Selmeczy et al., "Adenosine A_{2A} receptor activation inhibits T helper 1 and T helper 2 cell development and effector function," *The FASEB Journal*, vol. 22, no. 10, pp. 3491–3499, 2008.

[6] L. Oliveira, M. A. Timóteo, and P. Correia-de-Sá, "Tetanic depression is overcome by tonic adenosine A2A receptor facilitation of L-type Ca^{2+} influx into rat motor nerve terminals," *Journal of Physiology*, vol. 560, no. 1, pp. 157–168, 2004.

[7] J. B. Noronha-Matos, T. Morais, D. Trigo et al., "Tetanic failure due to decreased endogenous adenosine A_{2A} tonus operating neuronal Ca_v1 (L-type) influx in *Myasthenia gravis*," *Journal of Neurochemistry*, vol. 117, no. 5, pp. 797–811, 2011.

[8] N. Li, L. Mu, J. Wang et al., "Activation of the adenosine A2A receptor attenuates experimental autoimmune myasthenia gravis severity," *European Journal of Immunology*, vol. 42, no. 5, pp. 1140–1151, 2012.

[9] S. Huang, S. Apasov, M. Koshiba, and M. Sitkovsky, "Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion," *Blood*, vol. 90, no. 4, pp. 1600–1610, 1997.

[10] P. E. Zarek, C.-T. Huang, E. R. Lutz et al., "A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells," *Blood*, vol. 111, no. 1, pp. 251–259, 2008.

[11] R. A. Cunha, P. Correia-de-Sá, A. M. Sebastião, and J. A. Ribeiro, "Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides," *British Journal of Pharmacology*, vol. 119, no. 2, pp. 253–260, 1996.

[12] P. Correia-de-Sá, M. A. Timóteo, and J. A. Ribeiro, "Presynaptic A1 inhibitory/A(2A) facilitatory adenosine receptor activation

balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm," *Journal of Neurophysiology*, vol. 76, no. 6, pp. 3910–3919, 1996.

[13] M. T. Magalhães-Cardoso, M. F. Pereira, L. Oliveira, J. A. Ribeiro, R. A. Cunha, and P. Correia-de-Sá, "Ecto-AMP deaminase blunts the ATP-derived adenosine A2A receptor facilitation of acetylcholine release at rat motor nerve endings," *Journal of Physiology*, vol. 549, no. 2, pp. 399–408, 2003.

[14] G. Borsellino, M. Kleinewietfeld, D. Di Mitri et al., "Expression of ectonucleotidase CD39 by Foxp3$^+$ Treg cells: hydrolysis of extracellular ATP and immune suppression," *Blood*, vol. 110, no. 4, pp. 1225–1232, 2007.

[15] F. Baggi, A. Annoni, F. Ubiali et al., "Breakdown of tolerance to a self-peptide of acetylcholine receptor α-subunit induces experimental myasthenia gravis in rats," *Journal of Immunology*, vol. 172, no. 4, pp. 2697–2703, 2004.

[16] G. Giusti, "Adenosine deaminase," in *Methods of Enzymatic Analysis*, H. U. Bergmeyer, Ed., pp. 1092–1099, Academic Press, New York, NY, USA, 1974.

[17] E. Van Lunteren, M. Moyer, and H. J. Kaminski, "Adverse effects of myasthenia gravis on rat phrenic diaphragm contractile performance," *Journal of Applied Physiology*, vol. 97, no. 3, pp. 895–901, 2004.

[18] A. R. Pinheiro, D. Paramos-de-Carvalho, M. Certal et al., "Histamine induces ATP release from human subcutaneous fibroblasts, via pannexin-1 hemichannels, leading to Ca^{2+} mobilization and cell proliferation," *The Journal of Biological Chemistry*, vol. 288, no. 38, pp. 27571–27583, 2013.

[19] M. Fausther, J. Lecka, E. Soliman et al., "Coexpression of ecto-5′-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 302, no. 4, pp. G447–G459, 2012.

[20] H. Link and B. G. Xiao, "Rat models as tool to develop new immunotherapies," *Immunological Reviews*, vol. 184, pp. 117–128, 2001.

[21] S. Chiba, M. Saitoh, M. Kashiwagi, N. Kobayashi, and H. Matsumoto, "Isozyme analysis of the high serum adenosine deaminase activity in patients with myasthenia gravis," *Internal Medicine*, vol. 34, no. 2, pp. 81–84, 1995.

[22] Y. Zhang, H.-B. Wang, L.-J. Chi, and W.-Z. Wang, "The role of FoxP3$^+$CD4$^+$CD25^{hi} Tregs in the pathogenesis of myasthenia gravis," *Immunology Letters*, vol. 122, no. 1, pp. 52–57, 2009.

[23] A. Shore, H.-M. Dosch, and E. W. Gelfand, "Role of adenosine deaminase in the early stages of precursor T cell maturation," *Clinical and Experimental Immunology*, vol. 44, no. 1, pp. 152–155, 1981.

[24] S. Gertel-Lapter, K. Mizrachi, S. Berrih-Aknin, S. Fuchs, and M. C. Souroujon, "Impairment of regulatory T cells in myasthenia gravis: Studies in an experimental model," *Autoimmunity Reviews*, vol. 12, no. 9, pp. 894–903, 2013.

[25] L. Mu, B. Sun, Q. Kong et al., "Disequilibrium of T helper type 1, 2 and 17 cells and regulatory T cells during the development of experimental autoimmune myasthenia gravis," *Immunology*, vol. 128, no. 1, pp. e826–e836, 2009.

[26] A. Balandina, S. Lécart, P. Dartevelle, A. Saoudi, and S. Berrih-Aknin, "Functional defect of regulatory CD4$^+$CD25$^+$ T cells in the thymus of patients with autoimmune myasthenia gravis," *Blood*, vol. 105, no. 2, pp. 735–741, 2005.

[27] A. V. Sauer, I. Brigida, N. Carriglio et al., "Alterations in the adenosine metabolism and CD39/CD73 adenosinergic machinery cause loss of Treg cell function and autoimmunity in ADA-deficient SCID," *Blood*, vol. 119, no. 6, pp. 1428–1439, 2012.

[28] V. Macdermot, "The changes in the motor end-plate in myasthenia gravis," *Brain*, vol. 83, no. 1, pp. 24–36, 1960.

[29] V. C. Juel and J. M. Massey, "Myasthenia gravis," *Orphanet Journal of Rare Diseases*, vol. 2, no. 1, article 44, 2007.

[30] E. Tüzün and P. Christadoss, "Unraveling myasthenia gravis immunopathogenesis using animal models," *Drug Discovery Today: Disease Models*, vol. 3, no. 1, pp. 15–20, 2006.

[31] M. Mandapathil, B. Hilldorfer, M. J. Szczepanski et al., "Generation and accumulation of immunosuppressive adenosine by human CD4$^+$CD25^{high}FOXP3$^+$ regulatory T Cells," *The Journal of Biological Chemistry*, vol. 285, no. 10, pp. 7176–7186, 2010.

[32] S. Deaglio, K. M. Dwyer, W. Gao et al., "Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression," *Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1257–1265, 2007.

[33] V. Nessi, S. Nava, C. Ruocco et al., "Naturally occurring CD4$^+$CD25$^+$ regulatory T cells prevent but do not improve experimental myasthenia gravis," *Journal of Immunology*, vol. 185, no. 9, pp. 5656–5667, 2010.

[34] C. Vieira, F. Ferreirinha, I. Silva, M. Duarte-Araújo, and P. Correia-de-Sá, "Localization and function of adenosine receptor subtypes at the longitudinal muscle—myenteric plexus of the rat ileum," *Neurochemistry International*, vol. 59, no. 7, pp. 1043–1055, 2011.

[35] P. Correia-de-Sá and J. A. Ribeiro, "Adenosine uptake and deamination regulate tonic A_{2a} receptor facilitation of evoked [^{3}H]acetylcholine release from the rat motor nerve terminals," *Neuroscience*, vol. 73, no. 1, pp. 85–92, 1996.

[36] J. J. Plomp, G. T. van Kempen, and P. C. Molenaar, "Adaptation of quantal content to decreased postsynaptic sensitivity at single endplates in α-bungarotoxin-treated rats," *Journal of Physiology*, vol. 458, pp. 487–499, 1992.

[37] M. Faria, L. Oliveira, M. A. Timóteo, M. G. Lobo, and P. Correia-de-Sá, "Blockade of neuronal facilitatory nicotinic receptors containing $\alpha 3\beta 2$ subunits contribute to tetanic fade in the rat isolated diaphragm," *Synapse*, vol. 49, no. 2, pp. 77–88, 2003.

[38] S.-F. Ko, C.-C. Huang, M.-J. Hsieh et al., "31P MR spectroscopic assessment of muscle in patients with myasthenia gravis before and after thymectomy: initial experience," *Radiology*, vol. 247, no. 1, pp. 162–169, 2008.

[39] D. O. Smith, "Sources of adenosine released during neuromuscular transmission in the rat," *Journal of Physiology*, vol. 432, pp. 343–354, 1991.

[40] S. Buvinic, G. Almarza, M. Bustamante et al., "ATP released by electrical stimuli elicits calcium transients and gene expression in skeletal muscle," *Journal of Biological Chemistry*, vol. 284, no. 50, pp. 34490–34505, 2009.

[41] A. El-Tayeb, J. Iqbal, A. Behrenswerth et al., "Nucleoside-5′-monophosphates as prodrugs of adenosine A2A receptor agonists activated by ecto-5′-nucleotidase," *Journal of Medicinal Chemistry*, vol. 52, no. 23, pp. 7669–7677, 2009.

[42] U. Flögel, S. Burghoff, P. L. E. M. Van Lent et al., "Selective activation of adenosine A_{2A} receptors on immune cells by a CD73-dependent prodrug suppresses joint inflammation in experimental rheumatoid arthritis," *Science Translational Medicine*, vol. 4, no. 146, Article ID 146ra108, 2012.

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Review Article

Regulation of Hemichannels and Gap Junction Channels by Cytokines in Antigen-Presenting Cells

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Autocrine and paracrine signals coordinate responses of several cell types of the immune system that provide efficient protection against different challenges. Antigen-presenting cells (APCs) coordinate activation of this system via homocellular and heterocellular interactions. Cytokines constitute chemical intercellular signals among immune cells and might promote pro- or anti-inflammatory effects. During the last two decades, two membrane pathways for intercellular communication have been demonstrated in cells of the immune system. They are called hemichannels (HCs) and gap junction channels (GJCs) and provide new insights into the mechanisms of the orchestrated response of immune cells. GJCs and HCs are permeable to ions and small molecules, including signaling molecules. The direct intercellular transfer between contacting cells can be mediated by GJCs, whereas the release to or uptake from the extracellular milieu can be mediated by HCs. GJCs and HCs can be constituted by two protein families: connexins (Cxs) or pannexins (Panxs), which are present in almost all APCs, being Cx43 and Panx1 the most ubiquitous members of each protein family. In this review, we focus on the effects of different cytokines on the intercellular communication mediated by HCs and GJCs in APCs and their impact on purinergic signaling.

1. Introduction

An efficient immune response against pathogens and other challenges requires efficient coordination between different cell types, making cell-cell interaction a key step [1, 2]. To this end, the immune system uses different types of cellular communication, being the autocrine and paracrine signaling mediated by cytokines two of the most studied ones [3]. These types of signaling allow communication not only among immune cells, but also with resident cells of challenged tissues [4]. This coordination plays a pivotal role in antigen-presenting cells (APCs) activation because they specifically trigger activation of other cells through immunological synapse, such as T- and B-cell activation that mediate adaptive immunity [5], and the cytokines released at this stage determine the onset of the immune response [6].

Cytokines are soluble or membrane-attached proteins that have pro- or anti-inflammatory properties and are produced by immune and nonimmune cells. As expected, the abnormal release of cytokines promotes the development and progression of pathological conditions with rather diverse etiologies, including rheumatoid arthritis, cancer, and even depression [7–9]. In addition, cytokines favor other types of cellular communication through the expression of cell surface molecules [10] and/or release of soluble molecules, as we discuss in the next section. Both of these alternative mechanisms of cellular communication, which are dependent or independent of cellular contacts, might occur through membrane channels constituted by connexins (Cxs) or pannexins (Panxs).

Nowadays, immunologists' rising interest in Cx- and Panx-based channels is evident in the literature. One of the relevant findings that put GJCs in the center of the immunology field is the contribution to inflammation, antigen presentation, tolerance, HIV sensing, and tumoral immunity [11–17]. Here, we review the cytokine regulation of GJCs and HCs in different APCs.

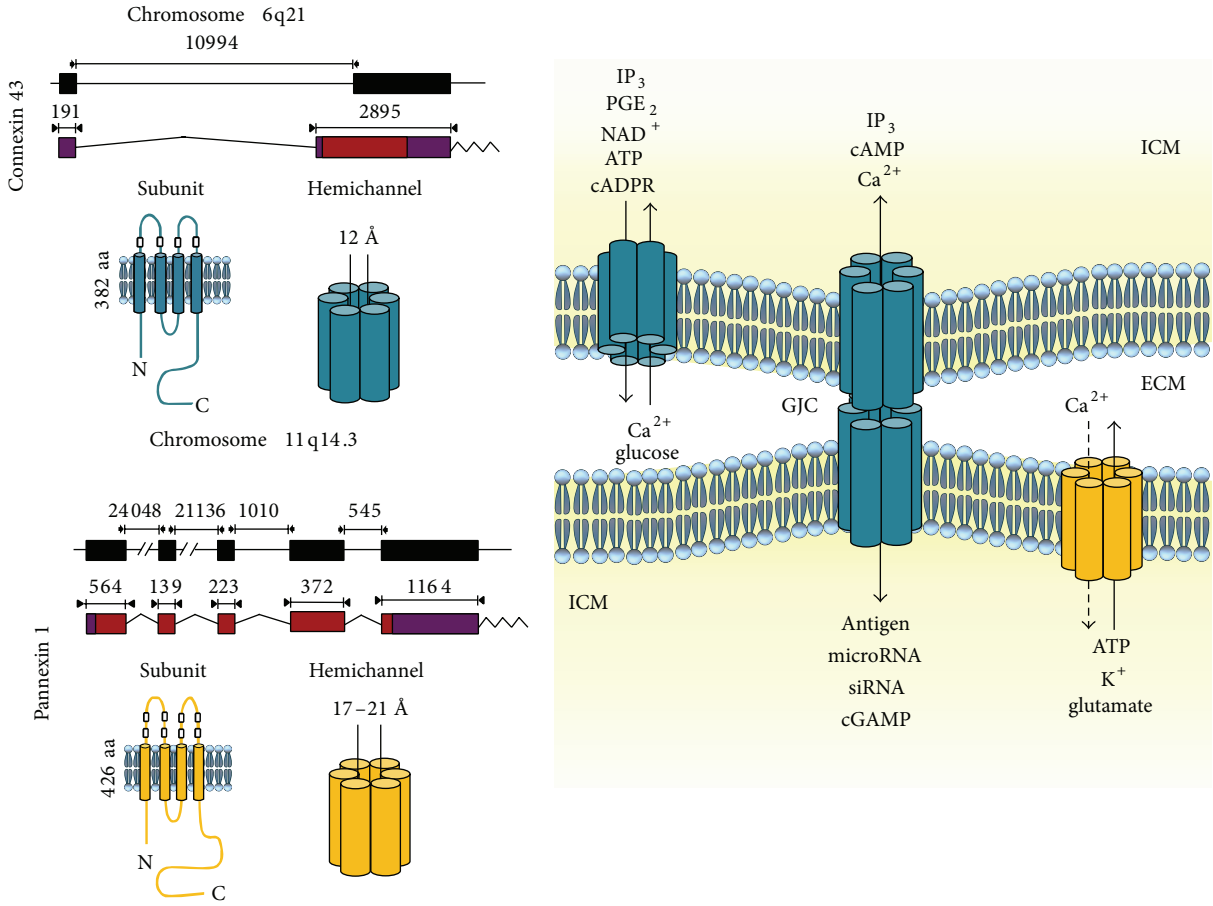

Figure 1: Connexin 43 and pannexin1 at gene and protein levels. Left: a diagram depicting the genomic regions, mRNA, and membrane topology of human connexin 43 (Cx43, top left) and pannexin 1 (Panx1, bottom left). Genomic loci are represented by black boxes that stand for the corresponding exons. mRNA diagrams representing the exons as coding protein regions (red boxes) and 3′- and 5′-non-coding areas (purple boxes) are shown. The intron lengths are indicated in the schemes of genomic loci, and exon sizes are indicated in the mRNA diagrams. In the membrane topology the white squares indicate extracellular cysteine residues of each protein. Six protein subunits constitute a hemichannel (HC), which has different pore sizes. Right: two adjoining cells forming a gap junction channel (GJC) at the cell interface. Each cell presents HCs formed by Cx43 or Panx1. Arrows denote the bidirectional communication with the intracellular milieu (ICM) for GJCs and the extracellular milieu (ECM) for HCs; some immunorelevant molecules are shown. Dotted line for Ca^{2+} permeating Panx1 HCs indicates that this phenomenon is not fully demonstrated.

1.1. Gap Junction Channels and Hemichannels. The most studied mechanism of intercellular communication that depends on close cell-cell contact is mediated by gap junction channels (GJCs) [18]. Since most immune cells are generally sparse within tissues, it is possible that this feature delayed the studies on GJCs. Members of the Cx family share the membrane topology and number of units that oligomerize in a GJC (dodecamer) and show high homology in primary sequence (Figure 1) [18–20]. These GJCs are formed by the docking of two adjacent hemichannels (HCs, hexamers) and allow direct contact-dependent cellular communication because they are permeable to ions and small compounds including immunorelevant molecules [13, 21–26].

The turnover of Cxs is between 2 and 3 h indicating that the strength of intercellular communication can be quickly affected by changes in rate of synthesis and/or degradation of GJC protein subunits. In addition, closure of GJCs can be induced in a few seconds by changes in the state of phosphorylation of Cxs [18]. Therefore, the high plasticity of GJCs is compatible with transient as well as stable gap junctional communication between contacting cells.

Recently, another family of proteins named Panxs and constituted by only three members (Panx1–3) was proposed to form GJCs. Exogenous expression of Panx1 alone or with Panx2 establish GJCs in oocytes [27]. Similar findings were obtained with Panx1 expression in mammalian cells [28]. Moreover, Panx3 has been proposed to form GJCs between osteoblasts and to contribute to the differentiation of C_2C_{12} cells into osteoblasts [29]. However, the expression of functional Panx GJCs still remains controversial [30]. Panxs share their membrane topology but show only very little homology in their primary sequence (Figure 1). In addition, Cx and Panx HCs are oligohexamers [18], but Panx2 has been shown to form octamers [31].

HCs are the least studied autocrine/paracrine intercellular communication pathway mainly due to their rather recent discovery. They correspond to one-half of a GJC, and Cx and/or Panx HCs are present in the cell surface of all cells so far studied, allowing the exchange of ions and small molecules between the intra- and extracellular compartments [20]. Cx and Panx HCs differ in their regulation and pore size [18, 20, 31]. Panx1 HCs exhibit a bigger pore vestibule, but the pore neck seems to be more selective than that of Cx HCs since they are not permeable to anionic molecules >250 Daltons [32], whereas several Cx HCs are permeable to Evans blue (−4 negative charge and ~950 Daltons) [33]. HCs allow communication in a cell-cell contact-independent manner because they permit the release or uptake of small molecules [34, 35].

Several conditions increase the open probability of Cx HCs including reduction of extracellular or increase of intracellular Ca^{2+} concentration [36]. In contrast, Panx1 HCs are not directly affected by changes in extracellular Ca^{2+} concentration, but extracellular ATP activation of some P2Y or $P2X_7$ receptors induces opening of Panx1 HCs [37]. Several GJC blockers also inhibit Cx and/or Panx HCs. Extracellular La^{3+} does not block Cx GJCs [38] or Panx HCs [39] but blocks all Cx HCs so far studied. Nevertheless the use of La^{3+} should be accompanied by using other blockers because it has been shown to block other membrane channels [40].

Cx43 and Panx1, the most ubiquitous members of each family of HC forming proteins, are expressed in APC [14, 20, 34, 41, 42]. Cytokine regulation of intercellular communication through GJCs and HCs might contribute to a rapid amplification and coordination of activating or inhibitory signals among neighboring cells. Here, we summarize the current knowledge on the regulation of both types of cellular communication by cytokines.

1.2. Immunorelevant Molecules and Cx- and Panx-Mediated Cell-Cell Communication. The study of GJCs began in the early 60s with the description of the structure responsible for intercellular electrical transmission [43]. These studies showed current transference between contacting excitable cells and were the first to use the term gap junction to identify this structure [44–47]. In the 70s, the permeation of different immunorelevant molecules was described. These studies included small peptides [48], IP_3 [49], and cAMP [50], but the study of Cxs and Panxs in immune cells had to wait for almost 30 years to be reported.

Although the presence of GJCs at ultrastuctural level was shown at the end of the 80s during antigen presentation [38, 51, 52], immunologists put an eye on the GJC field after the demonstration of antigen transfer (linear peptides up to 1,800 kDa) through GJCs in APCs [25]. This direct antigen transfer through GJCs allows cross-presentation, which corresponds to presentation of antigens in major histocompatibility complex (MHC) class I molecules by APCs that acquire antigens from infected or tumoral cells and after presentation to T cells initiating an effective immune response [24, 25]. Following this, our group and collaborators were able to show the transference of tumoral antigens between dendritic cells after stimulation with tumoral necrosis factor-α (TNF-α) and tumoral lysate [24]. In addition, GJCs allow cell-cell transference of single- and double-stranded RNA [53], as well as specific single-stranded microRNA [23], which has a high impact on immune responses [54]. Recently, the cell-cell transference of two different microRNAs (miR-142 and -223) between macrophages was demonstrated [55]. These data open new unexplored fields in the study of GJCs, which might be used for specific delivery of microRNA and siRNA. Whether Cx or Panx HCs allow the transfer of single- or double-stranded RNA has to be studied.

Ca^{2+} signaling plays a pivotal role in immune cells and contributes to all stages of the immune response. In APCs, it contributes directly to their migration, maturation, and cell death [56]. The transference of second messengers associated with Ca^{2+} signaling, such as IP_3, through GJCs was demonstrated several years ago [22, 49]. In addition, IP_3 is released through HCs [57], and intracellular IP_3 contributes to increase HC activity [58]. IP_3 contributes to different steps of the immune response [59] and plays an important role in migration of dendritic cells (DCs) [60]. Then, it is possible to hypothesize that transference of IP_3 between communicating DCs or release/uptake through HCs might have an impact on the phenotype of DCs. Moreover, direct Ca^{2+} transfer occurs via GJCs [26], and it is possible that similar Ca^{2+} communication occurs through DC-T-cell GJCs during immune synapse [13, 61]. In addition, it was shown recently that Cx [36, 62–67] and Panx HCs [28, 29] provide a new route for Ca^{2+} entry into the cell. Then, the functional expression of HCs in immune cells might also contribute to the Ca^{2+} signaling.

CD38 is an ectoenzyme expressed by myeloid and lymphoid cells that use NAD^+ to generate cADPR and ADP-ribose, which contribute to several immune cell responses [68]. Interestingly, NAD^+ permeates GJCs [22, 69] and Cx HCs [22, 70], and upon activation of $P2X_7$ receptors increase the opening of Panx1 HCs [71]. It can be anticipated that NAD^+ transfer or release through these channels might contribute to cell-cell communication in different immune cells. In addition, cADPR uptake occurs through Cx43 HCs [72], which in turn contributes to microglial survival [73].

ATP is a recognized DAMP that activates immune cells and also contributes to autocrine and paracrine activation when released from cells [34, 74, 75]. The contribution of Cx and Panx HCs to purinergic signaling has been reported and was recently revised [76]. Thus, Cx and Panx HC-mediated ATP release might play a role in all steps of the immune response. In contrast to ATP, prostaglandins (PGs) are small soluble molecules that seem to contribute to anti-inflammation in APCs, although this feature depends on the microenvironmental signals [77]. In particular, PGE_2 contributes to induce gap junctional communication [78] and also is released through Cx43 HCs [79]. Moreover, PGE_2 and purinergic signaling contribute to interleukin (IL) 1β release from macrophages [80]. Thus, PGE_2 and other metabolites produced by cyclooxygenase-2 might be released from APCs (and/or other immune cells), which produce a different signature in the involved cells depending on the inflammatory mediators that coexist with them.

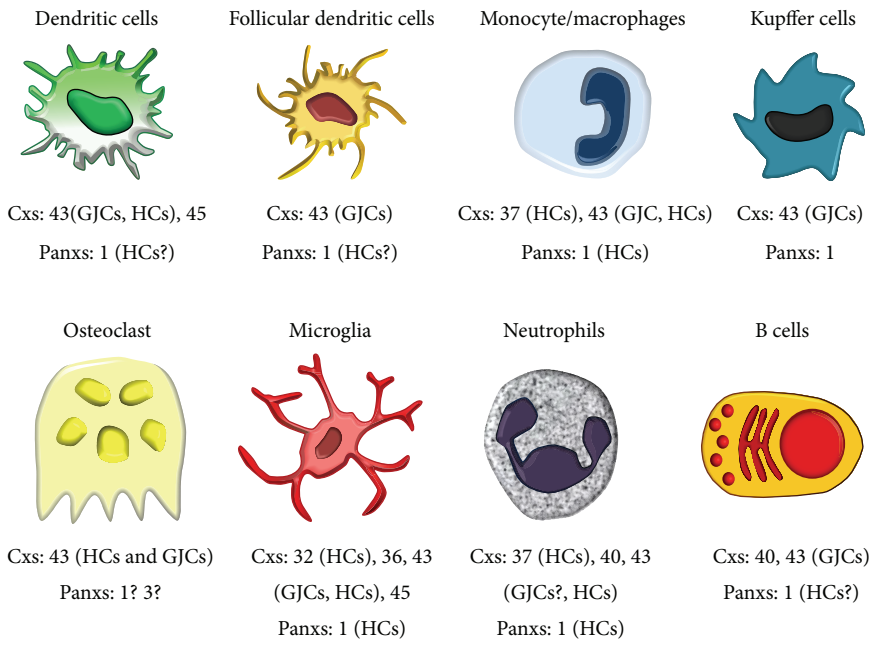

Figure 2: Connexin and pannexin expression in antigen-presenting cells. Summary scheme shows the expression of gap junction channels (GJCs) and hemichannels (HCs) formed by connexins (Cxs) and pannexins (Panxs) in different antigen-presenting cells (APCs). Question marks next to a protein (Cx or Panx) or channel type (GJC or HC) indicate that the expression or function remains unknown or is not fully shown.

Description of functional GJCs in T cells occurred almost 4 decades ago [81, 82]. However, a rising interest in T-cell GJCs began very recently after the discovery of their role in regulatory T cells- (Tregs-) mediated tolerance [12]. GJCs allow cAMP transfer from Tregs to naïve T cells and provide immunosuppression [12]. In addition, GJCs between DCs and Tregs contribute to prevent the activation of $CD8^+$ T cells [15], showing that GJCs provide amplification of activating or inhibitory signals.

The role of Cx- and Panx-based channels in infectious diseases is well documented [83], but an unexpected role was recently shown in the development of HIV infection. Cytosolic DNA-sensing occurs through an enzyme called cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) [84], which produces the second messenger cGAMP that enables DCs to sense HIV [85]. Importantly, transfer of cGAMP occurs through GJCs between Cx43 and Cx45 overexpressing cells [21]. This spreading of cGAMP activates STING (from stimulator of IFN genes) in the receiving cell, which in turn produces interferon (IFN) [21]. Since DCs and other APCs express Cx43 and Cx45 (Figure 2) [24, 86, 87], it is possible that gap junctional communication between these cells contributes to the HIV immune response.

2. Expression of Cxs in Antigen-Presenting Cells

Although GJCs in immune cells were described in the early 70s by Hülser and Peters who reported gap junctional communication between T cells [81, 82], the study of Cxs in APCs had to wait until the end of the decade when expression of GJCs and gap junctional communication was shown in macrophages [88, 89]. Later, they were found in DCs [51, 52, 90] and follicular DCs [91–93]. On the other hand, the study of HCs in the immune system started several years ago. Later in the 90s, Alves et al. (1996) showed ATP-induced dye uptake in macrophages, which was suggested to be mediated by HCs [94]. This study was followed by studies in microglia, neutrophils, and T cells several years later [34, 41, 95, 96].

2.1. Dendritic Cells (DCs). Ralph Steinman in the early 70s discovered the DCs [97], which emerge in the bone marrow from a myeloid common precursor and populate different organs [98]. In these cells, the expression of Cxs has been demonstrated, but the expression of Panxs remains unknown. However, the expression of Panx1 might be predicted by the ATP-induced dye uptake observed in these cells [99–102]. In addition, Panx1 expression has been detected at the mRNA level in DCs under resting conditions, while its upregulation has been demonstrated upon exposure to bacterial lipopolysaccharide (LPS) or IFN-γ in DCs [103, 104]. LPS-induced IL-1β release in DCs occurs in a $P2X_7$ receptor-independent way [105], suggesting that $P2X_7$ receptor-mediated opening of Panx1 might not contribute to inflammasome activation. However, whether Panx1 might contribute to other responses in DCs has not been reported yet. Here, we present evidence of Panx1 presence in $CD11c^+$ DCs from mouse spleen (Figure 3).

In murine and human DCs (primary cultures and cell lines), the expression of Cx43 and Cx45 has been demonstrated at the mRNA and protein levels (Figure 2) [13, 15,

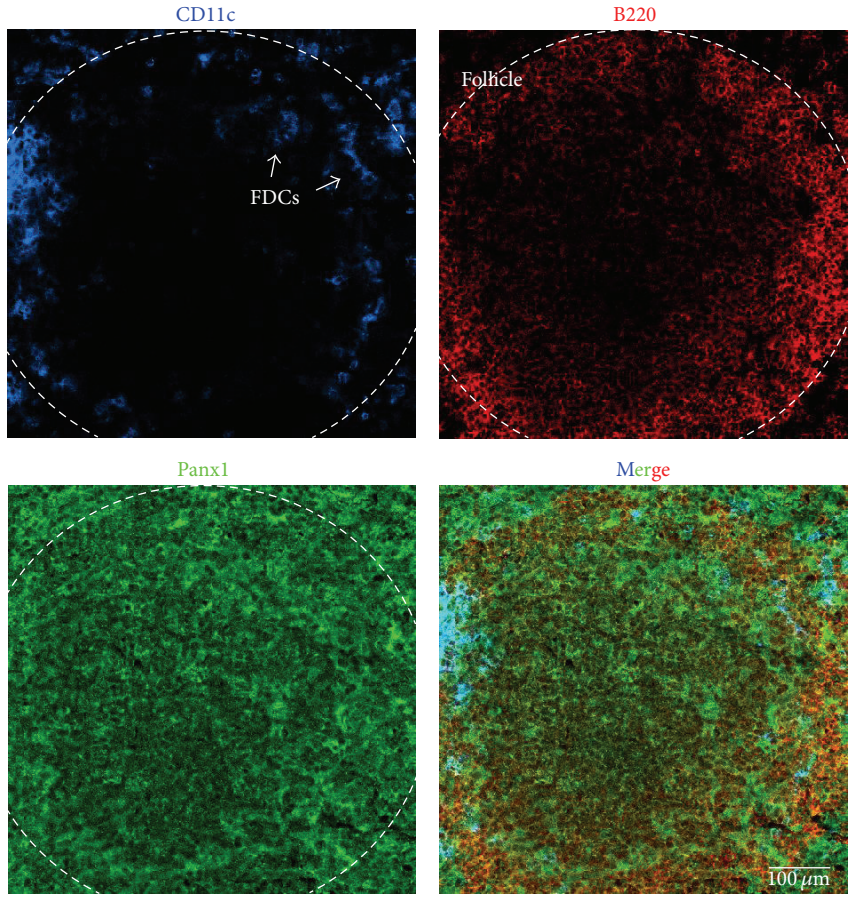

FIGURE 3: Dendritic and B cells of mouse spleen present pannexin1. Immunofluorescence of adult mice spleen cryosections (8 μm thick) fixed in ethanol (70% v/v) at −20°C for 20 min, mounted in Fluoromount-G and observed in a confocal microscope (Olympus, FluoView FV1000). Pannexin1 (Panx1 in green: primary antibody: rabbit anti-Panx1 antibody and secondary antibody goat anti-rabbit IgG conjugated to FITC) immunoreactivity is shown. Cells were identified by their reactivity to specific antigens as follows: dendritic cells (DCs) by CD11c (blue, monoclonal mouse antibody conjugated to allophycocyanin) and B cells by B220 (red, monoclonal mouse antibody conjugated to phycoerythrin) in a follicle. Arrows denote follicular DCs (arrows). Merge is also shown. Scale bar: 100 μm.

24, 61, 86, 87, 93, 106, 107]. In addition, migratory DEC205$^+$ DCs, found at draining lymph nodes after muscle damage, show increased immunoreactivity for Cx43 and Cx45 [86]. Consistent with the requirement of cell activation for Cx expression, Cx43 was not detected in skin DCs under resting conditions [108]. Similarly, Cx43 was found to contribute to the establishment of oral tolerance, because it mediates antigen transfer from CD103$^+$ DCs to macrophages in murine intestine [106]. Accordingly, expression of Cxs and functional state of GJCs are modulated by different cytokines (Table 1).

TNF-α is a proinflammatory cytokine and possibly the most relevant one because it is the first cytokine released by different cell types, including DCs, after exposure to different stimuli, such as cell damage or infection, and its receptor is expressed by all APCs [109]. However, TNF-α alone does not increase Cx43 total protein levels in murine or human DCs [24, 86] but potentiates the expression of functional GJCs between cultured DCs in combination with IL-1β or a tumoral lysate (Table 1) [24, 86]. Whether TNF-α induces HC activity in DCs remains unknown.

IL-1β, another proinflammatory cytokine released by different cell types including APCs, is maintained as an inactive precursor and after cleavage is released as a mature bioactive form to the extracellular milieu [110]. Similar to TNF-α, IL-1β alone does not induce gap junctional communication or Cxs expression but, in combination with TNF-α, induces GJCs and increases Cx43 and Cx45 levels in DCs (Table 1) [86]. The possible effect of IL-1β on the expression of HCs in DCs has not been reported yet.

IFN-γ contributes to the control of viral infections and is mostly produced by T and natural killer (NK) cells, but it is also produced and released by DCs [111–114]. Similar to TNF-α and IL-1β, treatment with IFN-γ does not induce gap junctional communication or increase in Cx43 levels [87] but, in combination with TNF-α and IL-1β, promotes a synergic response on Cx43 and Cx45 levels in DCs [86]. Moreover, in combination with LPS, IFN-γ potentiates the functional expression of GJCs in DCs [87] and prolongs the TNF-α/IL-1β-induced dye coupling [86], showing that IFN-γ is an enhancer rather than inducer of gap junctional communication. In addition, we show here that IFN-γ induces dye

Table 1: Effect of different cytokines on GJCs and HCs in different antigen-presenting cells.

Cytokine(s)	Cell type	Effect on Cx or Panx expression	Effect on GJCs, HCs and technique used
TNF-α	DCs	=Cx43 (Wb) [24]	=GJCs (DT) [24, 86]
		+MCL: ↑Cx43 (Wb) [24]	+MCL ↑GJCs [24]
	Mo	NE	=GJCs (DT) [140]
	Microglia	↑Cx32 (FC) [187]	↑HCs (MR, DU) [187, 192]
		↑Cx43 (Wb) [192]	↑GJCs (SL) [192]
		+ATP: ↑Cx43, Panx1 (Wb) [96]	+LPS ↑GJCs [196]
			=GJCs [96] +ATP ↑GJCs (DT) [96] +ATP ↔HCs (DU) [96]
	Neutrophils	+CM: ↑Cx37, 40, 43 (Wb, IF) [217]	+CM ↑GJCs (DT) [217]
IL-1β	DCs	NE	=GJCs (DT) [86]
	Microglia	NE	=GJCs (DT) [96]
IFN-γ	DCs	=Cx43 (Wb) [87]	=GJCs (DT) [86]
			↑HCs (DU)
			+LPS ↑GJCs [87]
	Mo	NE	=GJCs (DT) [140]
			+LPS ↑GJCs [140]
	KCs	=Cx43 (RT, Wb) [168]	+LPS ↑GJCs (DT, IF) [168]
	Microglia	NE	=GJCs (DT) [96]
IL-6	DCs	NE	=GJCs (DT) [86]
			↑HCs (DU)
	Microglia	NE	=GJCs (DT) [96]
RANKL	OCs	NE	↑GJCs? ↑HCs? [178]
CXCL12	B cell	↑Cx43 Phosphorylation (Wb)	=HCs
RANKL/M-CSF	OCs	↑Cx43 (RT, Wb) [176]	↑GJCs (IF) [176]
IFN-γ/IL-6	DCs	ND	=HCs (DU)
TNF-α/IL-β	DCs	↑Cx43 (RT, Wb) [86]	↑GJCs (DT) [86]
	Microglia	↑Cx43, Panx1 (Wb) [96]	↑GJCs (DT) [96]
TNF-α/IFN-γ	DCs	ND	=GJCs (DT) [86]
	Mo	↑Cx43 (Wb) [140]	↑GJCs (DT, IF) [140]
	Microglia	↑Cx43, Panx1 (Wb) [96]	↑GJCs (DT) [96, 196] ↑HCs (DU) [96]
TNF-α/IL-β/IFN-γ	DCs	↑Cx43 (RT, Wb) [86]	↑GJCs (DT) [86]
TNF-α/IL-β/IL-6	DCs	NE	↓GJCs (DT) [86]
	Microglia	↓Cx43, Panx1 (Wb) [96]	↓GJCs (DT) [96] ↓HCs (DU) [96]
TNF-α/IFN-γ/IL-6	DCs	NE	↓GJCs (DT)
	Microglia	↓Cx43, Panx1 (Wb) [96]	↓GJCs (DT) [96] ↓HCs (DU) [96]
TNF-α/IL-1β/IFN-γ/IL-6	DCs	NE	↓GJCs (DT) [86]

CM: conditioned medium, DCs: dendritic cells, DT: dye transfer, DU: dye uptake, FC: flow cytometry, IF: immunofluorescence, KCs: Kupffer cells, LPS: bacterial lipopolysaccharide, MCL: melanoma cell lysate, Mo: monocyte, NE: not evaluated, OCs: osteoclasts, RT: reverse transcription polymerase chain reaction, SL: scrape loading, and Wb: Western blot. Effect on HC or GJC activity: no effect (=), upregulation (↑), and downregulation of induced activity (↓).

uptake sensitive to La^{3+}, suggesting that the IFN-γ-induced dye uptake is mediated by Cx HCs (Figures 4(a) and 4(b)).

IL-6, described initially as a stimulating factor for IgG production in B cells, is a cytokine produced by almost all nucleated cells [115] and drives T helper 17 (Th17) differentiation and inhibits Tregs [116, 117]. However, IL-6 also shows anti-inflammatory effects as it decreases the reducing immune response and promotes the release of anti-inflammatory cytokines after exercise, such as IL-10 and transforming growth factor-β (TGF-β) [118, 119]. From

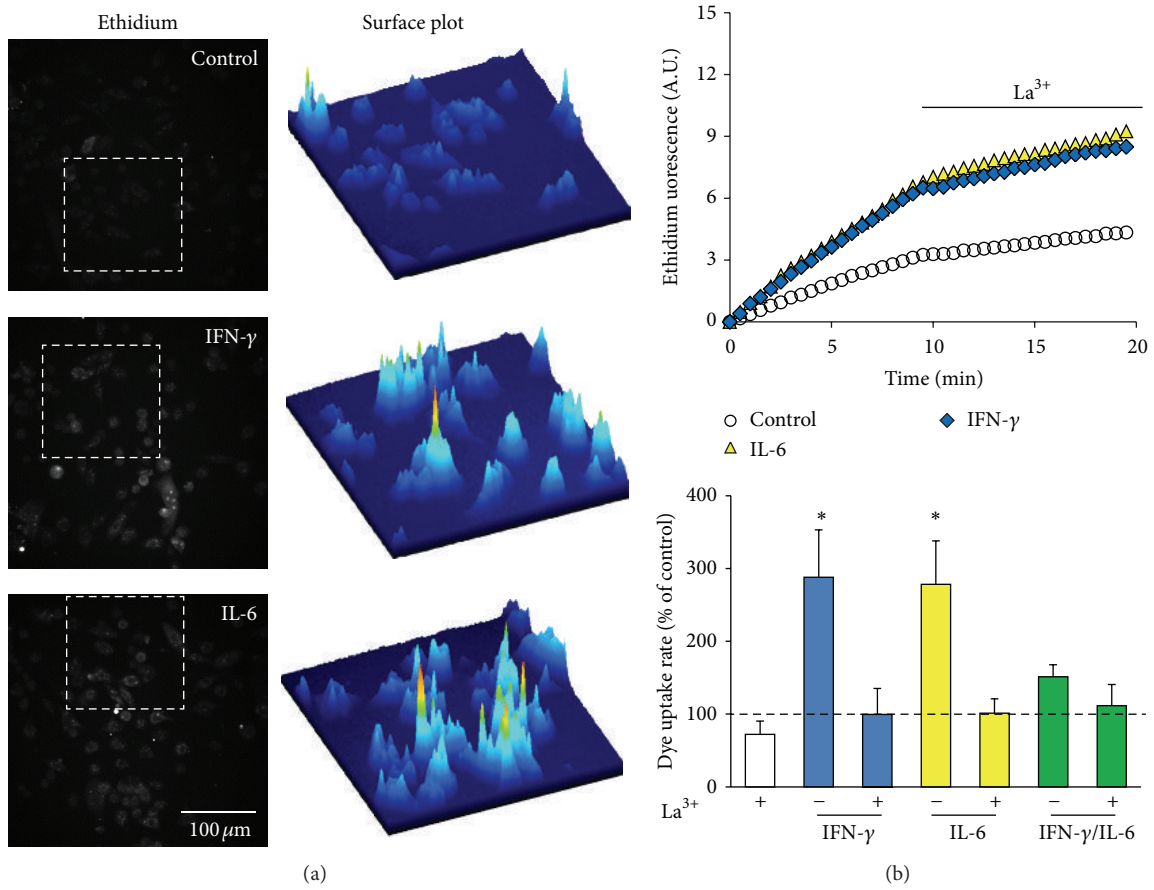

FIGURE 4: IFN-γ or IL-6 increases dye uptake in dendritic cells. Bone-marrow-derived dendritic cells (BMDCs) from balb/c mice were differentiated with 40 ng/mL GM-CSF and IL-4 for 7 days. At day 7, BMDCs were treated for 6 h with IFN-γ (10 ng/mL), IL-6 (10 ng/mL), or both, and ethidium uptake was evaluated in time-lapse experiments (Olympus BX 51W1I). (a) Left: fluorescence images of ethidium after 9 min of uptake. Scale bar: 50 μm. Right: ImageJ surface plot analysis of fluorescence intensity of the region indicated in the field (dotted square). (b) Top: time-lapse ethidium uptake under control conditions (white circles) or after 6 h treatment with IL-6 (yellow triangles) or IFN-γ (blue diamonds). Each point corresponds to the mean of 30 cells. After 10 min of recording, La^{3+} (200 μM) was added to the bath solution to block connexin hemichannels. Bottom: graph showing the basal dye uptake rate and the effect of La^{3+} on BMDCs after treatment with IFN-γ (blue bars), IL-6 (yellow bars), or both (green bars). Each bar corresponds to the mean ± SE (% of control condition, dotted line) of 3 independent experiments.

the GJCs perspective, IL-6 has an anti-inflammatory effect because it prevents the TNF-α/IL-β- and TNF-α/IL-β/IFN-γ-induced gap junctional communication in DCs [86]. Similar findings related to cytokine-regulation in microglia are discussed below. In this review, we present relevant data showing that IL-6 induces dye uptake in DCs in a similar way to IFN-γ and is blocked by La^{3+}, consistent with Cx HC-mediated response (Figure 4(b)). Interestingly, IL-6 antagonizes IFN-γ-induced dye uptake, which is correlated with its role in the maintenance of immature DCs [120]. This phenomenon might be promoted by downstream signaling pathways triggered by these cytokines that activate different suppressors of cytokine signaling proteins [121]. These data suggest that the effect of IL-6 on HC activity of DCs depends on the cytokine context present in the cellular microenvironment.

With these findings, it is plausible to anticipate that T-cell polarization is determined by the cytokine profile of the microenvironment, as well as by molecules directly exchanged and/or released to the extracellular milieu via GJCs and/or HCs, respectively, expressed by DCs and T cells.

2.2. Langerhans Cells (LCs). These cells were described almost 150 years ago by Paul Langerhans [122], but their role remains elusive over almost 100 years until they were described as leukocyte derived cells [123]. LCs reside in skin epidermis and represent the first barrier against pathogens and external noxa [124]. Although LCs are less motile than dermal DCs [125], they are better APCs [126], suggesting their important role in antigen presentation. LCs are characterized by the expression of the nonpolymorphic class I MHC molecule CD1a and C-type lectin Langerin, as well as the presence of Birbeck granules, which are tennis-racquet-shaped intracytoplasmic organelles [127–129]. When LCs capture antigens, they migrate to skin draining lymph nodes

(LNs) where they present antigens to naïve T cells [129] and might induce or suppress the immune response [130]. Early studies performed by Concha et al. observed at ultrastuctural level that physical interactions between LCs and T cells during allogeneic antigen presentation includes the presence of GJC-like structures [51, 52, 90].

Cx43 immunoreactivity was found in LC-like cells in human tissue with LC histiocytosis [91] and in MHCII$^+$ epidermal LC-like cells from human epidermis [25]. However, Zimmerli et al. detect no Cx43 immunoreactivity in LCs (CD1a$^+$ epidermal cells) from normal human skin [108]. This discrepancy could be explained in part by the inflammatory state of the tissue. Whether the tissue is under resting state or inflammation might affect the Cx43 expression, as occurs with the upregulation of Cx43 expression after stimulation in other immune cells. In support of the Cx expression, gap junctional communication between LCs has been shown to allow the transfer of antigenic peptides in a Cx43-dependent manner [25]. However, the possible functional expression of Cx HCs remains unknown.

Panx1 and Panx3 expression have been reported in murine epidermis [131], but their expression in LCs has not been documented. However, functional expression of Panx HCs is suggested by ATP-induced dye uptake in murine and human LCs [102, 132]. Since LCs express several purinergic receptors that contribute to the LC-mediated immune response [133], it is conceivable to suggest that Panx HCs might also contribute to cytokine release and activation of LCs.

2.3. Follicular Dendritic Cells. Unlike DCs, follicular DCs (FDCs) present a low phagocytic activity but high retention of antigen and immune complexes on their surfaces. They reside at follicles of secondary lymphoid organs [134], where they present antigens to B cells [135]. The origin of FDCs is a controversial topic because some evidences show that they emerge from bone marrow, while other studies propose that they derive from mesenchymal cells [134]. This controversy might have contributed to delay the establishment of primary cultures of FDCs and the subsequent demonstration of cell-cell communication mechanisms mediated by Cx- and Panx-based channels.

In situ hybridization studies showed Cx43 mRNA in human tonsils [93]. In addition, it was demonstrated that Cx43 colocalizes with FDC markers (CD21 and CD35) at germinal centers of human tonsils and spleen [91–93]. Moreover, gap junctional communication among FDCs and between FDCs and B cells has been demonstrated at functional and ultrastructural levels [91–93]. Here, we show that FDCs (CD11c$^+$) found in mouse spleen follicles present Panx1 immunoreactivity (Figure 3). The expression of functional HCs on FDCs remains unknown, but currently it is possible to speculate that TNF-α [134], crucial cytokine for development of FDCs, might modulate the expression of GJCs and HCs, as it occurs in other APCs. Similarly, IL-6 might affect HC activity in FDCs because these cells are the main source of this cytokine at germinal centers [134].

2.4. Monocyte/Macrophages. Monocytes emerge from the same precursor of DCs in the bone marrow and circulate in the blood [98]. Upon tissue injury, they rapidly extravasate and differentiate in DCs or macrophages, depending on the cytokine pattern present in the microenvironment [136, 137]. Studies on GJCs in APCs started with demonstrations of gap junctional communication between macrophages [88, 89], and information on the expression of Cxs and Panxs in these cells is increasing progressively [55, 106, 138–143]. Recently, it was shown that tumor-associated macrophages express Cx43, and it seems that they form GJCs in long networks [139]. Similarly, alveolar macrophages form communicating networks with epithelial cells in the alveoli where they coordinate Ca^{2+} signaling [144]. This cell-cell communication might be protective effect because specific deletion of Cx43 in macrophages increases the release of proinflammatory cytokines [144]. In addition, monocytes and macrophages form heterocellular GJCs with CD103$^+$ DCs, endothelial cells, and T cells [106, 140–142, 145].

Resting monocytes express Cx37 and, after activation, they also express Cx43. These Cxs regulate their adhesion and extravasation, respectively (Figure 2) [140, 142, 146, 147]. In support of this notion, TNF-α has been shown to increase Cx43 expression, adhesion, and extravasation of monocyte/macrophages [140, 142]. Treatment with TNF-α alone does not induce functional expression of GJCs in monocytes, but it remains to be demonstrated whether it induces Cx43 HC activity, which might be involved in cell adhesion [142, 146], as it has been demonstrated for Cx37 HCs [147]. IFN-γ does not induce the expression of HCs or GJCs but increases Cx43 levels, gap junctional communication, and *in vitro* migration when combined with LPS or TNF-α (Table 1) [140].

The expression of Panxs in monocytes was first suggested by ATP-induced dye uptake [148]. Recently, it was demonstrated that human monocytes express Panx1 under resting conditions, and its total levels are upregulated after treatment with LPS [138]. In monocytes, LPS induces functional expression of Panx1 HCs, which contributes to ATP release and consequently to IL-1β release [138].

Peritoneal, alveolar, and cell lines derived from macrophage express Cx37 and Cx43 under resting conditions, and upregulation of Cx43 expression is observed after activation [55, 72, 94, 144, 147, 149–155]. In macrophages, Cx37 negatively regulates cell adhesion as in monocytes [147], while Cx43 has been proposed to play a role in phagocytosis [150]. However, the latter remains controversial [153]. These particularities might rely on the different genetic background (mice strain, heterozygotes, or K.O.) and protocols used. Moreover, Cx43 HCs allow the release of small signaling molecules including ATP and NAD$^+$ and also contribute to IL-1β release in macrophages infected with *Bacillus anthracis* [72, 149, 156]. In addition, it has been recently shown nitric oxide release through HCs [157] and thus, it is possible that Cx37 and/or Cx43 HCs allow nitric oxide release in activated monocyte/macrophages [158]. Macrophages also express Panx1 HCs, which are activated by extracellular ATP [159]. This finding was suggested previously in studies where HC blockers were shown to reduce the ATP-induced dye uptake in peritoneal macrophages and in a macrophage cell line [94, 152].

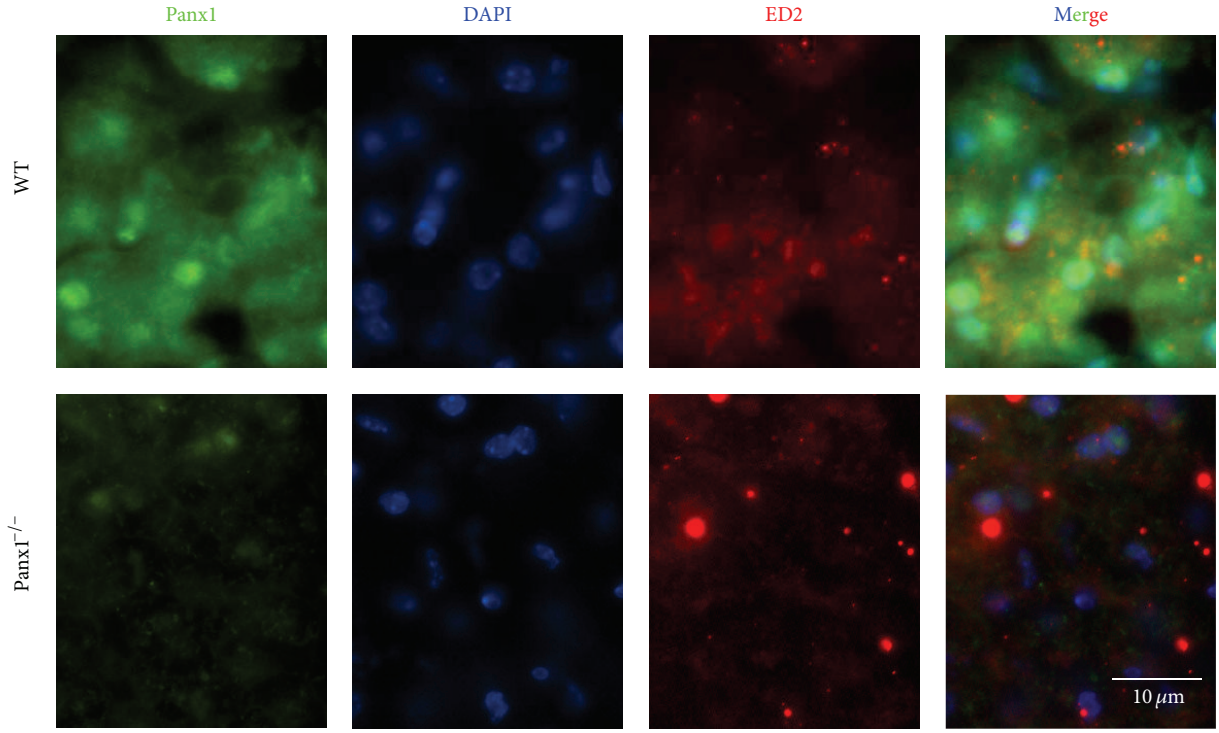

Figure 5: Expression of pannexin1 (Panx1) in Kupffer cells. Immunofluorescence analysis of liver cryosections (8 μm thick) obtained from adult wild-type (WT) and Panx1$^{-/-}$ adult (C57/BL6) mice was performed to analyze reactivity of Panx1 (green, primary antibody: rabbit anti-Panx1 antibody and secondary antibody goat anti-rabbit IgG conjugated to FITC) in ED2 (red: goat polyclonal antibody and secondary antibody mouse anti-goat conjugated to Cy3) positive cells, which correspond to Kupffer cells. Top panels correspond to a liver section of a WT mouse and bottom panels correspond to a liver section of a Panx1$^{-/-}$ mouse. No specific Panx1 reactivity was detected in Panx1$^{-/-}$ liver, but ED2 positive cells were evident. DAPI stain was used to visualize nuclei (blue), and merge is also shown. Panx1$^{-/-}$ mice were kindly donated by Dr. Hanna Monyer (University of Heidelberg, Germany). Bar: 10 μm.

In macrophages, Panx1 HCs contribute to IL-1β release through a pathway independent of their permeability [37], but to our knowledge the possible functional regulation of these channels by cytokines has not been described. However, an interesting suggestion of the possible regulation of Panx1 by cytokines was investigated by gene expression pattern in macrophage polarization [160]. Macrophages present different phenotypes depending on the stimuli and the microenvironment cytokine signature. Then, "classic" activation of macrophages with LPS or cytokines such as TNF-α or IFN-γ leads to a proinflammatory profile, which is named M1 [161]. Conversely, "alternative" activation after exposure to IL-4, IL-10, or IL-13 or particular Toll-like receptor agonists leads to macrophage differentiation with an anti-inflammatory profile, which is named M2 [161]. Interestingly, while M1 polarization induces downregulation of Panx1 expression in macrophages, M2 polarization induces some upregulation [160]. These observations suggest the involvement of Panx1 in the anti-inflammatory response of M2 macrophages, but whether functional Panx1 HC activity is increased in M2 has not been published yet. Altogether, these data suggest that Cx and Panx HCs play an important role in macrophage activation; their possible regulation by pro- or anti-inflammatory cytokines is a vast unexplored field of research.

2.5. Kupffer Cells. Kupffer cells (KCs) are the largest population of resident macrophages in the body, the liver being their organ of residence [137, 162]. These cells have the ability to present antigens, undergo fusion, form large multinucleated cells, and induce Tregs activation and degradation of intravascular debris [137, 163–165]. Under resting state, KCs release anti-inflammatory cytokines promoting tolerance [163], but after stimulation they release proinflammatory cytokines and might present antigens to CD4$^+$ (helper) and CD8$^+$ (cytotoxic) T cells [166, 167].

In the liver, KCs are sparse, but under proinflammatory conditions they form aggregates and present increased Cx43 immunoreactivity at KC-KC interface, suggesting GJC formation *in vivo* [168, 169]. In support of this notion, cultured KCs express low Cx43 mRNA and protein levels but do not communicate through GJCs under resting conditions (Figure 2). However, after exposure to LPS/IFN-γ, cultured KCs enhance the expression of Cx43 (Table 1) that is located at KC-KC interface allowing gap junctional communication [168]. Neither Panxs nor Cx HCs have been demonstrated in KCs. Here, we show the presence of Panx1 in KCs recognized by their ED2 reactivity in wild-type mice (Figure 5). Consistently, Panx1 was not detected in ED2 reactive cells in liver sections of Panx1$^{-/-}$ mice (Figure 5). Functional expression of

Panx1 HCs in KCs and their possible regulation by cytokines still remain unknown.

2.6. Osteoclasts. Osteoclasts (OCs) are large multinucleated macrophages located in bones. They can be derived from bone marrow precursors or monocytes and have bone-resorbing activity [137, 162, 170]. Because autoimmune diseases lead to bone destruction (e.g., rheumatoid arthritis) [170], a rising interest in the study of the interplay between skeleton and the immune system (osteoimmunology) has taken place during the last decade. Several cytokines, including IL-17, type I and II IFNs, and receptor activator of nuclear factor kappaB ligand (RANKL), have the ability to induce osteoclastogenesis, the process that modulates bone remodeling [170, 171]. Conversely, under noninflammatory conditions, OCs present antigens to CD4 and CD8 T cells, which differentiate into regulatory T cells and inhibit bone resorption [171].

Cx43 mRNA and protein have been detected in cultured OCs and also at the bone (Figure 2) [172–182]. OCs derived from bone marrow precursors or monocytes that undergo fusion and form multinucleated tartrate-resistant acid phosphatase (TRAP) positive cells with bone-resorbing activity express Cx43, which contributes to fusion as observed by the use of Cx43 blockers [180, 181]. Considering the involvement of Cx43 in fusion of OC precursor and the fact that osteoclastogenesis is inhibited by osteoprotegerin released from stromal/osteoblast lineage cells [173, 175], it is possible that under normal conditions osteoprotegerin downregulates Cx43 and then prevents fusion of precursors. Interestingly, a cytokine member of the TNF family named RANKL induces osteoclastogenesis in combination with macrophage colony-stimulating factor (M-CSF) and also increases Cx43 expression (Table 1) [176].

Ultrastructural evidence of GJCs between OCs has been reported [177, 182], but the functional expression of GJCs has been only suggested. The contribution of GJCs to the bone-resorbing activity has been addressed by using HC blockers [178–181], but still leaving open the possibility that OCs may also express Cx or Panx HCs. For instance, the expression of Panx1 HCs could be feasible because these contribute to macrophage fusion, which leads to multinucleated cell formation [183]. In addition, immunofluorescence analysis of bones shows that most cells presented Panx3 at the growth plate [29], suggesting that OCs might express this protein. Finally, Cx43, forming either GJCs or HCs, is involved in the development of rheumatoid arthritis because silencing Cx43 in rat lower limbs reduces the number of OCs and delays the onset of this disease [174]. This suggests that Cx43 expression by OCs might contribute to the development of this disease and might be a relevant target for its treatment.

2.7. Microglia. Microglia, the main resident macrophage of the central nervous system, remove dead cells and monitor cell microenvironment. After injury or infection, activated microglia secrete proinflammatory cytokines and present antigens. In addition, deregulation of their activation is a hallmark of neurodegenerative diseases [184–186].

The study of Cxs and Panxs in microglia has been extensive. The expression of Cxs 32, 36, and 43 and Panx1 has been reported. Some of these proteins form functional GJCs and HCs that contribute to cell-cell communication, migration, and neuronal death (Figure 3) [41, 95, 96, 187–197]. In addition, the mRNA of Cx45 was found in mouse but was not detectable in human microglia [194]. Cx43 seems to play a relevant role because its total protein levels are upregulated in microglia activated by advanced glycation endproducts, amyloid-β peptide, DAMPs, PAMPs, cytokines, and a Ca^{2+} ionophore [96, 189, 192, 193, 195–197]. Indeed, microglia treated with advanced glycation endproducts, proinflammatory cytokines, PAMPs, and a Ca^{2+} ionophore form GJCs presumably constituted by Cx43 [192, 193, 195]. In support of this position, the specific blockade or lack of Cx43 in microglia of Cx43 K.O. mice abrogates the cytokine-induced GJCs [96, 196].

Gap junctional communication between microglia is tightly regulated by several cytokines (Table 1). In fact, intercellular communication mediated by GJCs is increased in microglia after treatment with TNF-α, TNF-α/IFN-γ, and TNF-α/IL-1β [96, 192, 196]. Shaikh et al. [192] demonstrated gap junctional communication after treatment with TNF-α in a microglial cell line, but a recent study performed by Sáez et al. [96] showed that TNF-α does not induce dye coupling in primary cultures of microglia. However, there are several differences that might explain this discrepancy: (1) one study evaluated dye coupling through scrape loading while the other used microinjection; (2) both studies used different TNF-α concentrations; and (3) one study used a microglial cell line and the other used primary cultures of microglia. Consequently, the interpretation of these results should be taken cautiously and the protocols reconsidered.

Recently, it was shown that extracellular ATP is required by the cytokine-induced GJCs and forces the early onset of this gap junctional communication [96], showing a synergistic effect between cytokines and DAMPs. As observed in DCs by Corvalán et al. [86], IL-6 prevents the induction of GJCs in microglia by preventing upregulation of Cx43 and Panx1, as well as by increasing free intracellular Ca^{2+} levels [96]. Furthermore, it is possible that IL-6 might disrupt cell adhesion between microglia as shown in other cells [198], and consequently it might also prevent the formation of GJCs. Recently, absence of dye transfer between microglia *in vivo* and between microglia and other brain cells has been shown in both resting and injury conditions [143]. This study assessed dye transfer by using sulforhodamine B and previous studies that demonstrated gap junctional communication in microglia used Lucifer yellow [96, 192, 193, 195, 196]. The difference in the method used to evaluate functional gap junctional communication is relevant because GJCs are selective to molecules with different size and charges. In particular, Cx43 GJCs are less permeable to cationic than anionic dyes [199–201]. In addition, microglial GJCs were recently identified at ultrastructural level *in situ* between microglia and neural cell progenitors and also with nonidentified cells [202]. These data correlate with immunoreactivity of Cx43 at sites of apposition between the aforementioned

cells [202]. Finally, whether microglia establish GJCs *in vivo* allowing permeation of signaling or immunorelevant molecules remains controversial.

Recently, the expression of functional Cx and Panx HCs has been shown in microglia [73, 96, 187, 189, 203, 204]. Treatment with amyloid-β peptide increases Cx43 HC activity in microglial response, which in turn allows glutamate and ATP release [189]. Cx43 HC activity and ATP release are also increased by TNF-α/IFN-γ, but these two reactions are prevented by IL-6 [96]. These studies show that ATP is released through Cx43 HCs, although ATP might also be released by exocytosis [205]. In addition, Cx32 HC activity is increased in microglia treated with TNF-α and/or LPS, which induce glutamate release [187, 203, 204]. These findings suggest that a similar outcome in HC activity results from the action of different stimuli that trigger different intracellular signaling cascades.

A similar mechanism commands Panx1 HC activity, which can be enhanced by amyloid-β peptide and contributes to glutamate and ATP release [189]. In addition, TNF-α/IFN-γ increases Panx1 HC activity, leading to ATP release [96]. Moreover, microglial Panx1 HCs present an increased activity after exposure to high concentrations of ATP, which favor microglial migration [96, 190, 191]. Although exposure to TNF-α/IFN-γ or TNF-α/IL-1β does not affect the basal ATP-induced HC activity in microglia, IL-6 prevents the induction of Panx HC activity in cells treated with proinflammatory cytokines [96]. This inhibitory effect of IL-6 might downregulate microglial migration, as shown by arachidonic acid that closes Panx1 HCs [188]. Conversely to migration, Panx1 does not contribute to microglia proliferation at embryonic stages [206]. To sum up, these results suggest that microglia might migrate toward amyloid-β peptide plaques or ATP foci in a Panx1-dependent manner.

In addition, several studies show increased dye uptake or molecule release (e.g., ATP, glutamate) in activated microglia [192, 204, 207–209], but the use of Cx and Panx HC blockers (e.g., carbenoxolone) does not dissect the molecular entity that mediates the dye uptake. However, these experiments unveil that Cx and Panx HCs may contribute to neuronal death and host defense against pathogen infections. The latter seems to be mediated by IL-1 [208, 209]. In addition, recent studies show that HC blockers delay the development of Alzheimer' disease, amyotrophic lateral sclerosis, and multiple sclerosis in murine models of these diseases [204, 207], suggesting that HC blockers might be useful as a therapeutical approach to the treatment of these diseases. Interestingly, it was shown that carbenoxolone delays the onset of multiple sclerosis in mice by preventing the release of IL-23 from microglia and the polarization of Th17 cells [210]. Related to this last study, it may be possible that microglia communicate with T cells through Cx- and Panx1-based channels, determining the polarization of T cells. However, the heterocellular expression of GJCs between microglia and T cells, or the regulation of Cx- and Panx-based channels by IL-17, has not been addressed yet.

2.8. Neutrophils. These circulating leukocytes are the most abundant in the blood (50–70%), the first cells that arrive at the injury site after detection of chemokines and cytokines, and the first responders to most injuries sites. In addition, a new role has been shown in the maintenance of long-lived B cells by interacting at marginal zone in spleen [211, 212]. Although neutrophils express low or no MHC II and costimulatory molecules under resting conditions exposure to different cytokines, as occurring in chronic pathologies, leads to upregulation of MHC II expression in neutrophils and they acquire APC characteristics [213, 214]. Moreover, neutrophils perform MHC I-mediated cross-presentation and MHC II-mediated antigen presentation to T cells [214, 215]. In addition, murine neutrophils act as APCs and contribute to Th1 and Th17 cells polarization *in vitro* in absence of exogenous cytokines, and as expected those effects were MHC II-dependent [216]. Importantly, neutrophil-T-cell interaction promotes Th17 cell polarization independent of TGF-β and IL-6, suggesting that contact-dependent intercellular communication plays an important role in this process [216]. Thus, it is currently considered that neutrophils participate not only in early stages of innate immune responses, but also in further stages of adaptive immune responses, making their cellular interactions key steps for coordinating immune responses.

The study of Cxs in neutrophils began just two decades ago, and now it has been expanded to Panxs. Although no Cxs are detected in mouse or human circulating neutrophils, they expressed Cxs 37, 40, and Cx43 at mRNA and protein level after activation [17, 217–219]. However, some studies did not detect Cx43 in human blood neutrophils [220, 221]. However, this was expected considering that neutrophils were not stimulated.

Neutrophils form aggregates and communicate to each other through GJCs only after LPS or TNF-α exposure and in the presence of a cytokine containing endothelial cell-conditioned medium [217]. Nevertheless, the exact cytokine (or cytokine mixture) that induces expression of GJCs in neutrophils remains unknown. Additionally, neutrophils form functional GJCs with endothelial cells, which favor their neutrophil migration [142, 219]. In fact, there is ultrastructural evidence of gap junction formation between neutrophils and endothelial cells after ischemic injury [141]. Interestingly, TNF-α increases the neutrophil adhesion to endothelial cells as well as the migration *in vivo* in a Cx43-dependent manner [142]. However, *in vitro* studies have shown that TNF-α reduces the gap junctional communication between these cells [219], probably through a downregulation of endothelial Cxs. However, this apparent controversy might be due to differences between *in vivo* and *in vitro* studies, as well as the endothelial cell type used, timing of the response, stage of recruited neutrophil, and differences in microenvironment signals that command the inflammatory process. Similar differences occur in studies of neutrophil interactions with epithelial cells. While *in vivo* studies show that Cx43 contributes to neutrophil migration across an alveolar epithelial barrier in response to LPS [220], *in vitro* studies show absence of gap junctional communication between neutrophils and airway epithelial cells [221]. In addition, and supporting the contribution of Cx43 to cell-cell communication between the endothelium and neutrophils during extravasation, in several

studies downregulation of Cx43 reduces levels of neutrophil extravasation after burn injury, wound healing, and spinal cord damage [222–224]. Conversely, Cx40 deletion did not affect neutrophil migration [225], and the contribution of this protein to neutrophil activation is still unknown.

The expression of Cx and Panx HCs has been demonstrated in neutrophils. After activation, neutrophils present Cx43 reactive puncta on their surface [217] and release ATP through Cx43 HCs that favor migration without effect on adhesion to endothelial cells [218, 219]. Moreover, Panx1 HCs play a key role during neutrophil chemotaxis because their surface expression is polarized toward the leading edge where they allow ATP release and thus provide guidance for neutrophil migration [34, 226, 227]. It remains to be studied whether cytokines regulate Cx or Panx HC activity in neutrophils.

2.9. B Cells. B cells are also APCs because they present antigens in MHC II to $CD4^+$ T cells, which induce antibody production [135]. During this activation, B cells polarize toward the synapse, which determines whether the cell becomes effector or memory B cell [228, 229].

Expression of Cxs 40 and 43 has been demonstrated in isolated human B cells and at germinal centers of tonsil [92, 93]. Cx43 is also expressed in splenic B cells and some cell lines [230, 231]. Although endogenous functional expression of HCs remains unknown, Cx43 overexpression increases membrane permeability in a B cell line as expected [232]. Cx43 contributes to B cell spreading and adhesion. In fact, mutations that block the channel function of Cx43 impair the B-cell receptor- (BCR-) mediated spreading [230, 232]. However, Cx43 mutant expressed by B cells retained the ability to rearrange the cytoskeleton, conversely to B cells expressing a Cx43 with deletion of C-terminal. Unexpectedly, in this study no increase in dye uptake in resting or activated wild type B cells was found. In addition, blockade of HCs did not produce changes in BCR-induced cell spreading [232], suggesting that in these cells Cx43 contributes with a role to the intracellular signaling. It is worth mentioning that Cx43 colocalizes with actin in B cells and acts as a downstream signal for CXCL12-induced activation of Rap1 [233]. Moreover, downregulation of Cx43 impairs the CXCL12-induced migration and transendothelial migration [233], but whether HC activity contributes to B cell migration has not been studied yet.

Panx1 expression in B cells has not been reported, and unlike T cells there is no further evidence to suggest that B cells increase membrane permeability under certain conditions. Here, we present evidence that Panx1 is expressed in $B220^+$ B cells in mouse spleen (Figure 3). Moreover, freshly isolated murine B cells present Panx1 at the cell surface, suggesting that it might form functional HCs (Figure 6). Finally, although ATP stimulation did not induce dye uptake in B cells, it remains to be demonstrated whether antigen triggering affects the activity of Panx1 HCs.

Early studies showed the formation of GJCs between B cells and T cells that contribute to IgM synthesis [231], and also between B cells and FDCs [92, 93], suggesting a role for GJCs in B cell activation at immunological synapses. However, it is still unknown whether cytokines affect GJCs or HCs in B cells. Moreover, it remains to be elucidated whether other soluble cytokines such as IL-6, APRIL, BAFF, and TNF-α regulate the functional state of GJCs and/or HCs.

3. Concluding Remarks

The immune response efficiency relies on several homocellular and heterocellular interactions, which provide amplification to this response. Immune cells use different types of cellular communications, such as cytokines [3], exosomes [234], tunneling nanotubes [235], GJCs [17], and HCs. As shown here, all APCs express Cxs and/or Panxs and, in general, they are upregulated or redistributed after activation. GJCs and HCs contribute to almost all stages of the classical innate and adaptive immune response (Figure 7).

After injury, GJCs and HCs contribute to leukocyte extravasation [140–142, 146, 147, 219]. Panx1 HCs contribute to the recruitment of neutrophils and microglia toward the injury site [191, 227]. Although it remains controversial, it has been proposed that Cx43 contributes to phagocytosis [158]. Moreover, activated DCs, monocytes, macrophages, neutrophils, and microglia can communicate through GJCs [24, 86, 87, 96, 168, 176, 192, 196, 217], and HCs have been demonstrated in some of them. At this step, gap junctional communication might amplify the immune response because APCs might share specific information as antigen peptides [24, 25], which will increase the number of responding cells. Migratory DCs that arrive to lymph nodes present increased levels of Cx43 and Cx45 [86]. Recently, the expression of functional GJCs between DCs and T cells during immune synapse was shown to contribute to T-cell activation [13, 61], as it was previously suggested (Figure 7) [42, 52, 90, 236]. Prior to DC-T cell interaction, guidance of T-cell migration by extracellular signals induce specific Ca^{2+} dynamics that allow the establishment of kinapses and synapses, which correspond to short and long lasting interactions between these cells, respectively [237, 238]. Interestingly, recently it was shown that paracrine purinergic signaling modulates Ca^{2+} signaling in T cells in a $P2X_4$ and $P2X_7$ receptor-dependent manner, which ultimately reduce their motility [239]. Then, it is possible to anticipate that HCs might contribute to ATP release from mature DCs, which in the lymph nodes will help to establish DC-T-cell contact leading to antigen presentation.

It has been reported *in vitro* as well as *in situ* that human naïve $CD8^+$ T cells establish GJCs with melanoma target cells, contributing to their activation, but not to their lytic function [240]. Conversely, human NK cells establish GJCs with DCs and tumor cells in a Cx43-dependent process that contributes to NK cell-mediated lysis and further antitumoral immunity (Figure 7) [107]. Moreover, GJCs between polarized T cells (Th1 or Th2) have also been demonstrated [145]. Interestingly, Th1 and Th2 cells form GJCs with macrophages, but Th2 cells present lower levels of Cx43 [145], suggesting the possible involvement of other Cxs in this process. Similar to Th2 cells, in Th17 cells Cx43 is absent [241]. However, the expression of GJCs in these cells has not been shown. In addition, here

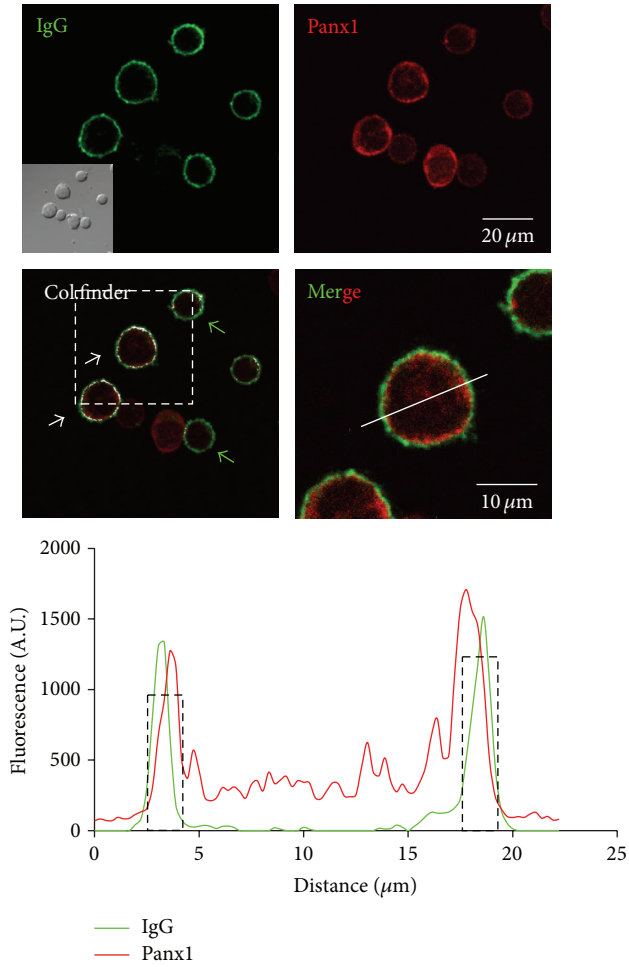

Figure 6: B cells present pannexin1 at the cell surface. Confocal images (Olympus, FluoView FV1000) of immunofluorescence analysis of freshly isolated B cells fixed in ethanol (70%). B cells were isolated from peripheral lymph nodes by positive selection from balb/c mice. Top left: B cells were identified with IgG (conjugated to FITC, green); the inset shows the bright field. Top right: pannexin1 (Panx1) immunoreactivity (red, primary antibody: rabbit anti-Panx1 antibody and secondary antibody goat anti-rabbit IgG conjugated to Cy3) is shown. Bar: 20 μm. Middle left: using ImageJ colocalization finder, it can be seen that Panx1 colocalizes with IgG (white) at the cell surface in some B cells (white arrows). B cells with low or no colocalization are indicated (green arrows). Middle right: zoom and merge of IgG and Panx1 labeling in a B cell denoted by a dotted square at middle left panel. The white line denotes the region used for the line scan. Bar: 10 μm. Bottom: ImageJ line scan analysis shows the fluorescence intensity of each channel through the white line in the middle of each cell. The peak coincidence (denoted by dotted squares) is an index of colocalization between the different fluorophores.

we showed that two polarizing cytokines (IFN-γ and IL-6) induce HC activity, but in combination they have antagonistic effects. This last fact is very important because it suggests that Cx GJCs and HCs might be involved in Th polarization, and different Cx profiles could be associated with a different phenotype.

During T-cell activation, expression of GJCs and HCs mainly constituted by Cx43 contributes to T-cell proliferation [81, 82, 231, 242]. In addition, it has been recently demonstrated that T cells also express functional Panx1 HCs during activation [243–246]. Indeed, GJCs are formed during T cell-B cell interactions [231, 247], as well as between B cells [231, 247], promoting immunoglobulin secretion. Here, we show that Panx1 is at the cell surface of B cell and might form HCs that might contribute to B cell activation. To produce high affinity antibodies, B cells must interact with FDCs, and GJCs contribute to this process (Figure 7) [92, 93].

In the peak of an immune response, lymphocytes should arrive at the affected tissue where GJCs are observed between T cells and endothelial cells (Figure 7) [248]. Also, Cx43 contributes to B cell spreading and adhesion [230, 232]. Consequently, it is possible that Cx43 and GJCs might be involved in this process *in vivo*. Moreover, Cx43 contributes to the development of Tregs [241], which transfer cAMP through GJCs and inhibit T-cell activation during resolution of immune response or immune suppression by Tregs [12]. Interestingly, GJCs between Tregs and DCs prevent the development of contact hypersensitivity reactions mediated by CD8 T cells [15]. Modulation of immune responses using "educated" immune cells was recently used to prevent allergy

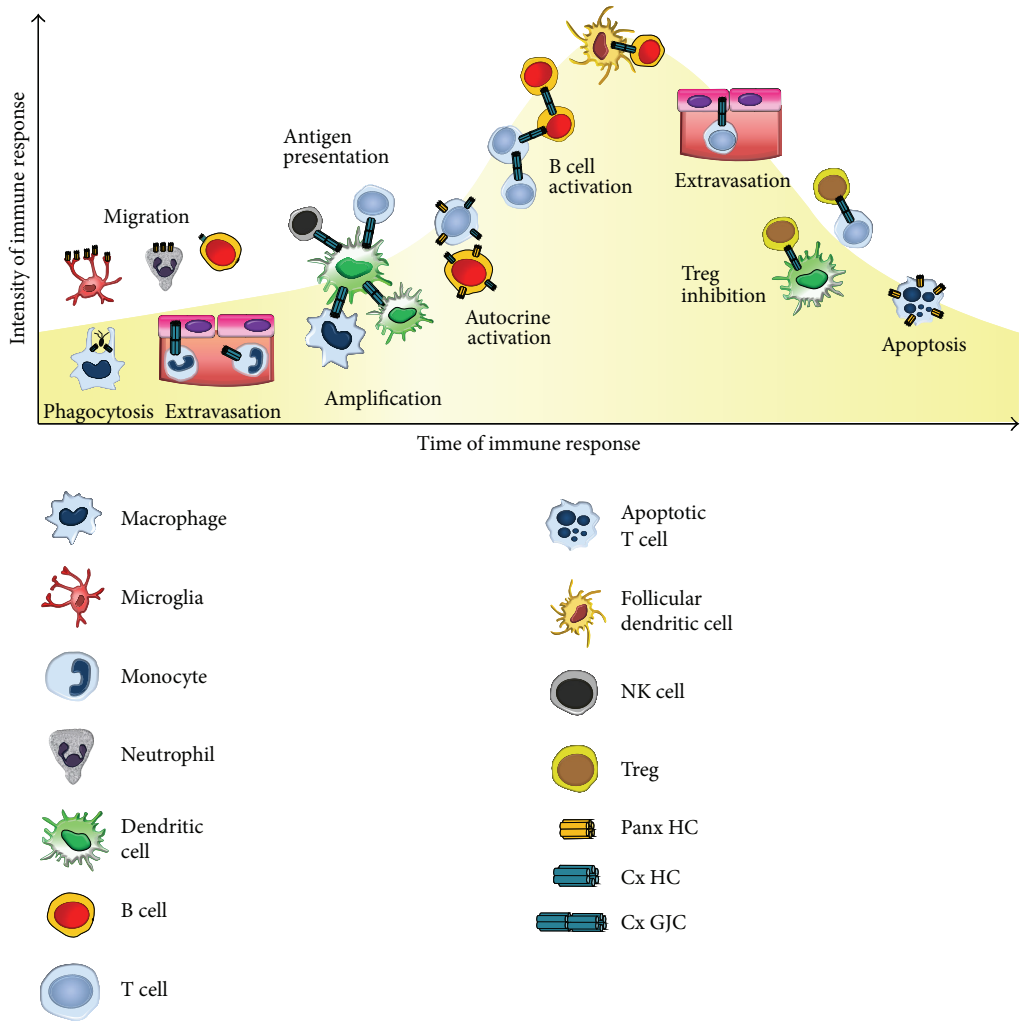

FIGURE 7: Scheme of different stages of classical immune response. The reported role for connexin- and pannexin-based channels is depicted in different immune cells functions as migration, antigen presentation, clonal expansion, and apoptosis.

reactions in mice. This effect was based on the generation of tolerogenic DCs after gap junctional communication with Tregs [249]. Another recent study showed that Tregs through GJCs are involved in controlling the HIV replication in T cells [250], opening an unexplored way to modulate the HIV infection.

We have summarized data showing that cytokines regulate both GJCs and HCs, which participate during most, if not all, steps of adaptive immune response. GJCs seem to be involved mainly in antigen presentation, whereas HCs are involved in functions such as migration or autocrine and paracrine activation. As presented here, during the lasts years a rising interest by immunologist in the field of cell-cell communication mediated by Cx- and Panx-based channels has driven many of the developments in the field. However, there is still much work to do because of more required technology transfer and collaboration between immunologists and "gap junctionologists." When the latter occurs, the GJC and HC regulation by cytokines might be used to provide an efficient immune response or to prevent or inhibit deleterious immune activation. Until recently, an important issue was the lack of specific tools to evaluate the role of GJC and HC activity *in vivo* during the immune response. A first interesting approach was used with the reconstitution of a mice previously irradiated [251]. In this chimeric mouse, a slight effect was observed during inflammation, and no gene dosage was observed [251], suggesting the possibility of gene compensation. However, recently two different murine models were developed to study the role of Cx43 in $CD11c^{+}$ cells, such as DCs and macrophages [106, 144]. These studies used *in vivo* imaging and tissue analysis to show the relevance of gap junctional communication between APCs and APCs or between APCs and epithelial cells [106, 144]. These tools have started a new age in the study of Cx43 in the immune response, even when a cell-specific K.O. for Panxs is still missing. However, compensation by other proteins might occur in these mice because the immune response should not rely only on the function of one protein, so the use of these tools should be analyzed in depth to avoid misinterpretations.

Finally, there is another possibility for the use of specific drug delivery to inhibit GJCs and HCs during *in vivo* responses, but the field of Cx- and Panx-based channel blockers is under development and mimetic peptides are not much specific [252]. However, new approaches are rising,

such as with the antibody $Cx43^{(E2)}$ which inhibits Cx43 HCs [253, 254]. After the development of these and other tools, the regulation by cytokines will open new possibilities to adjust the innate and adaptive immune responses.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] F. D. Batista and M. L. Dustin, "Cell: cell interactions in the immune system," *Immunological Reviews*, vol. 251, no. 1, pp. 7–12, 2013.

[2] G. Gasteiger and A. Y. Rudensky, "Interactions between innate and adaptive lymphocytes," *Nature Reviews Immunology*, vol. 14, pp. 631–639, 2014.

[3] R. Debets and H. F. J. Savelkoul, "Cytokines as cellular communicators," *Mediators of Inflammation*, vol. 5, no. 6, pp. 417–423, 1996.

[4] A. Miyajima, T. Kitamura, N. Harada, T. Yokota, and K. Arai, "Cytokine receptors and signal transduction," *Annual Review of Immunology*, vol. 10, pp. 295–331, 1992.

[5] J. Xie, C. M. Tato, and M. M. Davis, "How the immune system talks to itself: the varied role of synapses," *Immunological Reviews*, vol. 251, no. 1, pp. 65–79, 2013.

[6] M. L. Kapsenberg, "Dendritic-cell control of pathogen-driven T-cell polarization," *Nature Reviews Immunology*, vol. 3, no. 12, pp. 984–993, 2003.

[7] R. Dantzer, J. C. O'Connor, G. G. Freund, R. W. Johnson, and K. W. Kelley, "From inflammation to sickness and depression: when the immune system subjugates the brain," *Nature Reviews Neuroscience*, vol. 9, no. 1, pp. 46–56, 2008.

[8] I. B. McInnes and G. Schett, "Cytokines in the pathogenesis of rheumatoid arthritis," *Nature Reviews Immunology*, vol. 7, no. 6, pp. 429–442, 2007.

[9] G. Dranoff, "Cytokines in cancer pathogenesis and cancer therapy," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 11–22, 2004.

[10] A. Meager, "Cytokine regulation of cellular adhesion molecule expression in inflammation," *Cytokine and Growth Factor Reviews*, vol. 10, no. 1, pp. 27–39, 1999.

[11] V. Hornung, R. Hartamann, A. Ablasser, and K. P. Hopfner, "OAS proteins and cGAS: unifying concepts in sensing and responding to cytosolic nucleic acids," *Nature Reviews Immunology*, vol. 14, pp. 521–528, 2014.

[12] T. Bopp, C. Becker, M. Klein et al., "Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression," *The Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1303–1310, 2007.

[13] R. Elgueta, J. A. Tobar, K. F. Shoji et al., "Gap junctions at the dendritic cell-T cell interface are key elements for antigen-dependent T cell activation," *The Journal of Immunology*, vol. 183, no. 1, pp. 277–284, 2009.

[14] J. Neijssen, B. Pang, and J. Neefjes, "Gap junction-mediated intercellular communication in the immune system," *Progress in Biophysics and Molecular Biology*, vol. 94, no. 1-2, pp. 207–218, 2007.

[15] S. Ring, S. Karakhanova, T. Johnson, A. H. Enk, and K. Mahnke, "Gap junctions between regulatory T cells and dendritic cells prevent sensitization of $CD8^+$ T cells," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 1, pp. 237.e7–246.e7, 2010.

[16] F. Saccheri, C. Pozzi, F. Avogadri et al., "Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity," *Science Translational Medicine*, vol. 2, no. 44, Article ID 44ra57, 2010.

[17] J. C. Sáez, R. Araya, M. C. Brañes et al., "Chapter 25: gap junctions in inflammatory responses: connexins, regulation and possible functional roles," in *Current Topics in Membranes*, vol. 49 of *Gap Junctions—Molecular Basis of Cell Communication in Health and Disease*, pp. 555–579, 2000.

[18] J. C. Sáez, V. M. Berthoud, M. C. Brañes, A. D. Martínez, and E. C. Beyer, "Plasma membrane channels formed by connexins: their regulation and functions," *Physiological Reviews*, vol. 83, pp. 1359–1400, 2003.

[19] C. D'Hondt, J. Iyyathurai, M. Vinken et al., "Regulation of connexin- and pannexin-based channels by post-translational modifications," *Biology of the Cell*, vol. 105, no. 9, pp. 373–398, 2013.

[20] C. D'hondt, R. Ponsaerts, H. de Smedt, G. Bultynck, and B. Himpens, "Pannexins, distant relatives of the connexin family with specific cellular functions?" *BioEssays*, vol. 31, no. 9, pp. 953–974, 2009.

[21] A. Ablasser, J. L. Schmid-Burgk, I. Hemmerling et al., "Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP," *Nature*, vol. 503, pp. 530–534, 2013.

[22] A. L. Harris, "Connexin channel permeability to cytoplasmic molecules," *Progress in Biophysics and Molecular Biology*, vol. 94, no. 1-2, pp. 120–143, 2007.

[23] M. Katakowski, B. Buller, X. Wang, T. Rogers, and M. Chopp, "Functional microRNA is transferred between glioma cells," *Cancer Research*, vol. 70, no. 21, pp. 8259–8263, 2010.

[24] A. Mendoza-Naranjo, P. J. Saéz, C. C. Johansson et al., "Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells," *The Journal of Immunology*, vol. 178, no. 11, pp. 6949–6957, 2007.

[25] J. Neijssen, C. Herberts, J. W. Drijfhout, E. Reits, L. Janssen, and J. Neefjes, "Cross-presentation by intercellular peptide transfer through gap junctions," *Nature*, vol. 434, no. 7029, pp. 83–88, 2005.

[26] O. Tour, S. R. Adams, R. A. Kerr et al., "Calcium Green FlAsH as a genetically targeted small-molecule calcium indicator," *Nature Chemical Biology*, vol. 3, no. 7, pp. 423–431, 2007.

[27] R. Bruzzone, S. G. Hormuzdi, M. T. Barbe, A. Herb, and H. Monyer, "Pannexins, a family of gap junction proteins expressed in brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13644–13649, 2003.

[28] F. Vanden Abeele, G. Bidaux, D. Gordienko et al., "Functional implications of calcium permeability of the channel formed by pannexin 1," *The Journal of Cell Biology*, vol. 174, no. 4, pp. 535–546, 2006.

[29] M. Ishikawa, T. Iwamoto, T. Nakamura, A. Doyle, S. Fukumoto, and Y. Yamada, "Pannexin 3 functions as an ER Ca^{2+} channel, hemichannel, and gap junction to promote osteoblast differentiation," *Journal of Cell Biology*, vol. 193, no. 7, pp. 1257–1274, 2011.

[30] G. E. Sosinsky, D. Boassa, R. Dermietzel et al., "Pannexin channels are not gap junction hemichannels," *Channels*, vol. 5, no. 3, pp. 193–197, 2011.

[31] C. Ambrosi, O. Gassmann, J. N. Pranskevich et al., "Pannexin1 and pannexin2 channels show quaternary similarities to connexons and different oligomerization numbers from each other," *The Journal of Biological Chemistry*, vol. 285, no. 32, pp. 24420–24431, 2010.

[32] R. A. Romanov, M. F. Bystrova, O. A. Rogachevskaya, V. B. Sadovnikov, V. I. Shestopalov, and S. S. Kolesnikov, "The ATP permeability of pannexin 1 channels in a heterologous system and in mammalian taste cells is dispensable," *Journal of Cell Science*, vol. 125, no. 22, pp. 5514–5523, 2012.

[33] L. A. Cea, B. A. Cisterna, C. Puebla, M. Frank, and X. F. Figueroa, "De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, pp. 16229–16234, 2013.

[34] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[35] A. Baroja-Mazo, M. Barberà-Cremades, and P. Pelegrín, "The participation of plasma membrane hemichannels to purinergic signaling," *Biochimica et Biophysica Acta: Biomembranes*, vol. 1828, no. 1, pp. 79–93, 2013.

[36] E. Decrock, M. Vinken, M. Bol et al., "Calcium and connexin-based intercellular communication, a deadly catch?" *Cell Calcium*, vol. 50, no. 3, pp. 310–321, 2011.

[37] P. Pelegrin and A. Surprenant, "The P2X7 receptor—pannexin connection to dye uptake and IL-1β release," *Purinergic Signalling*, vol. 5, no. 2, pp. 129–137, 2009.

[38] J. E. Contreras, H. A. Sánchez, E. A. Eugenin et al., "Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 1, pp. 495–500, 2002.

[39] P. Pelegrin and A. Surprenant, "Pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated P2X7 receptor," *EMBO Journal*, vol. 25, no. 21, pp. 5071–5082, 2006.

[40] K. A. Schalper, N. Palacios-Prado, J. A. Orellana, and J. C. Sáez, "Currently used methods for identification and characterization of hemichannels," *Cell Communication and Adhesion*, vol. 15, no. 1-2, pp. 207–218, 2008.

[41] A. Koulakoff, X. Mei, J. A. Orellana, J. C. Sáez, and C. Giaume, "Glial connexin expression and function in the context of Alzheimer's disease," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1818, no. 8, pp. 2048–2057, 2012.

[42] J. C. Sáez, M. C. Brañes, L. A. Corvalán et al., "Gap junctions in cells of the immune system: structure, regulation and possible functional roles," *Brazilian Journal Of Medical and Biological Research*, vol. 33, pp. 447–455, 2000.

[43] J. D. Robertson, "The occurrence of a subunit pattern in the unit membranes of club endings in mauthner cell synapses in goldfish brains," *Journal of Cell Biology*, vol. 19, pp. 201–221, 1963.

[44] J. P. Revel, A. G. Yee, and A. J. Hudspeth, "Gap junctions between electrotonically coupled cells in tissue culture and in brown fat," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 68, no. 12, pp. 2924–2927, 1971.

[45] J. P. Revel and M. J. Karnovsky, "Hexagonal array of subunits in intercellular junctions of the mouse heart and liver," *The Journal of Cell Biology*, vol. 33, no. 3, pp. C7–C12, 1967.

[46] G. D. Pappas, Y. Asada, and M. V. Bennett, "Morphological correlates of increased coupling resistance at an electrotonic synapse," *Journal of Cell Biology*, vol. 49, no. 1, pp. 173–188, 1971.

[47] L. Barr, M. M. Dewey, and W. Berger, "Propagation of action potentials and the structure of the nexus in cardiac muscle," *The Journal of General Physiology*, vol. 48, pp. 797–823, 1965.

[48] I. Simpson, B. Rose, and W. R. Loewenstein, "Size limit of molecules permeating the junctional membrane channels," *Science*, vol. 195, no. 4275, pp. 294–296, 1977.

[49] J. C. Sáez, J. A. Connor, D. C. Spray, and M. V. L. Bennett, "Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 8, pp. 2708–2712, 1989.

[50] R. W. Tsien and R. Weingart, "Inotropic effect of cyclic AMP in calf ventricular muscle studied by a cut end method," *Journal of Physiology*, vol. 260, no. 1, pp. 117–141, 1976.

[51] C. U. Brand, T. Hunziker, T. Schaffner, A. Limat, H. A. Gerber, and L. R. Braathen, "Activated immunocompetent cells in human skin lymph derived from irritant contact dermatitis: an immunomorphological study," *The British Journal of Dermatology*, vol. 132, no. 1, pp. 39–45, 1995.

[52] M. Concha, A. Vidal, G. Garces, C. D. Figueroa, and I. Caorsi, "Physical interaction between Langerhans cells and T-lymphocytes during antigen presentation *in vitro*," *Journal of Investigative Dermatology*, vol. 100, no. 4, pp. 429–434, 1993.

[53] V. Valiunas, Y. Y. Polosina, H. Miller et al., "Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions," *Journal of Physiology*, vol. 568, no. 2, pp. 459–468, 2005.

[54] E. Tsitsiou and M. A. Lindsay, "microRNAs and the immune response," *Current Opinion in Pharmacology*, vol. 9, no. 4, pp. 514–520, 2009.

[55] A. Aucher, D. Rudnicka, and D. M. Davis, "MicroRNAs transfer from human macrophages to hepato-carcinoma cells and inhibit proliferation," *The Journal of Immunology*, vol. 191, pp. 6250–6260, 2013.

[56] M. Vig and J. Kinet, "Calcium signaling in immune cells," *Nature Immunology*, vol. 10, no. 1, pp. 21–27, 2009.

[57] D. G. Gossman and H.-B. Zhao, "Hemichannel-mediated inositol 1,4,5-trisphosphate (IP_3) release in the cochlea: a novel mechanism of IP3 intercellular signaling," *Cell Communication & Adhesion*, vol. 15, no. 4, pp. 305–315, 2008.

[58] K. Braet, W. Vandamme, P. E. M. Martin, W. H. Evans, and L. Leybaert, "Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26," *Cell Calcium*, vol. 33, no. 1, pp. 37–48, 2003.

[59] A. T. Miller, P. P. Chamberlain, and M. P. Cooke, "Beyond IP3: roles for higher order inositol phosphates in immune cell signaling," *Cell Cycle*, vol. 7, no. 4, pp. 463–467, 2008.

[60] W. Yang, M. K. Nurbaeva, E. Schmid et al., "Akt2- and ETS1-dependent IP3 receptor 2 expression in dendritic cell migration," *Cellular Physiology and Biochemistry*, vol. 33, pp. 222–236, 2014.

[61] A. Mendoza-Naranjo, G. Bouma, C. Pereda et al., "Functional gap junctions accumulate at the immunological synapse and contribute to T cell activation," *Journal of Immunology*, vol. 187, no. 6, pp. 3121–3132, 2011.

[62] H. A. Sánchez, J. A. Orellana, V. K. Verselis, and J. C. Sáez, "Metabolic inhibition increases activity of connexin-32 hemichannels permeable to Ca2+ in transfected HeLa cells," *The American Journal of Physiology—Cell Physiology*, vol. 297, no. 3, pp. C665–C678, 2009.

[63] M. C. Fiori, V. Figueroa, M. E. Zoghbi, J. C. Saéz, L. Reuss, and G. A. Altenberg, "Permeation of calcium through purified connexin 26 hemichannels," *The Journal of Biological Chemistry*, vol. 287, no. 48, pp. 40826–40834, 2012.

[64] V. Figueroa, P. J. Sáez, J. D. Salas et al., "Linoleic acid induces opening of connexin26 hemichannels through a PI3K/Akt/Ca^{2+}-dependent pathway," *Biochimica et Biophysica Acta*, vol. 1828, no. 3, pp. 1169–1179, 2013.

[65] M. De Bock, N. Wang, M. Bol et al., "Connexin 43 hemichannels contribute to cytoplasmic Ca^{2+} oscillations by providing a bimodal Ca^{2+}-dependent Ca^{2+} entry pathway," *Journal of Biological Chemistry*, vol. 287, no. 15, pp. 12250–12266, 2012.

[66] K. A. Schalper, H. A. Sánchez, S. C. Lee, G. A. Altenberg, M. H. Nathanson, and J. C. Sáez, "Connexin 43 hemichannels mediate the Ca^{2+} influx induced by extracellular alkalinization," *The American Journal of Physiology: Cell Physiology*, vol. 299, no. 6, pp. C1504–C1515, 2010.

[67] H. A. Sánchez, G. Meşe, M. Srinivas, T. W. White, and V. K. Verselis, "Differentially altered Ca2+ regulation and Ca2+ permeability in Cx26 hemichannels formed by the A40V and G45E mutations that cause keratitis ichthyosis deafness syndrome," *Journal of General Physiology*, vol. 136, no. 1, pp. 47–62, 2010.

[68] F. Malavasi, S. Deaglio, A. Funaro et al., "Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology," *Physiological Reviews*, vol. 88, no. 3, pp. 841–886, 2008.

[69] S. Bruzzone, L. Franco, L. Guida et al., "A self-restricted CD38-connexin 43 cross-talk affects NAD+ and cyclic ADP-ribose metabolism and regulates intracellular calcium in 3T3 fibroblasts," *Journal of Biological Chemistry*, vol. 276, no. 51, pp. 48300–48308, 2001.

[70] S. Bruzzone, L. Guida, E. Zocchi, L. Franco, and A. de Flora, "Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells," *The FASEB Journal*, vol. 15, no. 1, pp. 10–12, 2001.

[71] X. J. Xu, M. Boumechache, L. E. Robinson et al., "Splice variants of the P2X7 receptor reveal differential agonist dependence and functional coupling with pannexin-1," *Journal of Cell Science*, vol. 125, no. 16, pp. 3776–3789, 2012.

[72] E. Song, S. Rah, Y. Lee et al., "Connexin-43 hemichannels mediate cyclic ADP-ribose generation and its Ca^{2+}-mobilizing activity by NAD^+/cyclic ADP-ribose transport," *The Journal of Biological Chemistry*, vol. 286, no. 52, pp. 44480–44490, 2011.

[73] Y. Ma, W. Cao, L. Wang et al., "Basal CD38/cyclic ADP-ribose-dependent signaling mediates ATP release and survival of microglia by modulating connexin 43 hemichannels," *Glia*, vol. 62, no. 6, pp. 943–955, 2014.

[74] M. J. L. Bours, E. L. R. Swennen, F. di Virgilio, B. N. Cronstein, and P. C. Dagnelie, "Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation," *Pharmacology and Therapeutics*, vol. 112, no. 2, pp. 358–404, 2006.

[75] H. Kono and K. L. Rock, "How dying cells alert the immune system to danger," *Nature Reviews Immunology*, vol. 8, no. 4, pp. 279–289, 2008.

[76] N. Wang, M. de Bock, E. Decrock et al., "Paracrine signaling through plasma membrane hemichannels," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1828, no. 1, pp. 35–50, 2013.

[77] M. Rodriguez, E. Domingo, C. Municio, Y. Alvarez, and E. Hugo, "Polarization of the innate immune response by prostaglandin E2: a puzzle of receptors and signals," *Molecular Pharmacology*, vol. 85, pp. 187–197, 2014.

[78] B. Cheng, Y. Kato, S. Zhao et al., "PGE2 is essential for gap junction-mediated Intercellular communication between osteocyte-like MLO-Y4 cells in response to mechanical strain," *Endocrinology*, vol. 142, no. 8, pp. 3464–3473, 2001.

[79] J. X. Jiang and P. P. Cherian, "Hemichannels formed by connexin 43 play an important role in the release of protaglandin E2 by osteocytes in response to mechanical strain," *Cell Communication and Adhesion*, vol. 10, no. 4–6, pp. 259–264, 2003.

[80] M. Barbera-Cremades, A. Baroja-Mazo, A. I. Gomez, F. Machado, F. Di Virgilio, and P. Pelegriń, "P2X7 receptor-stimulation causes fever via PGE2 and IL-1β release," *The FASEB Journal*, vol. 26, no. 7, pp. 2951–2962, 2012.

[81] D. F. Hülser and J. H. Peters, "Intercellular communication in phytohemagglutinin-induced lymphocyte agglutinates," *European Journal of Immunology*, vol. 1, no. 6, pp. 494–495, 1971.

[82] D. F. Hülser and J. H. Peters, "Contact cooperation in stimulated lymphocytes. II. Electrophysiological investigations on intercellular communication," *Experimental Cell Research*, vol. 74, no. 2, pp. 319–326, 1972.

[83] E. A. Eugenín, "Role of Connexin/Pannexin containing channels in infectious diseases," *FEBS Letters*, vol. 588, no. 8, pp. 1389–1395, 2014.

[84] D. Gao, J. Wu, Y. Wu et al., "Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses," *Science*, vol. 341, no. 6148, pp. 903–906, 2013.

[85] X. Lahaye, T. Satoh, M. Gentili et al., "The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells," *Immunity*, vol. 39, no. 6, pp. 1132–1142, 2013.

[86] L. A. Corvalán, R. Araya, M. C. Brañes et al., "Injury of skeletal muscle and specific cytokines induce the expression of gap junction channels in mouse dendritic cells," *Journal of Cellular Physiology*, vol. 211, pp. 649–660, 2007.

[87] H. Matsue, J. Yao, K. Matsue et al., "Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs," *The Journal of Immunology*, vol. 176, no. 1, pp. 181–190, 2006.

[88] M. Porvaznik and T. J. MacVittie, "Detection of gap junctions between the progeny of a canine macrophage colony-forming cell in vitro," *Journal of Cell Biology*, vol. 82, no. 2, pp. 555–564, 1979.

[89] J. A. Levy, R. M. Weiss, E. R. Dirksen, and M. R. Rosen, "Possible communication between murine macrophages oriented in linear chains in tissue culture," *Experimental Cell Research*, vol. 103, no. 2, pp. 375–385, 1976.

[90] M. Concha, C. D. Figueroa, and I. Caorsi, "Ultrastructural characteristics of the contact zones between Langerhans cells and lymphocytes," *Journal of Pathology*, vol. 156, no. 1, pp. 29–36, 1988.

[91] T. Krenacs and M. Rosendaal, "Immunohistological detection of gap junctions in human lymphoid tissue: connexin43 in follicular dendritic and lymphoendothelial cells," *Journal of Histochemistry and Cytochemistry*, vol. 43, no. 11, pp. 1125–1137, 1995.

[92] T. Krenacs and M. Rosendaal, "Gap-junction communication pathways in germinal center reactions," *Developmental Immunology*, vol. 6, no. 1-2, pp. 111–118, 1998.

[93] T. Krenacs, M. Van Dartel, E. Lindhout, and M. Rosendaal, "Direct cell/cell communication in the lymphoid germinal center: connexin43 gap junctions functionally couple follicular dendritic cells to each other and to B lymphocytes," *European Journal of Immunology*, vol. 27, no. 6, pp. 1489–1497, 1997.

[94] L. A. Alves, R. Coutinho-Silva, P. M. Persechini, D. C. Spray, W. Savino, and A. C. Campos de Carvalho, "Are there functional gap junctions or junctional hemichannels in macrophages?" *Blood*, vol. 88, no. 1, pp. 328–334, 1996.

[95] J. A. Orellana, P. J. Sáez, K. F. Shoji et al., "Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration," *Antioxidants and Redox Signaling*, vol. 11, no. 2, pp. 369–399, 2009.

[96] P. J. Sáez, K. F. Shoji, M. A. Retamal et al., "ATP is required and advances cytokine-induced gap junction formation in microglia in vitro," *Mediators of Inflammation*, vol. 2013, Article ID 216402, 16 pages, 2013.

[97] R. M. Steinman and Z. A. Cohn, "Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution," *The Journal of Experimental Medicine*, vol. 137, no. 5, pp. 1142–1162, 1973.

[98] K. Liu and M. C. Nussenzweig, "Origin and development of dendritic cells," *Immunological Reviews*, vol. 234, no. 1, pp. 45–54, 2010.

[99] R. Sluyter and J. S. Wiley, "Extracellular adenosine 5′-triphosphate induces a loss of CD23 from human dendritic cells via activation of $P2X_7$ receptors," *International Immunology*, vol. 14, no. 12, pp. 1415–1421, 2002.

[100] O. K. Nihei, A. C. C. de Carvalho, W. Savino, and L. A. Alves, "Pharmacologic properties of P_{2Z}/$P2X_7$ receptor characterized in murine dendritic cells: role on the induction of apoptosis," *Blood*, vol. 96, no. 3, pp. 996–1005, 2000.

[101] R. Coutinho-Silva, P. M. Persechini, R. Da Cunha Bisaggio et al., "P(2Z)/P2X7 receptor-dependent apoptosis of dendritic cells," *The American Journal of Physiology—Cell Physiology*, vol. 276, no. 5, pp. C1139–C1147, 1999.

[102] J. G. Georgiou, K. K. Skarratt, S. J. Fuller et al., "Human epidermal and monocyte-derived langerhans cells express functional P2X7 receptors," *Journal of Investigative Dermatology*, vol. 125, no. 3, pp. 482–490, 2005.

[103] Y. Kurashima, T. Amiya, T. Nochi et al., "Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors," *Nature Communications*, vol. 3, article 1034, 2012.

[104] V. I. Shestopalov and Y. Panchin, "Pannexins and gap junction protein diversity," *Cellular and Molecular Life Sciences*, vol. 65, no. 3, pp. 376–394, 2008.

[105] Y. He, L. Franchi, and G. Núñez, "TLR agonists stimulate Nlrp3-dependent IL-1β production independently of the purinergic P2X7 receptor in dendritic cells and in vivo," *Journal of Immunology*, vol. 190, no. 1, pp. 334–339, 2013.

[106] E. Mazzini, L. Massimiliano, G. Penna, and M. Rescigno, "Oral tolerance can be established via gap junction transfer of fed antigens from $CX3CR1^+$ macrophages to $CD103^+$ dendritic cells," *Immunity*, vol. 40, no. 2, pp. 248–261, 2014.

[107] A. Tittarelli, A. Mendoza-Naranjo, M. Farias et al., "Gap junction intercellular communications regulate NK cell activation and modulate NK cytotoxic capacity," *Journal of Immunology*, vol. 192, pp. 1313–1319, 2014.

[108] S. C. Zimmerli, F. Masson, J. Cancela, P. Meda, and C. Hauser, "Cutting edge: lack of evidence for connexin-43 expression in human epidermal langerhans cells," *The Journal of Immunology*, vol. 179, no. 7, pp. 4318–4321, 2007.

[109] L. S. Deluca and J. L. Gommerman, "Fine-tuning of dendritic cell biology by the TNF superfamily," *Nature Reviews Immunology*, vol. 12, no. 5, pp. 339–351, 2012.

[110] J. E. Sims and D. E. Smith, "The IL-1 family: regulators of immunity," *Nature Reviews Immunology*, vol. 10, no. 2, pp. 89–102, 2010.

[111] I. Fricke, D. Mitchell, J. Mittelstädt et al., "Mycobacteria induce IFN-γ production in human dendritic cells via triggering of TLR2," *The Journal of Immunology*, vol. 176, no. 9, pp. 5173–5182, 2006.

[112] D. M. Frucht, T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu, "IFN-γ production by antigen-presenting cells: mechanisms emerge," *Trends in Immunology*, vol. 22, no. 10, pp. 556–560, 2001.

[113] E. M. Coccia, "IFN regulation and functions in myeloid dendritic cells," *Cytokine and Growth Factor Reviews*, vol. 19, no. 1, pp. 21–32, 2008.

[114] L. C. Platanias, "Mechanisms of type-I- and type-II-interferon-mediated signalling," *Nature Reviews Immunology*, vol. 5, no. 5, pp. 375–386, 2005.

[115] T. Hirano, K. Yasukawa, H. Harada et al., "Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin," *Nature*, vol. 324, no. 6092, pp. 73–76, 1986.

[116] A. Kimura and T. Kishimoto, "IL-6: regulator of Treg/Th17 balance," *European Journal of Immunology*, vol. 40, no. 7, pp. 1830–1835, 2010.

[117] M. F. Neurath and S. Finotto, "IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer," *Cytokine and Growth Factor Reviews*, vol. 22, no. 2, pp. 83–89, 2011.

[118] B. K. Pedersen, "IL-6 signalling in exercise and disease," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1295–1297, 2007.

[119] Z. Xing, J. Gauldie, G. Cox et al., "IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses," *Journal of Clinical Investigation*, vol. 101, no. 2, pp. 311–320, 1998.

[120] S. J. Park, T. Nakagawa, H. Kitamura et al., "IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation," *Journal of Immunology*, vol. 173, no. 6, pp. 3844–3854, 2004.

[121] A. Yoshimura, T. Naka, and M. Kubo, "SOCS proteins, cytokine signalling and immune regulation," *Nature Reviews Immunology*, vol. 7, no. 6, pp. 454–465, 2007.

[122] P. Langerhans, "Ueber die Nerven der menschlichen Haut," *Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin*, vol. 44, no. 2-3, pp. 325–337, 1868.

[123] G. Stingl, K. Tamaki, and S. I. Katz, "Origin and function of epidermal Langerhans cells," *Immunological Reviews*, vol. 53, pp. 149–174, 1980.

[124] T. Hieronymus, M. Zenke, J. H. Baek, and K. Sere, "The clash of Langerhans cell homeostasis in skin: should I stay or should I go?" *Seminars in Cell & Developmental Biology*, 2014.

[125] L. G. Ng, A. Hsu, M. A. Mandell et al., "Migratory dermal dendritic cells act as rapid sensors of protozoan parasites," *PLoS Pathogens*, vol. 4, no. 11, p. e1000222, 2008.

[126] J. Banchereau, L. Thompson-Snipes, S. Zurawski et al., "The differential production of cytokines by human Langerhans cells and dermal CD14 + DCs controls CTL priming," *Blood*, vol. 119, no. 24, pp. 5742–5749, 2012.

[127] R. E. Hunger, P. A. Sieling, M. T. Ochoa et al., "Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells," *Journal of Clinical Investigation*, vol. 113, no. 5, pp. 701–708, 2004.

[128] N. Romani, A. Lenz, H. Glassel et al., "Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function," *Journal of Investigative Dermatology*, vol. 93, no. 5, pp. 600–609, 1989.

[129] M. Merad, F. Ginhoux, and M. Collin, "Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 935–947, 2008.

[130] N. Romani, P. M. Brunner, and G. Stingl, "Changing views of the role of langerhans cells," *Journal of Investigative Dermatology*, vol. 132, no. 3, pp. 872–881, 2012.

[131] S. J. Celetti, K. N. Cowan, S. Penuela, Q. Shao, J. Churko, and D. W. Laird, "Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation," *Journal of Cell Science*, vol. 123, no. 8, pp. 1363–1372, 2010.

[132] J. N. S. N. Tran, A. Pupovac, R. M. Taylor, J. S. Wiley, S. N. Byrne, and R. Sluyter, "Murine epidermal Langerhans cells and keratinocytes express functional P2X7 receptors," *Experimental Dermatology*, vol. 19, no. 8, pp. e151–e157, 2010.

[133] R. D. Granstein, W. Ding, J. Huang et al., "Augmentation of cutaneous immune responses by ATPγS: purinergic agonists define a novel class of immunologic adjuvants," *Journal of Immunology*, vol. 174, no. 4, pp. 7725–7731, 2005.

[134] M. E. M. El Shikh and C. Pitzalis, "Follicular dendritic cells in health and disease," *Frontiers in Immunology*, vol. 3, article 292, 2012.

[135] F. D. Batista and N. E. Harwood, "The who, how and where of antigen presentation to B cells," *Nature Reviews Immunology*, vol. 9, no. 1, pp. 15–27, 2009.

[136] C. Shi and E. G. Pamer, "Monocyte recruitment during infection and inflammation," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 762–774, 2011.

[137] S. Gordon and P. R. Taylor, "Monocyte and macrophage heterogeneity," *Nature Reviews Immunology*, vol. 5, no. 12, pp. 953–964, 2005.

[138] E. Asgari, G. Le Friec, H. Yamamoto et al., "C3a modulates IL-1beta secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation," *Blood*, vol. 122, pp. 3473–3481, 2013.

[139] B. Caillou, M. Talbot, U. Weyemi et al., "Tumor-Associated macrophages (TAMs) form an interconnected cellular supportive network in anaplastic thyroid carcinoma," *PLoS ONE*, vol. 6, no. 7, Article ID e22567, 2011.

[140] E. A. Eugenín, M. C. Brañes, J. W. Berman, and J. C. Sáez, "TNF-α plus IFN-γ induce connexin43 expression and formation of gap junctions between human monocytes/macrophages that enhance physiological responses," *The Journal of Immunology*, vol. 170, no. 3, pp. 1320–1328, 2003.

[141] P. I. Jara, M. P. Boric, and J. C. Sáez, "Leukocytes express connexin 43 after activation with lipopolysaccharide and appear to form gap junctions with endothelial cells after ischemia-reperfusion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 7011–7015, 1995.

[142] L. P. Véliz, F. G. González, B. R. Duling, J. C. Sáez, and M. P. Boric, "Functional role of gap junctions in cytokine-induced leukocyte adhesion to endothelium in vivo," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 3, pp. H1056–H1066, 2008.

[143] S. K. Wasseff and S. S. Scherer, "Activated microglia do not form functional gap junctions in vivo," *Journal of Neuroimmunology*, vol. 269, no. 1-2, pp. 90–93, 2014.

[144] K. Westphalen, G. A. Gusarova, M. N. Islam et al., "Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity," *Nature*, vol. 506, pp. 503–506, 2014.

[145] A. Bermudez-Fajardo, M. Ylihärsilä, W. H. Evans, A. C. Newby, and E. Oviedo-Orta, "CD4+ T lymphocyte subsets express connexin 43 and establish gap junction channel communication with macrophages in vitro," *Journal of Leukocyte Biology*, vol. 82, no. 3, pp. 608–612, 2007.

[146] D. Yuan, Q. Wang, D. Wu et al., "Monocyte-endothelial adhesion is modulated by Cx43-stimulated ATP release from monocytes," *Biochemical and Biophysical Research Communications*, vol. 420, no. 3, pp. 536–541, 2012.

[147] C. W. Wong, T. Christen, I. Roth et al., "Connexin37 protects against atherosclerosis by regulating monocyte adhesion," *Nature Medicine*, vol. 12, no. 8, pp. 950–954, 2006.

[148] B. J. Gu, W. Y. Zhang, L. J. Bendall, I. P. Chessell, G. N. Buell, and J. S. Wiley, "Expression of P2X(7) purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X7 receptors," *American Journal of Physiology: Cell Physiology*, vol. 279, no. 4, pp. C1189–C1197, 2000.

[149] S. R. Ali, A. M. Timmer, S. Bilgrami et al., "Anthrax toxin induces macrophage death by p38 MAPK inhibition but leads to inflammasome activation via ATP leakage," *Immunity*, vol. 35, no. 1, pp. 34–44, 2011.

[150] R. J. Anand, S. Dai, S. C. Gribar et al., "A role for connexin43 in macrophage phagocytosis and host survival after bacterial peritoneal infection," *Journal of Immunology*, vol. 181, no. 12, pp. 8534–8543, 2008.

[151] E. C. Beyer and T. H. Steinberg, "Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages," *The Journal of Biological Chemistry*, vol. 266, no. 13, pp. 7971–7974, 1991.

[152] F. S. A. Fortes, I. L. Pecora, P. M. Persechini et al., "Modulation of intercellular communication in macrophages: possible interactions between GAP junctions and P2 receptors," *Journal of Cell Science*, vol. 117, no. 20, pp. 4717–4726, 2004.

[153] A. M. Glass, B. J. Wolf, K. M. Schneider, M. F. Princiotta, and S. M. Taffet, "Connexin43 is dispensable for phagocytosis," *Journal of Immunology*, vol. 190, no. 9, pp. 4830–4835, 2013.

[154] M. M. Monick, A. B. Carter, P. K. Robeff, D. M. Flaherty, M. W. Peterson, and G. W. Hunninghake, "Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of β-catenin," *The Journal of Immunology*, vol. 166, no. 7, pp. 4713–4720, 2001.

[155] D. Polacek, R. Lal, M. V. Volin, and P. F. Davies, "Gap junctional communication between vascular cells: induction of connexin43 messenger RNA in macrophage foam cells of atherosclerotic lesions," *The American Journal of Pathology*, vol. 142, no. 2, pp. 593–606, 1993.

[156] G. Ayna, D. V. Krysko, A. Kaczmarek, G. Petrovski, P. Vandenabeele, and L. Fésüs, "ATP release from dying autophagic cells and their phagocytosis are crucial for inflammasome activation in macrophages," *PLoS ONE*, vol. 7, no. 6, Article ID e40069, 2012.

[157] X. F. Figueroa, M. A. Lillo, P. S. Gaete, M. A. Riquelme, and J. C. Sáez, "Diffusion of nitric oxide across cell membranes of the vascular wall requires specific connexin-based channels," *Neuropharmacology*, vol. 75, pp. 471–478, 2013.

[158] R. J. Anand, S. Dai, C. Rippel et al., "Activated macrophages inhibit enterocyte gap junctions via the release of nitric oxide," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 294, no. 1, pp. G109–G119, 2008.

[159] P. Pelegrin and A. Surprenant, "Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1β release through a dye uptake-independent pathway," *Journal of Biological Chemistry*, vol. 282, no. 4, pp. 2386–2394, 2007.

[160] G. Lopez-Castejón, A. Baroja-Mazo, and P. Pelegrín, "Novel macrophage polarization model: from gene expression to identification of new anti-inflammatory molecules," *Cellular and Molecular Life Sciences*, vol. 68, no. 18, pp. 3095–3107, 2011.

[161] F. O. Martínez and S. Gordon, "The M1 and M2 paradigm of macrophage activation: time for reassessment," *F1000Prime Reports*, vol. 6, article 13, 2014.

[162] M. J. Kiel and S. J. Morrison, "Uncertainty in the niches that maintain haematopoietic stem cells," *Nature Reviews Immunology*, vol. 8, no. 4, pp. 290–301, 2008.

[163] A. W. Thomson and P. A. Knolle, "Antigen-presenting cell function in the tolerogenic liver environment," *Nature Reviews Immunology*, vol. 10, no. 11, pp. 753–766, 2010.

[164] K. Pulford and R. L. Souhami, "Cell division and giant cell formation in Kupffer cell cultures," *Clinical and Experimental Immunology*, vol. 42, no. 1, pp. 67–76, 1980.

[165] H. Ishibashi, M. Nakamura, A. Komori, K. Migita, and S. Shimoda, "Liver architecture, cell function, and disease," *Seminars in Immunopathology*, vol. 31, no. 3, pp. 399–409, 2009.

[166] L. Beattie, A. Peltan, A. Maroof et al., "Dynamic imaging of experimental Leishmania donovani-induced hepatic granulomas detects kupffer cell-restricted antigen presentation to antigen-specific CD8 T cells," *PLoS Pathogens*, vol. 6, no. 3, Article ID e1000805, 2010.

[167] P. A. Knolle and G. Gerken, "Local control of the immune response in the liver," *Immunological Reviews*, vol. 174, pp. 21–34, 2000.

[168] E. A. Eugenín, H. E. González, H. A. Sanchez, M. C. Brañes, and J. C. Sáez, "Inflammatory conditions induce gap junctional communication between rat Kupffer cells both in vivo and in vitro," *Cellular Immunology*, vol. 247, pp. 103–110, 2007.

[169] H. E. González, E. A. Eugenín, and G. Garcés, "Regulation of hepatic connexins in cholestasis: possible involvement of Kupffer cells and inflammatory mediators," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 282, pp. G991–G1001, 2002.

[170] H. Takayanagi, "Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems," *Nature Reviews Immunology*, vol. 7, no. 4, pp. 292–304, 2007.

[171] Z. S. Buchwald and R. Aurora, "Osteoclasts and CD8 T cells form a negative feedback loop that contributes to homeostasis of both the skeletal and immune systems," *Clinical and Developmental Immunology*, vol. 2013, Article ID 429373, 9 pages, 2013.

[172] N. Batra, R. Kar, and J. X. Jiang, "Gap junctions and hemichannels in signal transmission, function and development of bone," *Biochimica et Biophysica Acta: Biomembranes*, vol. 1818, no. 8, pp. 1909–1918, 2012.

[173] M. Watkins, S. K. Grimston, J. Y. Norris et al., "Osteoblast connexin43 modulates skeletal architecture by regulating both arms of bone remodeling," *Molecular Biology of the Cell*, vol. 22, no. 8, pp. 1240–1251, 2011.

[174] S. Tsuchida, Y. Arai, T. Kishida et al., "Silencing the expression of connexin 43 decreases inflammation and joint destruction in experimental arthritis," *Journal of Orthopaedic Research*, vol. 31, no. 4, pp. 525–530, 2013.

[175] Y. Zhang, E. M. Paul, V. Sathyendra et al., "Enhanced osteoclastic resorption and responsiveness to mechanical load in gap junction deficient bone," *PLoS ONE*, vol. 6, no. 8, Article ID e23516, 2011.

[176] A. F. Schilling, S. Filke, T. Lange et al., "Gap junctional communication in human osteoclasts *in vitro* and *in vivo*," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 6A, pp. 2497–2504, 2008.

[177] K. Herde, S. Hartmann, R. Brehm et al., "Connexin 43 expression of foreign body giant cells after implantation of nanoparticulate hydroxyapatite," *Biomaterials*, vol. 28, no. 33, pp. 4912–4921, 2007.

[178] S. F. Matemba, A. Lie, and M. Ransjö, "Regulation of osteoclastogenesis by gap junction communication," *Journal of Cellular Biochemistry*, vol. 99, no. 2, pp. 528–537, 2006.

[179] M. Ransjö, J. Sahli, and A. Lie, "Expression of connexin 43 mRNA in microisolated murine osteoclasts and regulation of bone resorption in vitro by gap junction inhibitors," *Biochemical and Biophysical Research Communications*, vol. 303, no. 4, pp. 1179–1185, 2003.

[180] J. Ilvesaro, P. Tavi, and J. Tuukkanen, "Connexin-mimetic peptide Gap 27 decreases osteoclastic activity," *BMC Musculoskeletal Disorders*, vol. 2, article 10, 2001.

[181] J. Ilvesaro, K. Väänänen, and J. Tuukkanen, "Bone-resorbing osteoclasts contain gap-junctional connexin-43," *Journal of Bone and Mineral Research*, vol. 15, no. 5, pp. 919–926, 2000.

[182] S. J. Jones, C. Gray, H. Sakamaki et al., "The incidence and size of gap junctions between the bone cells in rat calvaria," *Anatomy and Embryology*, vol. 187, no. 4, pp. 343–352, 1993.

[183] I. Lemaire, S. Falzoni, B. Zhang, P. Pellegatti, and F. di Virgilio, "The P2X7 receptor and pannexin-1 are both required for the promotion of multinucleated macrophages by the inflammatory cytokine GM-CSF," *Journal of Immunology*, vol. 187, no. 7, pp. 3878–3887, 2011.

[184] U. Hanisch, "Microglia as a source and target of cytokines," *GLIA*, vol. 40, no. 2, pp. 140–155, 2002.

[185] R. M. Ransohoff and V. H. Perry, "Microglial physiology: unique stimuli, specialized responses," *Annual Review of Immunology*, vol. 27, pp. 119–145, 2009.

[186] H. Kettenmann, U. K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[187] H. Takeuchi, S. Jin, J. Wang et al., "Tumor necrosis factor-α induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner," *The Journal of Biological Chemistry*, vol. 281, no. 30, pp. 21362–21368, 2006.

[188] S. E. Samuels, J. B. Lipitz, J. Wang, G. Dahl, and K. J. Muller, "Arachidonic acid closes innexin/pannexin channels and thereby inhibits microglia cell movement to a nerve injury," *Developmental Neurobiology*, vol. 73, no. 8, pp. 621–631, 2013.

[189] J. A. Orellana, K. F. Shoji, V. Abudara et al., "Amyloid β-induced death in neurons involves glial and neuronal hemichannels," *Journal of Neuroscience*, vol. 31, no. 13, pp. 4962–4977, 2011.

[190] A. M. Fontainhas, M. Wang, K. J. Liang et al., "Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission," *PLoS ONE*, vol. 6, no. 1, Article ID e15973, 2011.

[191] D. Davalos, J. Grutzendler, G. Yang et al., "ATP mediates rapid microglial response to local brain injury *in vivo*," *Nature Neuroscience*, vol. 8, no. 6, pp. 752–758, 2005.

[192] S. B. Shaikh, B. Uy, A. Perera, and L. F. B. Nicholson, "AGEs-RAGE mediated up-regulation of connexin43 in activated human microglial CHME-5 cells," *Neurochemistry International*, vol. 60, no. 6, pp. 640–651, 2012.

[193] S. Garg, M. M. Syed, and T. Kielian, "Staphylococcus aureus-derived peptidoglycan induces Cx43 expression and functional gap junction intercellular communication in microglia," *Journal of Neurochemistry*, vol. 95, no. 2, pp. 475–483, 2005.

[194] K. Dobrenis, H.-Y. Chang, M. H. Pina-Benabou et al., "Human and mouse microglia express connexin36, and functional gap junctions are formed between rodent microglia and neurons," *Journal of Neuroscience Research*, vol. 82, no. 3, pp. 306–315, 2005.

[195] A. D. Martínez, E. A. Eugenín, M. C. Brañes, M. V. Bennett, and J. C. Sáez, "Identification of second messengers that induce expression of functional gap junctions in microglia cultured from newborn rats," *Brain Research*, vol. 943, pp. 191–201, 2002.

[196] E. A. Eugenín, D. Eckardt, M. Theis, K. Willecke, M. V. L. Bennett, and J. C. Sáez, "Microglia at brain stab wounds express connexin 43 and in vitro form functional gap junctions after treatment with interferon-γ and tumor necrosis factor-α," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 4190–4195, 2001.

[197] J. I. Nagy, W. Li, E. L. Hertzberg, and C. A. Marotta, "Elevated connexin43 immunoreactivity at sites of amyloid plaques in Alzheimer's disease," *Brain Research*, vol. 717, no. 1-2, pp. 173–178, 1996.

[198] K. S. Ásgeirsson, K. Ólafsdóttir, J. G. Jónasson, and H. M. Ógmundsdóttir, "The effects of IL-6 on cell adhesion and E-cadherin expression in breast cancer," *Cytokine*, vol. 10, no. 9, pp. 720–728, 1998.

[199] F. Cao, R. Eckert, C. Elfgang et al., "A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in *HeLa* transfectants and *Xenopus* oocytes," *Journal of Cell Science*, vol. 111, no. 1, pp. 31–43, 1998.

[200] C. Elfgang, R. Eckert, H. Lichtenberg-Frate et al., "Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells," *The Journal of Cell Biology*, vol. 129, no. 3, pp. 805–817, 1995.

[201] G. Kanaporis, P. R. Brink, and V. Valiunas, "Gap junction permeability: selectivity for anionic and cationic probes," *The American Journal of Physiology—Cell Physiology*, vol. 300, no. 3, pp. C600–C609, 2011.

[202] R. Talaveron, E. R. Matarredona, R. R. de la Cruz, D. Macias, V. Galvez, and A. M. Pastor, "Implanted neural progenitor cells regulate glial reaction to brain injury and establish gap junctions with host glial cells," *Glia*, vol. 62, no. 4, pp. 623–638, 2014.

[203] H. Takeuchi, S. Jin, H. Suzuki et al., "Blockade of microglial glutamate release protects against ischemic brain injury," *Experimental Neurology*, vol. 214, no. 1, pp. 144–146, 2008.

[204] H. Takeuchi, H. Mizoguchi, Y. Doi et al., "Blockade of gap junction hemichannel suppresses disease progression in mouse models of amyotrophic lateral sclerosis and Alzheimer's disease," *PLoS ONE*, vol. 6, no. 6, Article ID e21108, 2011.

[205] Y. Imura, Y. Morizawa, R. Komatsu et al., "Microglia release ATP by exocytosis," *GLIA*, vol. 61, no. 8, pp. 1320–1330, 2013.

[206] C. Rigato, N. Swinnen, R. Buckinx et al., "Microglia proliferation is controlled by P2X7 receptors in a pannexin-1-independent manner during early embryonic spinal cord invasion," *Journal of Neuroscience*, vol. 32, no. 34, pp. 11559–11573, 2012.

[207] J. Shijie, H. Takeuchi, I. Yawata et al., "Blockade of glutamate release from microglia attenuates experimental autoimmune encephalomyelitis in mice," *The Tohoku Journal of Experimental Medicine*, vol. 217, no. 2, pp. 87–92, 2009.

[208] J. Xiong, M. Burkovetskaya, N. Karpuk, and T. Kielian, "IL-1RI (interleukin-1 receptor type I) signalling is essential for host defence and hemichannel activity during acute central nervous system bacterial infection," *ASN Neuro*, vol. 4, no. 3, pp. 175–185, 2012.

[209] I. Yawata, H. Takeuchi, Y. Doi, J. Liang, T. Mizuno, and A. Suzumura, "Macrophage-induced neurotoxicity is mediated by glutamate and attenuated by glutaminase inhibitors and gap junction inhibitors," *Life Sciences*, vol. 82, no. 21-22, pp. 1111–1116, 2008.

[210] L. Endong, J. Shijie, Y. Sonobe et al., "The Gap-junction inhibitor Carbenoxolone suppresses the differentiation of Th17 cells through inhibition of IL-23 expression in antigen presenting cells," *Journal of Neuroimmunology*, vol. 240-241, pp. 58–64, 2011.

[211] A. Cerutti, M. Cols, and I. Puga, "Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes," *Nature Reviews Immunology*, vol. 13, no. 2, pp. 118–132, 2013.

[212] A. Mantovani, M. A. Cassatella, C. Costantini, and S. Jaillon, "Neutrophils in the activation and regulation of innate and adaptive immunity," *Nature Reviews Immunology*, vol. 11, no. 8, pp. 519–531, 2011.

[213] G. P. Sandilands, J. McCrae, K. Hill, M. Perry, and D. Baxter, "Major histocompatibility complex class II (DR) antigen and costimulatory molecules on in vitro and in vivo activated human polymorphonuclear neutrophils," *Immunology*, vol. 119, no. 4, pp. 562–571, 2006.

[214] I. Müller, M. Munder, P. Kropf, and G. M. Hänsch, "Polymorphonuclear neutrophils and T lymphocytes: strange bedfellows or brothers in arms?" *Trends in Immunology*, vol. 30, no. 11, pp. 522–530, 2009.

[215] C. Beauvillain, Y. Delneste, M. Scotet et al., "Neutrophils efficiently cross-prime naive T cells in vivo," *Blood*, vol. 110, no. 8, pp. 2965–2973, 2007.

[216] D. S. A. Abdallah, C. E. Egan, B. A. Butcher, and E. Y. Denkers, "Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation," *International Immunology*, vol. 23, no. 5, pp. 317–326, 2011.

[217] M. C. Brañes, J. E. Contreras, and J. C. Sáez, "Activation of human polymorphonuclear cells induces formation of functional gap junctions and expression of connexins," *Medical Science Monitor*, vol. 8, pp. R313–R323, 2002.

[218] H. K. Eltzschig, T. Eckle, A. Mager et al., "ATP release from activated neutrophils occurs via connexin 43 and modulates

adenosine-dependent endothelial cell function," *Circulation Research*, vol. 99, no. 10, pp. 1100–1108, 2006.

[219] S. Zahler, A. Hoffmann, T. Gloe, and U. Pohl, "Gap-junctional coupling between neutrophils and endothelial cells: a novel modulator of transendothelial migration," *Journal of Leukocyte Biology*, vol. 73, no. 1, pp. 118–126, 2003.

[220] M. Z. R. Sarieddine, K. E. L. Scheckenbach, B. Foglia et al., "Connexin43 modulates neutrophil recruitment to the lung," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 11-12, pp. 4560–4570, 2009.

[221] I. Scerri, O. Tabary, T. Dudez et al., "Gap junctional communication does not contribute to the interaction between neutrophils and airway epithelial cells," *Cell Communication & Adhesion*, vol. 13, no. 1-2, pp. 1–12, 2006.

[222] P. Coutinho, C. Qiu, S. Frank et al., "Limiting burn extension by transient inhibition of Connexin43 expression at the site of injury," *The British Journal of Plastic Surgery*, vol. 58, no. 5, pp. 658–667, 2005.

[223] M. Cronin, P. N. Anderson, J. E. Cook, C. R. Green, and D. L. Becker, "Blocking connexin43 expression reduces inflammation and improves functional recovery after spinal cord injury," *Molecular and Cellular Neuroscience*, vol. 39, no. 2, pp. 152–160, 2008.

[224] C. Qiu, P. Coutinho, S. Frank et al., "Targeting connexin43 expression accelerates the rate of wound repair," *Current Biology*, vol. 13, no. 19, pp. 1697–1703, 2003.

[225] S. Rignault, J. Haefliger, B. Waeber, L. Liaudet, and F. Feihl, "Acute inflammation decreases the expression of connexin 40 in mouse lung," *Shock*, vol. 28, no. 1, pp. 78–85, 2007.

[226] Y. Bao, Y. Chen, C. Ledderose, L. Li, and W. G. Junger, "Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils," *The Journal of Biological Chemistry*, vol. 288, no. 31, pp. 22650–22657, 2013.

[227] Y. Chen, Y. Yao, Y. Sumi et al., "Purinergic signaling: a fundamental mechanism in neutrophil activation," *Science Signaling*, vol. 3, no. 125, p. ra45, 2010.

[228] O. Thaunat, A. G. Granja, P. Barral et al., "Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity," *Science*, vol. 335, no. 6067, pp. 475–479, 2012.

[229] M.-I. Yuseff, P. Pierobon, A. Reversat, and A.-M. Lennon-Duménil, "How B cells capture, process and present antigens: a crucial role for cell polarity," *Nature Reviews Immunology*, vol. 13, no. 7, pp. 475–486, 2013.

[230] S. Machtaler, M. Dang-Lawson, K. Choi, C. Jang, C. C. Naus, and L. Matsuuchi, "The gap junction protein Cx43 regulates B-lymphocyte spreading and adhesion," *Journal of Cell Science*, vol. 124, no. 15, pp. 2611–2621, 2011.

[231] E. Oviedo-Orta, T. Hoy, and W. H. Evans, "Intercellular communication in the immune system: differential expression of connexin40 and 43, and perturbation of gap junction channel functions in peripheral blood and tonsil human lymphocyte subpopulations," *Immunology*, vol. 99, no. 4, pp. 578–590, 2000.

[232] L. Falk, M. Dang-Lawson, J. L. Vega et al., "Mutations of Cx43 that affect B cell spreading in response to BCR signaling," *Biology Open*, vol. 3, pp. 185–194, 2014.

[233] S. Machtaler, K. Choi, M. Dang-Lawson et al., "The role of the gap junction protein connexin43 in B lymphocyte motility and migration," *FEBS Letters*, vol. 588, no. 8, pp. 1249–1258, 2014.

[234] C. Théry, M. Ostrowski, and E. Segura, "Membrane vesicles as conveyors of immune responses," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 581–593, 2009.

[235] S. C. Watkins and R. D. Salter, "Functional connectivity between immune cells mediated by tunneling nanotubules," *Immunity*, vol. 23, no. 3, pp. 309–318, 2005.

[236] E. Oviedo-Orta and W. H. Evans, "Gap junctions and connexins: potential contributors to the immunological synapse," *Journal of Leukocyte Biology*, vol. 72, no. 4, pp. 636–642, 2002.

[237] H. D. Moreau and P. Bousso, "Visualizing how T cells collect activation signals in vivo," *Current Opinion in Immunology*, vol. 26, pp. 56–62, 2014.

[238] H. D. Moreau, F. Lemaître, E. Terriac et al., "Dynamic in situ cytometry uncovers T cell receptor signaling during immunological synapses and kinapses in vivo," *Immunity*, vol. 37, no. 2, pp. 351–363, 2012.

[239] C. M. Wang, C. Ploia, F. Anselmi, A. Sarukhan, and A. Viola, "Adenosine triphosphate acts as a paracrine signaling molecule to reduce the motility of T cells," *The EMBO Journal*, vol. 33, no. 12, pp. 1354–1364, 2014.

[240] H. Benlalam, T. Carré, A. Jalil et al., "Regulation of gap junctions in melanoma and their impact on Melan-A/MART-1-specific $CD8^+$ T lymphocyte emergence," *Journal of Molecular Medicine*, vol. 91, no. 10, pp. 1207–1220, 2013.

[241] M. Kuczma, J. R. Lee, and P. Kraj, "Connexin 43 signaling enhances the generation of Foxp3+ regulatory T cells," *Journal of Immunology*, vol. 187, no. 1, pp. 248–257, 2011.

[242] E. Oviedo-Orta, M. Perreau, W. H. Evans, and I. Potolicchio, "Control of the proliferation of activated $CD4^+$ T cells by connexins," *Journal of Leukocyte Biology*, vol. 88, no. 1, pp. 79–86, 2010.

[243] U. Schenk, A. M. Westendorf, E. Radaelli et al., "Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels," *Science Signaling*, vol. 1, no. 39, p. ra6, 2008.

[244] K. F. Shoji, PJ. Sáez, P. Harcha, H. L. Aguila, and J. C. Sáez, "Pannexin1 channels act downstream of P2X receptors in ATP-induced murine T-cell death," *Channels*, vol. 8, no. 2, pp. 142–156, 2014.

[245] T. Woehrle, L. Yip, A. Elkhal et al., "Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse," *Blood*, vol. 116, no. 18, pp. 3475–3484, 2010.

[246] T. Woehrle, L. Yip, M. Manohar et al., "Hypertonic stress regulates T cell function via pannexin-1 hemichannels and P2X receptors," *Journal of Leukocyte Biology*, vol. 88, no. 6, pp. 1181–1189, 2010.

[247] E. Oviedo-Orta, P. Gasque, and W. H. Evans, "Immunoglobulin and cytokine expression in mixed lymphocyte cultures is reduced by disruption of gap junction intercellular communication," *FASEB Journal*, vol. 15, no. 3, pp. 768–774, 2001.

[248] E. Oviedo-Orta, R. J. Errington, and W. H. Evans, "Gap junction intercellular communication during lymphocyte transendothelial migration," *Cell Biology International*, vol. 26, no. 3, pp. 253–263, 2002.

[249] U. Luckey, T. Schmidt, N. Pfender et al., "Crosstalk of regulatory T cells and tolerogenic dendritic cells prevents contact allergy in subjects with low zone tolerance," *The Journal of Allergy and Clinical Immunology*, vol. 130, no. 3, pp. 781.e11–797.e11, 2012.

[250] M. E. Moreno-Fernandez, C. M. Rueda, L. K. Rusie, and C. A. Chougnet, "Regulatory T cells control HIV replication in activated T cells through a cAMP-dependent mechanism," *Blood*, vol. 117, no. 20, pp. 5372–5380, 2011.

[251] T. D. Nguyen and S. M. Taffet, "A model system to study Connexin 43 in the immune system," *Molecular Immunology*, vol. 46, no. 15, pp. 2938–2946, 2009.

[252] G. Dahl, "Gap junction-mimetic peptides do work, but in unexpected ways," *Cell Communication and Adhesion*, vol. 14, no. 6, pp. 259–264, 2007.

[253] A. J. Siller-Jackson, S. Burra, S. Gu et al., "Adaptation of connexin 43-hemichannel prostaglandin release to mechanical loading," *The Journal of Biological Chemistry*, vol. 283, no. 39, pp. 26374–26382, 2008.

[254] M. A. Riquelme, R. Kar, S. Gu, and J. X. Jiang, "Antibodies targeting extracellular domain of connexins for studies of hemichannels," *Neuropharmacology*, vol. 75, pp. 525–532, 2013.

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Research Article

Nucleotides Regulate Secretion of the Inflammatory Chemokine CCL2 from Human Macrophages and Monocytes

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CCL2 is an important inflammatory chemokine involved in monocyte recruitment to inflamed tissues. The extracellular nucleotide signalling molecules UTP and ATP acting via the P2Y2 receptor are known to induce CCL2 secretion in macrophages. We confirmed this in the human THP-1 monocytic cell line showing that UTP is as efficient as LPS at inducing CCL2 at early time points (2–6 hours). Expression and calcium mobilisation experiments confirmed the presence of functional P2Y2 receptors on THP-1 cells. UTP stimulation of human peripheral CD14+ monocytes showed low responses to LPS (4-hour stimulation) but a significant increase above background following 6 hours of treatment. The response to UTP in human monocytes was variable and required stimulation >6 hours. With such variability in response we looked for single nucleotide polymorphisms in P2RY2 that could affect the functional response. Sequencing of P2RY2 from THP-1 cells revealed the presence of a single nucleotide polymorphism altering amino acid 312 from arginine to serine (rs3741156). This polymorphism is relatively common at a frequency of 0.276 (n = 404 subjects). Finally, we investigated CCL2 secretion in response to LPS or UTP in human macrophages expressing 312Arg-P2Y2 or 312Ser-P2Y2 where only the latter exhibited significant UTP-induced CCL2 secretion (n = 5 donors per group).

1. Introduction

The CCL2/CCR2 mediated recruitment of monocytes is necessary for fighting infections to microorganisms [1]. This chemokine signalling axis has also been implicated in a number of inflammatory disorders where monocyte infiltration is a key factor such as atherosclerosis, multiple sclerosis, and rheumatoid arthritis [2]. Understanding the cellular regulation of this important chemokine is therefore critical for understanding some of the early pathophysiology of inflammatory disorders.

P2Y receptors are members of the metabotropic family of purinergic receptors belonging to the larger family of G-protein coupled receptors. Eight subtypes of P2Y have been identified to date—P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14—with differences in both pharmacology and downstream signalling pathways [3, 4]. P2Y2 has a widespread distribution in the body including expression on glial cells, some neurons, endothelial cells, epithelial cells of many tissues, and myeloid immune cells including monocytes, macrophages, and dendritic cells [5–10]. Many studies have demonstrated activation of P2Y2 induces a transient calcium response [11, 12] but much less is known about the regulation of chemokine or cytokine production. Our previous work was the first to demonstrate a role for the P2Y2 receptor in regulation of CCL2 secretion from alveolar and peritoneal macrophages [10]. P2Y2 and the UDP-responsive P2Y6 receptor can also signal other chemokine productions including CXCL8 (IL-8) and CCL20 (MIP-3α) [9, 13, 14]. Knockout mouse studies have demonstrated that P2Y2 plays an important role in defence against lung infection with *Pseudomonas aeruginosa* [15] yet it can also play a role in allergic lung inflammation in various models [16, 17].

Extracellular nucleotide induction of CCL2 in cells expressing P2Y2 could be an important trigger for the initial recruitment of monocytes to an inflammatory site. Many inducers have been identified for CCL2 including lipopolysaccharide (LPS), growth factors such as platelet

derived growth factor (PDGF), and cytokines such as tumour necrosis factor-alpha (TNF-α), reviewed in [18]. Following tissue/cellular damage or regulated nucleotide release from cells or nerve terminals, the activation of P2Y2 by extracellular nucleotides could switch on a rapid production of CCL2. Whilst this may be beneficial for initiating repair to an injured site, uncontrolled or chronic nucleotide release may be detrimental and cause excessive tissue inflammation. Both ATP and UTP are known danger signals and act as immunomodulatory signals to communicate alarm messages to immune cells.

The aim of this study was to investigate extracellular nucleotide-induced chemokine production in human macrophages and monocytes with an emphasis on CCL2. Firstly we wanted to confirm our earlier findings in rodent alveolar macrophages using a human monocyte/macrophage cell line and secondly we wanted to compare P2Y2 induced CCL2 secretion with that of LPS, a known bacterial inducer of CCL2. Finally we wanted to perform a pilot study to determine whether we could measure nucleotide-induced CCL2 secretion from primary human cells and assess the responses to both P2Y2 agonists and LPS.

2. Materials and Methods

2.1. Materials. UTP, ATP, suramin, and LPS (strain 055:B5) were from Sigma-Aldrich (Ryde, Australia). 2-thio-UTP and UTPγS were from Tocris Biosciences (Bristol, UK). Interferon γ (IFN-γ) was from Roche Diagnostics. MwoI restriction enzyme was from NEB.

2.2. Cell Culture. The THP-1 monocytic cell line was maintained in RPMI 1640 media (Life Technologies) containing 10% foetal bovine serum (US origin, Lonza), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all Life Technologies) and grown under humidified conditions in a 5% CO_2 incubator. Cells were routinely passaged every 2-3 days. For differentiation to macrophages, THP-1 cells were plated at 1×10^6 cells/well in 24-well plates in 500 μL complete medium and stimulated with 1000 U/mL IFN-γ and 100 ng/mL LPS for 48 hours.

2.3. Human Monocyte Preparation. Peripheral venous blood was collected in lithium heparin tubes (Becton Dickinson) from healthy volunteers with informed consent (study approved by Nepean Blue Mountains Local Health Service Human Ethics committee). Mononuclear cells were isolated by Ficoll-Paque (GE Healthcare) gradient centrifugation and monocytes were isolated using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotech, Germany) as per manufacturer's instructions. The purity for CD14 monocyte isolations was routinely >90% by flow cytometry.

For macrophage experiments, peripheral blood mononuclear cells were plated in complete RPMI 1640 medium at a density of 2.5×10^6 cells per well. Cells were incubated for 2 hours at 37°C in order to adhere to plastic, nonadherent cells removed, and adherent PBMCs were cultured overnight in 1 mL complete media, washed once the following day, and cultured for 6 more days.

2.4. Calcium Measurements. THP-1 cells were harvested from flasks, pelleted at 300 ×g, and resuspended in Fluo-4 NW assay buffer (Life Technologies). THP-1 cells were plated at a density of 2×10^5 cells/well into a 96-well plate coated with poly-D-lysine (Merck Millipore) and were loaded for 30 minutes at 37°C. Human monocytes were plated at 2–4×10^5 cells/well and prepared in the same way. Calcium measurements were performed using a Fluostar OPTIMA plate reader (BMG Labtech) with excitation at 485 nm and emission at 520 nm. All measurements were made at 37°C using a gain setting of 40%.

2.5. ELISA Experiments. THP-1 cells were plated at 1×10^6 cells/well in a 24-well plate in RPMI 1640 media containing 1% serum (0.5 mL per well). Stimulations were performed in duplicate. LPS (1 or 10 μg/mL) or nucleotides (varying concentrations) were added directly into the media for 2, 4, 6, or 24 hours. Following stimulation media were removed from the wells into Eppendorfs and centrifuged to remove any contaminating cells. Supernatants were transferred to fresh Eppendorfs and frozen at −80°C. Freshly isolated human peripheral blood $CD14^+$ monocytes were plated at 5×10^5 cells/well in RPMI media containing 1% serum. Stimulations were performed in duplicate/triplicate as for THP-1 cells and cell-free supernatants collected after 4 or 6 hours.

Ninety-six well plates (NUNC) were coated with anti-MCP-1 capture antibody (clone 10F7, BD Biosciences) at a concentration of 2 μg/mL in sodium carbonate buffer pH 9.5. Samples and standards (recombinant human MCP-1) were diluted in media. Detection antibody, anti-MCP-1-biotin (clone 5D3-F7, BD Biosciences), was used at 0.5 μg/mL and followed by streptavidin-HRP at 1 μg/mL. TMB-Ultra (PerBioscience) was used for visualisation and 1 M H_2SO_4 was used as stop solution. Absorbance at 450 nm was read using a BMG Labtech Optima plate reader. Standard curves were fit with regression factor of $r^2 > 0.96$.

2.6. Real-Time PCR. Cells were stimulated as described above, collected into RNA Protect reagent (Qiagen), and stored at −80°C. Cells were then processed to extract total RNA using an RNEasy Mini Kit (Qiagen). RNA concentrations were measured using a spectrophotometer (Cary) using absorbance at 260 nm. 1 μg RNA was reverse transcribed using a Tetro cDNA synthesis kit (Bioline) as per manufacturer's instructions.

Primers for real-time PCR were optimised for concentrations over the range (0.125 μM to 1 μM) and primer efficiencies were determined. Primer sequences were β-actin forward 5′-GCC CTG GCA CCC AGC ACA AT-3′ and reverse 5′-GGA GGG GCC GGA CTC GTC AT-3′, GAPDH forward 5′-CGA GAT CCC TCC AAA ATC AA-3′ and reverse 5′-TTC ACA CCC ATG ACG AAC AT-3′, CCL2 forward 5′-CCC CAG TCA CCT GCT GTT AT-3′ and reverse 5′-GAG TTT GGG TTT GCT TGT CC-3′, CCL20 forward 5′-AAG TTG TCT GTG TGC GCA AAT CC-3′ and reverse 5′-CCA

TTC CAG AAA AGC CAC AGT TTT-3′, CCL3 forward 5′-ACT TTG AGA CGA GCA GCC AGTG-3′ and reverse 5′-TTT CTG GAC CCA CTC CTC ACTG-3′, and CXCL8 forward 5′-GAG AGT GAT TGA GAG TGG ACC AC-3′ and reverse 5′-CAC AAC CCT CTG CAC CCA GTT T-3′. Quantitative real-time PCR was performed using Sensimix SYBR green No ROX mastermix (Bioline) and 0.75 μM each primer on freshly prepared cDNA. PCR were performed in triplicate using a Rotorgene 2000 (Corbett Research) and analysed using a threshold of 0.003 to determine Ct values. Data was analysed using the $2^{-\Delta\Delta Ct}$ method.

2.7. Flow Cytometry. One million THP-1 or peripheral blood mononuclear cells were stained per flow tube. Cells were fixed with 2% paraformaldehyde buffer for 20 minutes on ice and permeabilised with 0.1% saponin in PBS. Rabbit IgG was used as the negative control and anti-P2Y2 (Sigma) was used at 1 : 100 dilution. Primary antibodies were incubated for 30 minutes in PBS/0.1% saponin containing 5% human AB serum. Cells were co-stained with mouse anti-human CD14-FITC (1 : 100 dilution). PBMCs were washed with PBS/saponin and incubated with goat anti-rabbit IgG-Alexa 647 at 1 : 100 dilution for 30 minutes on ice. After final washing with PBS/saponin, cells were resuspended in PBS and 30000 events acquired on a BD FACSCalibur flow cytometer. Monocytes were identified by CD14 expression and plots of P2Y2-Alexa-647 staining were generated using Weasel flow cytometry software (WEHI).

2.8. Genotyping. Genomic DNA was prepared from whole blood as previously described [19] and stored at −80°C. The P2Y2 gene was amplified using 0.025 U/mL recombinant *Taq* DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$, 100 μM dNTP, and 0.4 μM each of forward and reverse primers. Primer sequences were forward 5′-CTT TTG CCG TCA TCC TTG TCT-3′ and reverse 5′-CAT CTC GGG CAA AGC GTA-3′ yielding a product of 328 bp. The following cycling conditions were used: initial denaturation (95°C for 3 minutes), followed by 40 cycles of denaturation (95°C for 45 seconds), annealing (55°C for 30 seconds), and extension (72°C for 30 seconds) on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts, USA). The samples were then cooled at 4°C for 10 minutes.

A restriction assay was designed to determine the sequence at nucleotide 1269, G, or C. Mutation from G>C introduces an extra cut-site for the restriction enzyme MwoI. P2Y2 PCR product was incubated with 3 U MwoI for 1 hour at 60°C. Genomic DNA carrying G at nucleotide 1269 will be cut at 2 positions yielding 3 fragments of 195 bp, 130 bp, and 3 bp. Genomic DNA carrying C at nucleotide 1269 will be cut at 3 positions yielding 4 fragments of 181 bp, 130 bp, 14 bp, and 3 bp. The assay distinguishes between bands of 195 bp and 181 bp using a 3% agarose gel. Genotyping was verified using a commercial high-throughput method assay for rs3741156 (AGRF, Brisbane).

2.9. Statistical Analysis. Data plotted are means ± SEM of three to four experiments. Graphs and statistical analysis were performed using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. P2Y2 Regulates CCL2 Production in THP-1 Monocytes. It has been demonstrated by others that THP-1 cells express P2Y2 receptors [13, 20]. We confirmed P2Y2 expression on THP-1 using flow cytometry (Figure 1(a)) and used a functional measure of P2Y2 receptors by intracellular calcium measurements in response to a range of P2Y2 nucleotide agonists, ATP, UTP, 2-thio-UTP, UTPγS, and UDP (Figures 1(b) and 1(c)). A concentration-response curve was generated for UTP-induced calcium responses on THP-1 cells (Figure 1(d)). Calcium responses were reduced in the presence of suramin (100 μM), a broad P2 receptor antagonist known to block P2Y2 responses (Figure 1(b)).

We then stimulated THP-1 cells with the nucleotides UTP and ATPγS to stimulate P2Y2, or with LPS (1–10 μg/mL) to induce CCL2 chemokine production and secretion. We determined the amount of CCL2 secreted at three separate timepoints: 2 hours, 6 hours, and 24 hours (Figure 2). We found that UTP and ATPγS were as effective as LPS at stimulating CCL2 secretion after 2 hours. After 6 hours the UTP or ATPγS-induced CCL2 secretion remained elevated above basal and again was not significantly different to LPS. However, after 24 hours of stimulation there was a further increase in the LPS-induced CCL2 secretion, while the UTP or ATPγS-induced CCL2 secretion remained low (Figure 2). We decided to study the 2-hour timepoint to further investigate nucleotide-induced CCL2 secretion. A range of different nucleotide agonists were tested and many increased CCL2 levels above background levels (Figure 3). However, we found that only UTP or LPS treatments gave a significant difference compared to basal (one-way ANOVA with Dunnett's post hoc test, n = 3-4 experiments). The UTP signal was not blocked by suramin, a feature also observed when concentrations of UTP higher than 100 nM were used in calcium responses in THP-1 cells (data not shown). Using the THP-1 cell line we looked at CCL2 gene induction by UTP in comparison to LPS. We used quantitative real-time PCR and measured CCL2 relative to β-actin as the reference gene at 2 hours following stimulation with LPS, UTP, UDP, or media alone. We found that UTP induced an 8.8-fold increase in CCL2 expression compared to an 11-fold upregulation of CCL2 in response to LPS (Figure 4). In contrast UDP did not induce a significant upregulation of CCL2 levels (Figure 4). As expected, LPS induced other chemokines CCL20 (459-fold above media control) and CXCL8 (1121-fold above media control) in THP-1 cells, whereas UTP treatment induced a 4.1-fold upregulation of CCL20 and a 2.3-fold upregulation of CCL3 with respect to unstimulated cells. UTP induced some upregulation of CXCL8 expression (61-fold) but this was only 5% of the LPS response (1121-fold).

3.2. CCL2 Production in $CD14^+$ Primary Human Monocytes. Following our observations that UTP could induce a similar level of CCL2 secretion as LPS in the THP-1 monocytic

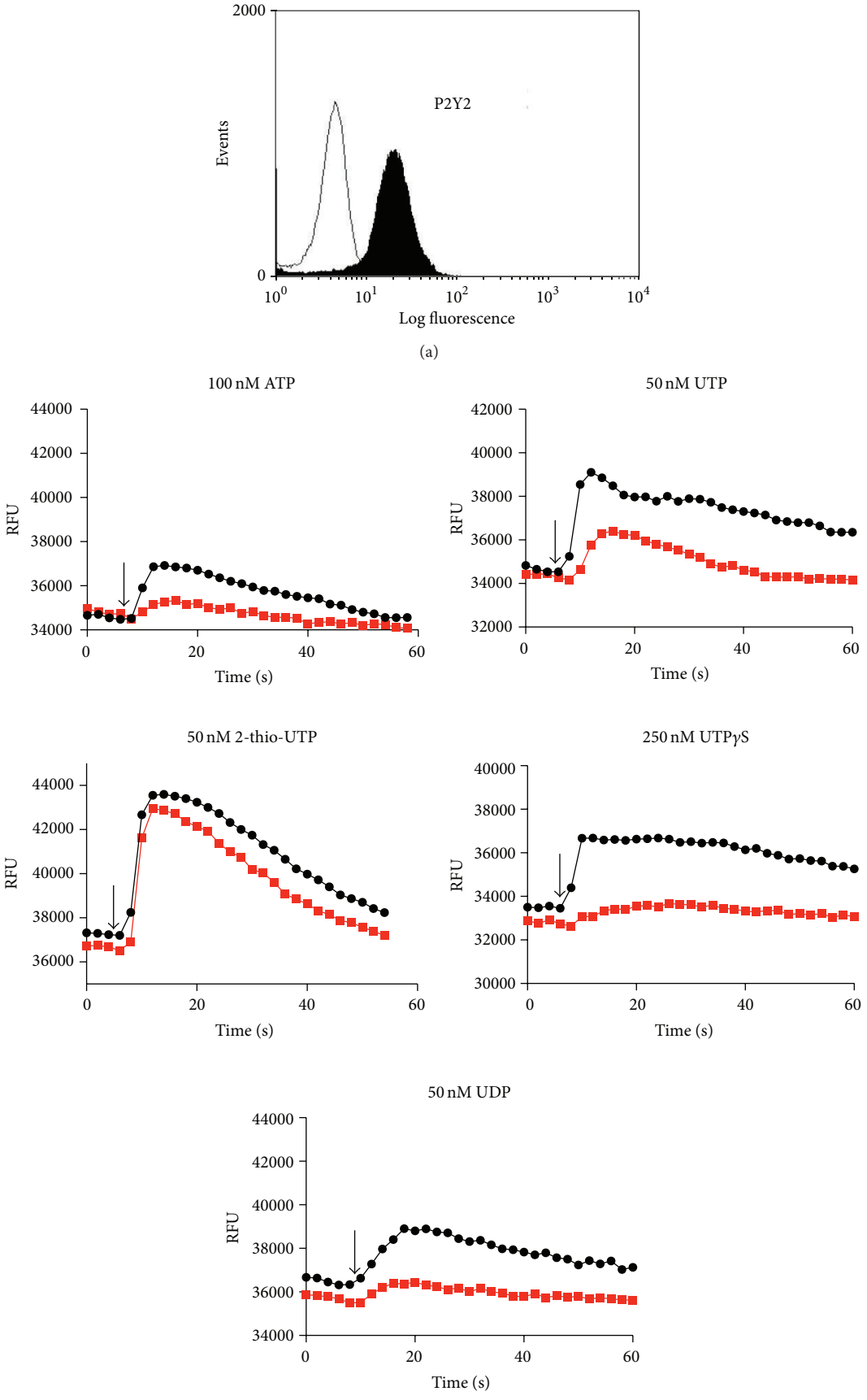

Figure 1: Continued.

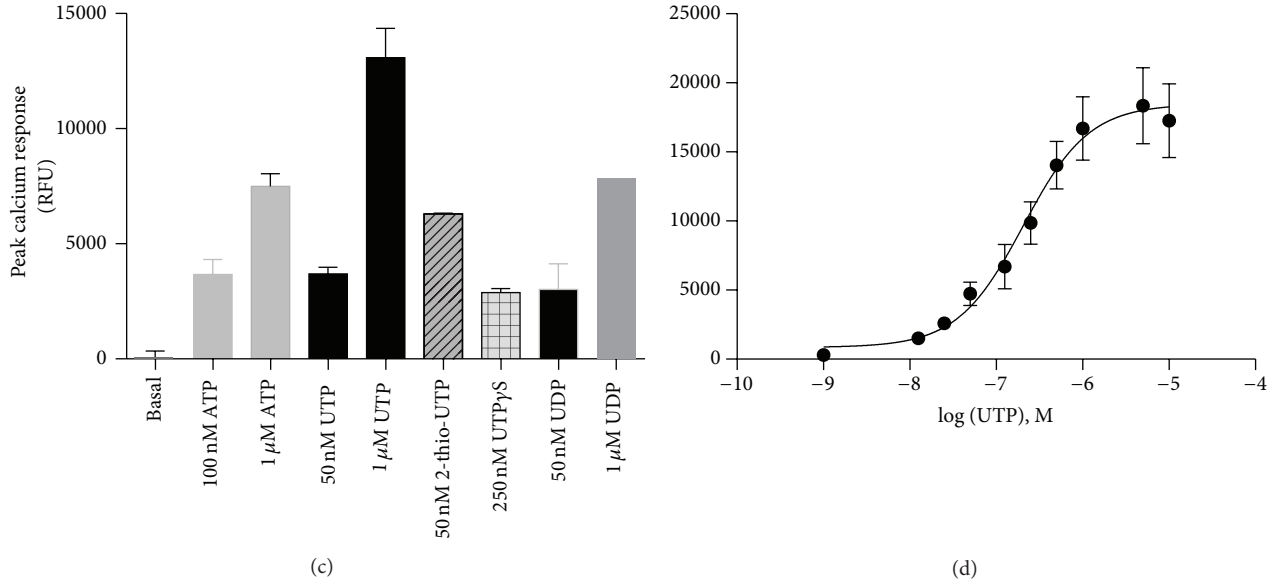

Figure 1: Nucleotides induce intracellular calcium responses in THP-1 cells. (a) Representative plot showing P2Y2 expression on THP-1 monocytes using a specific anti-P2Y2 antibody and a BD FACSCalibur flow cytometer. (b) THP-1 cells were loaded with Fluo-4 using a NW kit and plated at 2×10^5 cells/well in poly-D-lysine coated 96-well plates. Intracellular calcium responses were measured using a BMG Labtech Fluostar Optima plate reader for over 60 seconds at 37°C. Responses to 100 nM ATP, 50 nM UTP, 50 nM 2-thio-UTP, and 250 nM UTPγS are shown in black. Paired responses of cells pretreated with the P2 receptor antagonist suramin (30 μM; 30 minutes) are shown in red. (c) Mean calcium response data from several experiments. (d) A concentration-response curve was generated for UTP-induced calcium responses on THP-1 cells. Mean responses ± SEM are plotted from 4 separate experiments.

cell line, we wanted to address whether this was also a feature of primary human monocytes. We probed CD14-positive PBMCs for P2Y2 expression and found significant labelling relative to a nonspecific IgG control (Figure 5(a)). To assess the functionality of P2Y2 receptors on magnetically isolated CD14 positive monocytes we performed calcium measurements on Fluo-4AM loaded cells. UTP at both 10 μM and 100 μM induced large transient calcium responses in primary monocytes (Figure 5(b)).

We stimulated human $CD14^+$ monocytes with media, UTP (10 μM), or LPS (100 ng/mL) for 4 hours (n = 11 different donors). When analysing the data as collective, we found a small increase in CCL2 in response to LPS (mean 376 ± 88 pg/mL) above background CCL2 secretion in media alone (mean 223 ± 56 pg/mL). There was no difference above background with 10 μM UTP treatment (mean 215 ± 51 pg/mL) (Figure 5(c)). When looking at each individual donor we could see that in 4 out of 11 subjects UTP increased CCL2 levels above media alone treated cells. Subtracting the background CCL2 concentration showed a UTP response with a mean of 23 pg/mL (n = 4 subjects, range 8–49 pg/mL).

We then determined whether a longer incubation time of 6 hours would reveal an increased CCL2 secretory response to UTP. When analysing the data as a collective, we found a significant increase in CCL2 in response to LPS (mean 645 ± 55 pg/mL, n = 5 donors, $P < 0.05$ one-way ANOVA with Dunnett's post hoc test) compared to background (mean 154 ± 55 pg/mL, n = 5 donors). Treatment with 10 μM UTP (mean 134 ± 47 pg/mL) or 100 μM UTP (229 ± 72 pg/mL, n = 5 donors) was not significantly different from background CCL2 levels (Figure 5(d)). Again, when looking at each subject we could see that all 5 donors responded to 100 μM UTP with CCL2 levels ranging from 19 to 129 pg/mL above the basal secretion with a mean value of 76 pg/mL.

To determine whether we could measure CCL2 gene induction in primary human monocytes we performed qPCR analysis of CCL2 gene induction by UTP (100 μM) and LPS after a 6-hour stimulation. We found variable induction of CCL2 and CCL20 in response to either UTP or LPS (Figure 6, n = 3-4 donors).

3.3. Does P2Y2 Play a Role in CCL2 Secretion in Human Macrophages? With such small responses to UTP in monocytes and unstimulated THP-1 monocytic cells, we decided to differentiate THP-1 to macrophage-like cells using IFN-γ and LPS [20]. Our previous work was performed using an alveolar macrophage cell line and peritoneal macrophages and measured robust CCL2 production [10]. IFN-γ/LPS differentiated THP-1 cells (48-hour treatment) were stimulated with either LPS, UTP, or media control and CCL2 was measured in supernatants collected at 6 and 24 hours. A much higher basal secretion of CCL2 was measured compared to unstimulated THP-1 but both LPS and UTP could further increase CCL2 secretion at both timepoints (Figure 7).

To begin to address the variability in responses observed with primary monocytes we focused on nonsynonymous single nucleotide polymorphisms in P2Y2. There are three known SNPs in human P2Y2 which may affect functional responses [21, 22]. We first sequenced the P2Y2 gene from

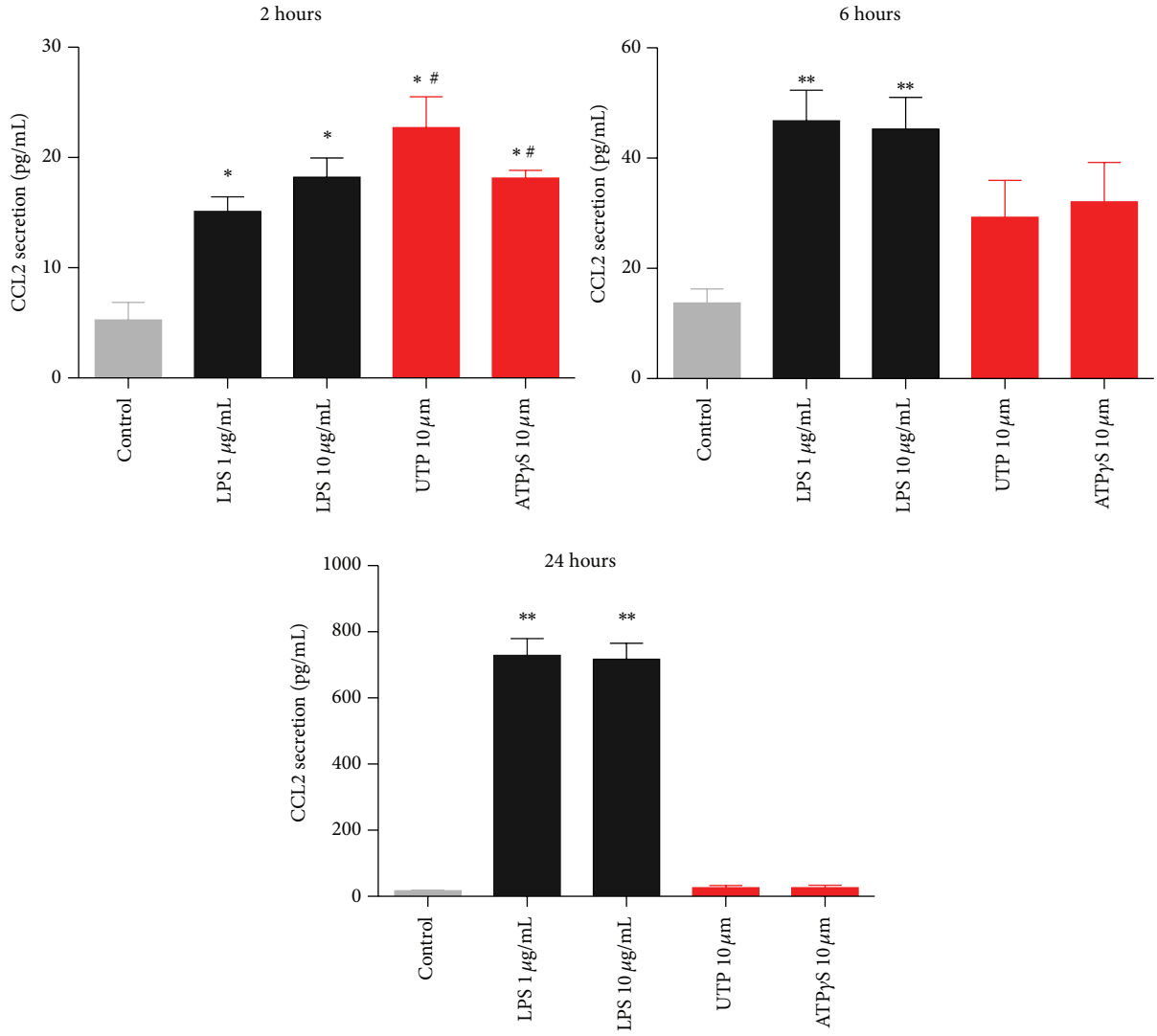

Figure 2: The effect of P2Y2 agonists compared with LPS on CCL2 secretion from unprimed THP-1 cells. THP-1 cells were plated in RPMI media containing 1% serum and stimulated with media alone, LPS (1 or 10 μg/mL), UTP (10 μM), or ATPγS (10 μM) for the times indicated above each graph. Mean raw data is plotted in pg/mL ± SEM from three independent experiments. Symbols: $*$ denotes $P < 0.05$ compared with control, # denotes no significant difference between treatments, and ns denotes not significant with respect to control (one-way ANOVA with Tukey's post hoc test). Standard curves were performed for each ELISA experiment with fits of $r > 0.95$.

THP-1 monocytes and found several synonymous SNPs and a single nonsynonymous SNP (rs3741156) altering amino acid 312 from arginine (R) to serine (S) (data not shown). We developed an in-house genotyping assay based on restriction enzyme analysis with MwoI. The presence of G or C at position 1269 (NM_002564) correlates with a cut-site for this enzyme. Wild-type individuals carrying G at this position would show 3 fragments of 195, 130, and 3 bp, whereas polymorphic individuals carrying C at this position would show 4 fragments in the PCR restriction assay of 181, 130, 14, and 3 bp (Figure 8(a)). We used this assay to genotype P2Y2 in healthy volunteers and confirmed the genotyping using a custom high-throughput SNP assay (Australian Genome Research Facility). From genotyping a total of 404 subjects we determined an allele frequency of 0.276 for rs3741156 ($n = 404$ subjects).

We then performed a pilot study to investigate the effect of genotype on chemokine secretion using individuals carrying wild-type (WT) P2Y2 or 312S-P2Y2 receptors (5 subjects per genotype). We stained PBMCs from WT and 312S-P2Y2 subjects for expression level of P2Y2 on CD14 positive cells and found no significant difference in mean fluorescence intensity ($n = 3$ donors/genotype) (Figure 8(b)). We cultured adherent PBMCs to macrophages over 7 days to differentiate monocytes to macrophages and stimulated cells with media, 10 μM UTP, or 1 μg/mL LPS for 4 hours ($n = 5$ donors per genotype group). Supernatants were tested for CCL2 by ELISA and results are shown in Figure 9 as a scatter graph. Responses to both LPS (mean 1054 ± 485 pg/mL) and UTP (mean 882 ± 341 pg/mL) were smaller in WT-P2Y2 macrophages and not significantly different from background CCL2 (mean 433 ± 181 pg/mL, $n = 5$ donors). In contrast,

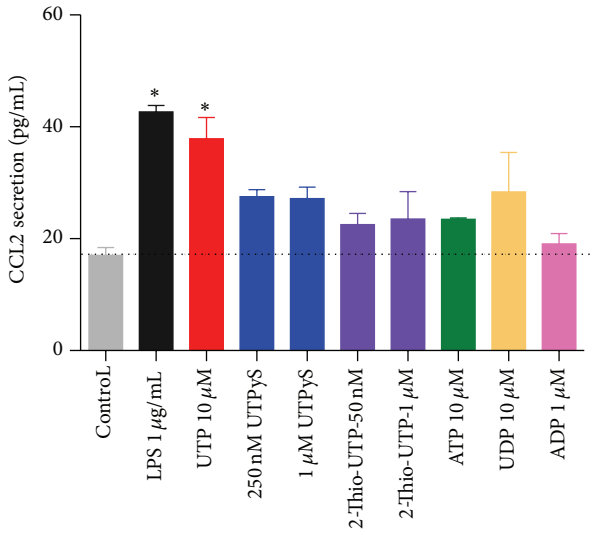

FIGURE 3: Nucleotides induce CCL2 secretion from THP-1 cells after 2-hour stimulation. THP-1 cells were plated in RPMI media containing 1% serum and stimulated with media alone, LPS (1 μg/mL), UTP (10 μM), UTPγS (250 nM and 1 μM), 2-thio-UTP (50 nM and 1 μM), ATP (10 μM), UDP (10 μM), or ADP (10 μM) for 2 hours. Mean raw data is plotted in pg/mL ± SEM. Standard curves were performed for each ELISA experiment with fits of $r > 0.95$.

responses to LPS (mean 1411 ± 280 pg/mL) and UTP (mean 1416 ± 401 pg/mL) were significantly different from background CCL2 (mean 682 ± 271 pg/mL, $n = 5$ donors, one-way ANOVA with Dunnett's post hoc test) in 312S-P2Y2 expressing macrophages.

4. Discussion

This study is the first to investigate UTP induced chemokine secretion from THP-1 cells and primary human monocytes and macrophages. Our main finding suggests that UTP can elicit comparable levels of CCL2 as stimulation with the bacterial product LPS in THP-1 cells. We also found that macrophages produce more CCL2 in response to nucleotides than monocytes even though levels of P2Y2 expression are similar.

It is well known in the literature that monocytes and macrophages express P2Y2 receptors amongst other P2Y and P2X receptors [3, 4, 11, 13]. Here we confirm expression of P2Y2 in THP-1 cells using flow cytometry and intracellular calcium measurements as an indirect measure of G protein-coupled receptor activation (Figure 1). The pharmacology for this receptor is relatively poor compared with other purinergic receptors. Several pieces of evidence suggest that the UTP-induced response in THP-1 cells is due to P2Y2 activation. UTP is only known to activate P2Y2, P2Y4, and P2Y6 receptors [23], and, of these, the only receptor responsive to ATP is P2Y2. ATP and UTP are equipotent at P2Y2 receptors and EC_{50} values for UTP and ATP-induced calcium responses were 97 ± 34 nM and 105 ± 59 nM, respectively, in THP-1 cells. Suramin could suppress UTP and ATP-induced calcium responses as well as the response elicited by UTPγS (Figure 1). However, suramin did not suppress responses induced by the P2Y2-selective agonist 2-thio-UTP (Figure 1). Furthermore, we found that suramin could not suppress calcium responses induced by high concentrations (>1 μM) of nucleotides and as such we did not use suramin in the subsequent CCL2 experiments.

The calcium experiments were used as an indication that functional P2Y receptors were present on THP-1 cells and we found that the P2Y6 receptor agonist UDP also elicited a suramin-sensitive calcium response in THP-1 cells (Figure 1). Whilst P2Y6 is likely expressed in THP-1 cells as shown by Yebdri et al. [13], experiments with CCL2 secretion suggest that UDP did not have a significant our effect (Figure 3). Furthermore, both UTPγS (P2Y2 and P2Y4 selective agonist) and 2-thio-UTP (P2Y2 selective) could induce CCL2 secretion above basal levels but not to the same degree as UTP (full agonist at P2Y2).

A major aim of this study was to compare nucleotide-induced chemokine secretion to a known proinflammatory signal such as LPS. We chose the THP-1 cell line to perform these experiments to limit variability in responses. The LPS-induced CCL2 secretory response in THP-1 monocytes was low (<100 pg/mL) at early timepoints such as 2 and 6 hours but increased over a 24-hour treatment period (Figure 2). The amount of constitutively produced CCL2 from THP-1 cells in our hands was similar to that measured by Steube et al. [24]. Steube et al. observed a low level of CCL2 secretion from THP-1 in response to LPS compared to other myelomonocytic cells lines [24]. This low constitutive CCL2 secretion is quite different from the high level of constitutive CCL2 expression seen in NR8383 alveolar macrophages in our previous study [10].

We compared the LPS-induced CCL2 response to that of UTP and ATPγS at concentrations known to induce large calcium responses (10 μM) in THP-1 cells. At an early timepoint, 2 hours, the nucleotide-induced CCL2 secretion was comparable to LPS-induced CCL2 secretion (Figure 2). However, the nucleotide-induced response remained stable between 2 and 6 hours and the LPS-induced response steadily increased over time. In primary human monocytes and macrophages the kinetics of CCL2 production appeared to be slower in response to either LPS or UTP (Figures 5 and 9). After 4–6 hours of stimulation the LPS-induced CCL2 response becomes significant above background CCL2 secretion, whereas overall the UTP-induced response remains not significantly elevated above basal levels (Figure 5). Thus in primary human monocytes we observed a large difference between the P2Y2-induced response and the LPS-induced response. Several factors may influence this data including genetic differences in membrane receptors, expression levels of receptors at the plasma membrane, and differences in intracellular signalling or in CCL2 mRNA stability. However, in human monocyte-derived macrophages the UTP-induced response was again similar to the LPS-induced response (Figure 9) and this was also true in IFN/LPS differentiated THP-1 macrophages after 6 hours (Figure 7).

We investigated whether nucleotides could also induce production of other inflammatory chemokines such as CCL20 (MIP-3α), CCL3 (MIP-1α), and CXCL8 (IL-8).

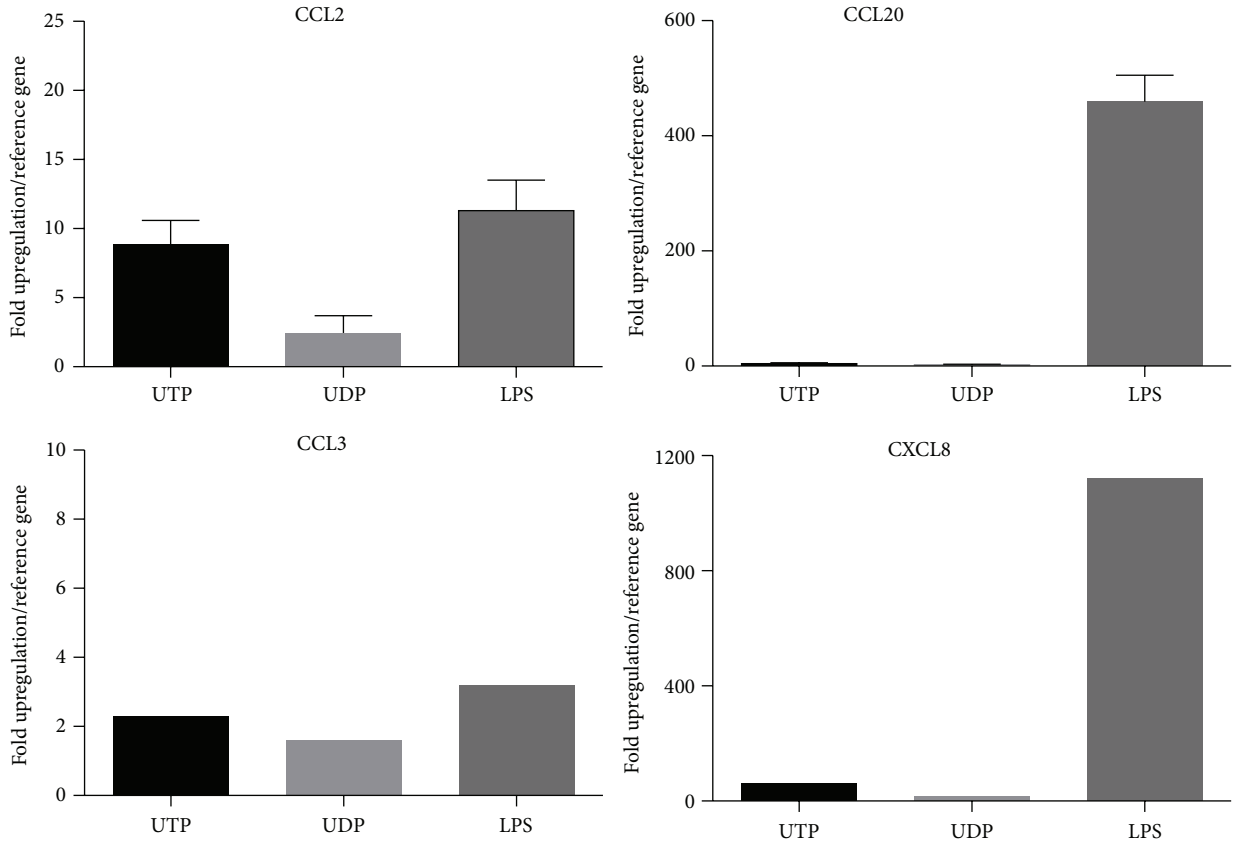

Figure 4: UTP induces CCL2 gene transcription in THP-1 monocytes. THP-1 cells were plated in RPMI media containing 1% serum and stimulated with media alone, LPS (1 μg/mL), UTP (10 μM), or UDP (10 μM) for 2 hours. RNA was extracted, reverse transcribed to cDNA, and probed with quantitative PCR primers for chemokines (CCL2, CCL20, CCL3, and CXCL8) and reference genes (β-actin and GAPDH). PCR was performed in triplicate using a Rotorgene 2000. Data was analysed using the $2^{-\Delta\Delta Ct}$ method using a threshold of 0.003.

Nucleotide-induced CCL20 production has been demonstrated in human dendritic cells (UDP and ATPγS) [9], CXCL8 production in monocytes [13], and CCL3 production in rodent microglia (UTP, UDP) [7]. Using quantitative PCR we found that LPS upregulated both CCL20 and CXCL8 in THP-1 cells; however UTP had no significant effect on CCL3, CCL20, and CXCL8 production (Figure 4). In primary human monocytes LPS significantly induced CCL20 gene expression in all 4 donors (42–427-fold increase). In comparison UTP and UDP nucleotides did not significantly induce CCL20 ($n = 4$ donors). Our data shows that CCL2 induction in primary human $CD14^+$ monocytes was variable in response to either LPS or UTP (Figure 6) confirming our variability in the protein secretion experiments.

The finding that UTP stimulation of P2Y2 receptors can elicit a similar CCL2 chemokine response to LPS stimulation of TLR4 is a novel finding and one with potential relevance for inflammation. UTP is a known danger signal, similar to ATP, and is likely to be present in areas of tissue damage without infection in addition to sites of infection. Such nucleotides may be a driving factor in sterile inflammation and may contribute to disease associated chronic inflammatory states. It is also important to hypothesise about why nucleotides may induce CCL2. In addition to the role of CCL2 as a chemokine for immune cells, it may be released as a mechanism for upregulating other receptors such as P2X4 receptors, as recently described by Toyomitsu et al. [25]. CCL2 may therefore act as an autocrine factor on monocytes/macrophages to prime further inflammatory signalling.

A second major aim of the current study was to investigate P2Y2 induced chemokine production in primary human monocytes and macrophages. We isolated monocytes from peripheral blood of a number of donors to determine if the nucleotide-induced CCL2 response was detectable. Our data demonstrates a degree of variability in constitutive and induced secretion of CCL2 from monocytes and macrophages. This type of variability has been observed previously for LPS where different individuals can be classified as high or low responders [26] and other studies have demonstrated variable LPS-induced responses including interleukin 1β (IL-1β) secretion [27] and CCL20 chemokine secretion from human dendritic cells and monocytes [9]. We and others have also previously shown variability in IL-1β secretion in response to P2X7 activation [19, 28]. One source of such variability in responses is genetic variation in the form of single nucleotide polymorphisms and three SNPs have been identified in the human *P2RY2* gene [21, 22]. Sequencing determined that one SNP, rs3741156, was present

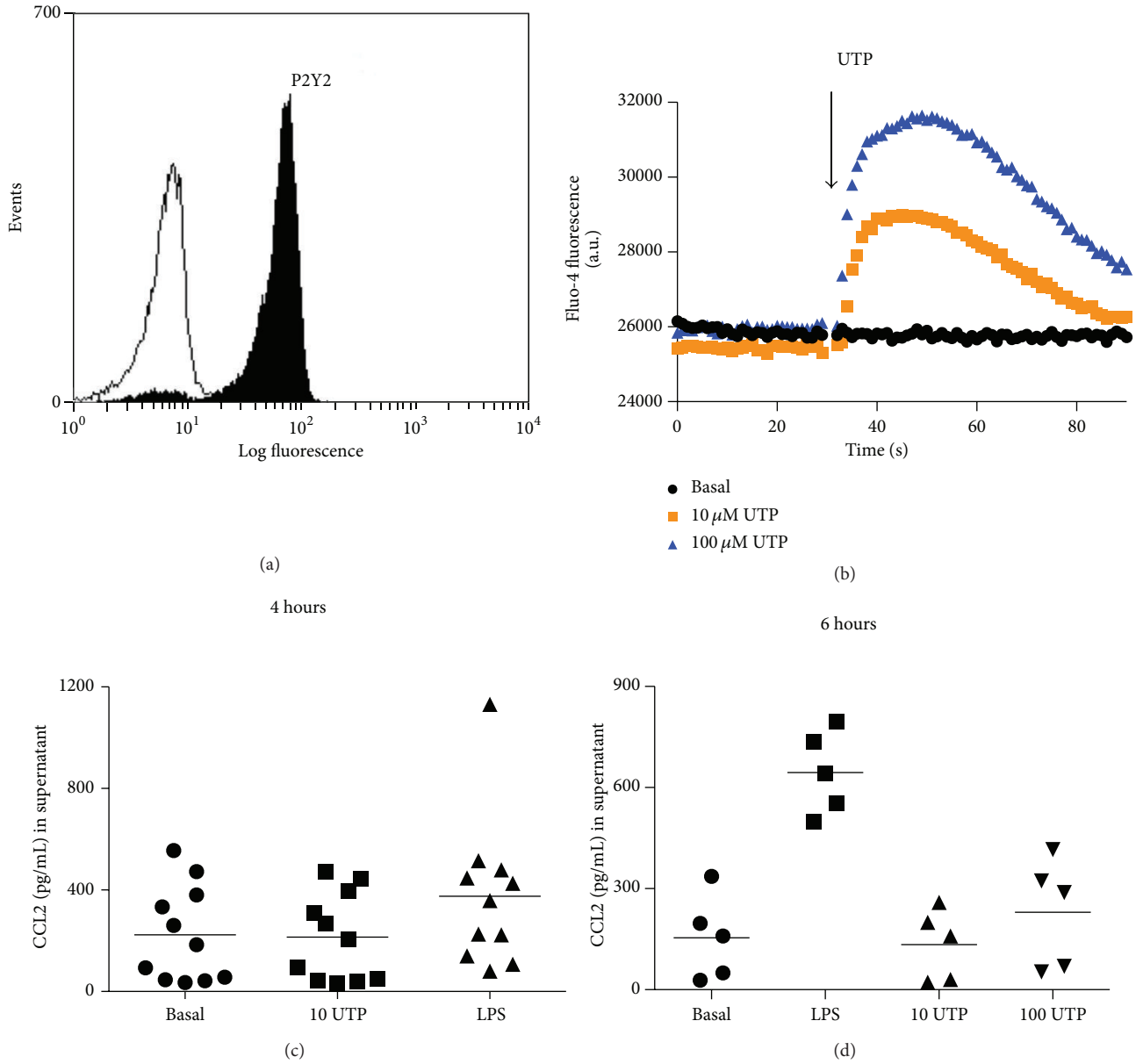

Figure 5: Primary human peripheral blood monocytes express P2Y2 but display variable CCL2 secretion to UTP. (a) Human PBMCs were labelled with conjugated CD14-FITC and rabbit anti-P2Y2 followed by goat anti-rabbit Alexa 647 using an intracellular staining protocol. (b) Human CD14 positive magnetically isolated monocytes were plated on poly-D-lysine plates and loaded with Fluo-4AM. UTP induced a transient calcium response at both 10 μM (orange) and 100 μM (blue). Calcium measurements were recorded using a Fluostar Optima plate reader at 37°C. (c) Human $CD14^+$ monocytes were plated at 5×10^5 cells/well and stimulated with media, LPS 100 ng/mL, or 10 μM UTP for 4 hours. Each symbol represents a different donor ($n = 11$ donors in total). (d) Human $CD14^+$ monocytes stimulated for 6 hours with media, LPS 100 ng/mL, 10 μM UTP, or 100 μM UTP. Each symbol represents a different donor ($n = 5$ different donors).

in the THP-1 cell line and other studies have demonstrated that this mutation altering amino acid 312 can affect UTP-induced calcium responses in transfected cells [21]. Other studies have indicated that P2Y induced calcium signalling is important in switching on CCL2 production in myeloid cells [7]. Monocyte-derived macrophages from individuals carrying the 312Ser-P2Y2 variant displayed a significant CCL2 secretory response compared to individuals expressing a 312Arg-containing receptor (Figure 9). Therefore, SNPs in P2Y2 may correlate with variability in responses. Future studies will investigate if other SNPs are present in the gene and whether they have a functional effect on receptor signalling.

5. Conclusions

Nucleotides are effective inducers of the chemokine CCL2 from THP-1 monocytic cell line similar to lipopolysaccharide.

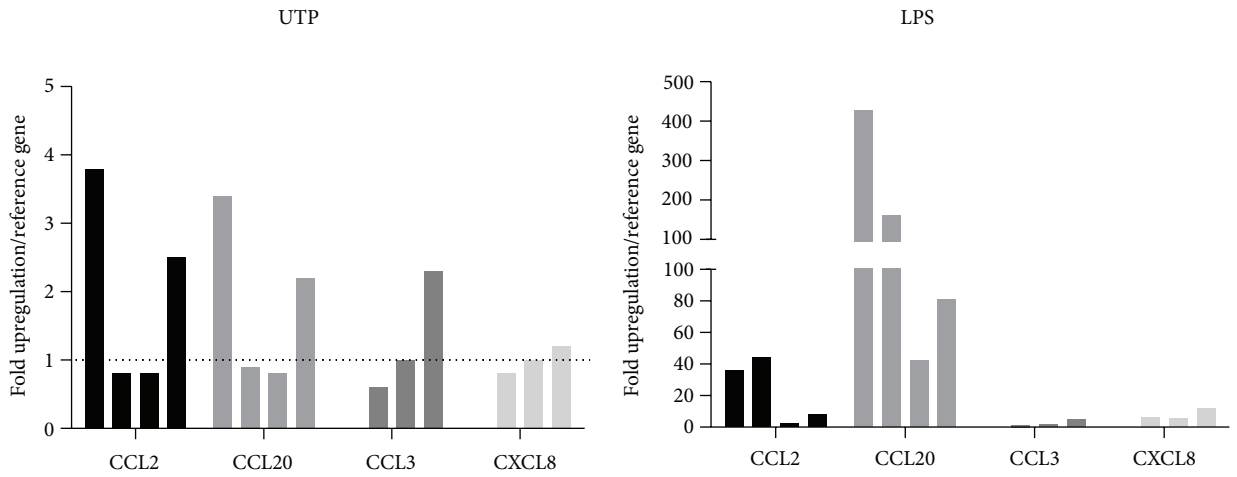

Figure 6: UTP induces chemokine gene transcription in human monocytes. Human CD14$^+$ monocytes were plated in RPMI media containing 1% serum and stimulated with media alone, LPS (1 μg/mL), UTP (10 μM), or UDP (10 μM) for 6 hours. RNA was extracted, reverse transcribed to cDNA, and probed with quantitative PCR primers for chemokines (CCL2, CCL20, CCL3, and CXCL8) and reference gene (β-actin). PCR was performed in triplicate using a Rotorgene 2000. Data was analysed using the $2^{-\Delta\Delta Ct}$ method using a threshold of 0.003 and fold upregulation with respect to the media control is plotted.

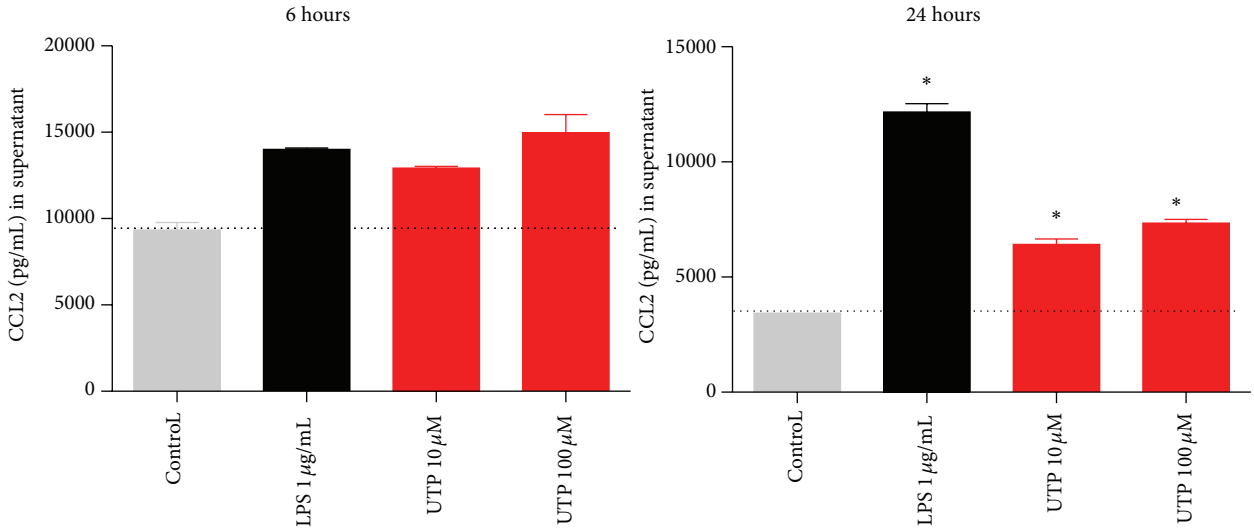

Figure 7: IFN/LPS differentiated THP-1 macrophages display increased CCL2 production to LPS or UTP. THP-1 cells were plated in RPMI media containing 10% serum and stimulated with IFN-γ and LPS for 48 hours. Media were removed and cells were challenged with either media alone (control), LPS (1 μg/mL), UTP (10 μM), or UTP (100 μM) for 4 hours. Mean raw data is plotted in pg/mL ± SEM. Standard curves were performed for each ELISA experiment with fits of $r > 0.95$.

Human primary macrophages display a more robust CCL2 response to nucleotides than human monocytes. Some of the variability in the CCL2 response to nucleotides could be explained by genetic variation in the P2Y2 gene with a mutation altering amino acid 312 demonstrating an increased chemokine response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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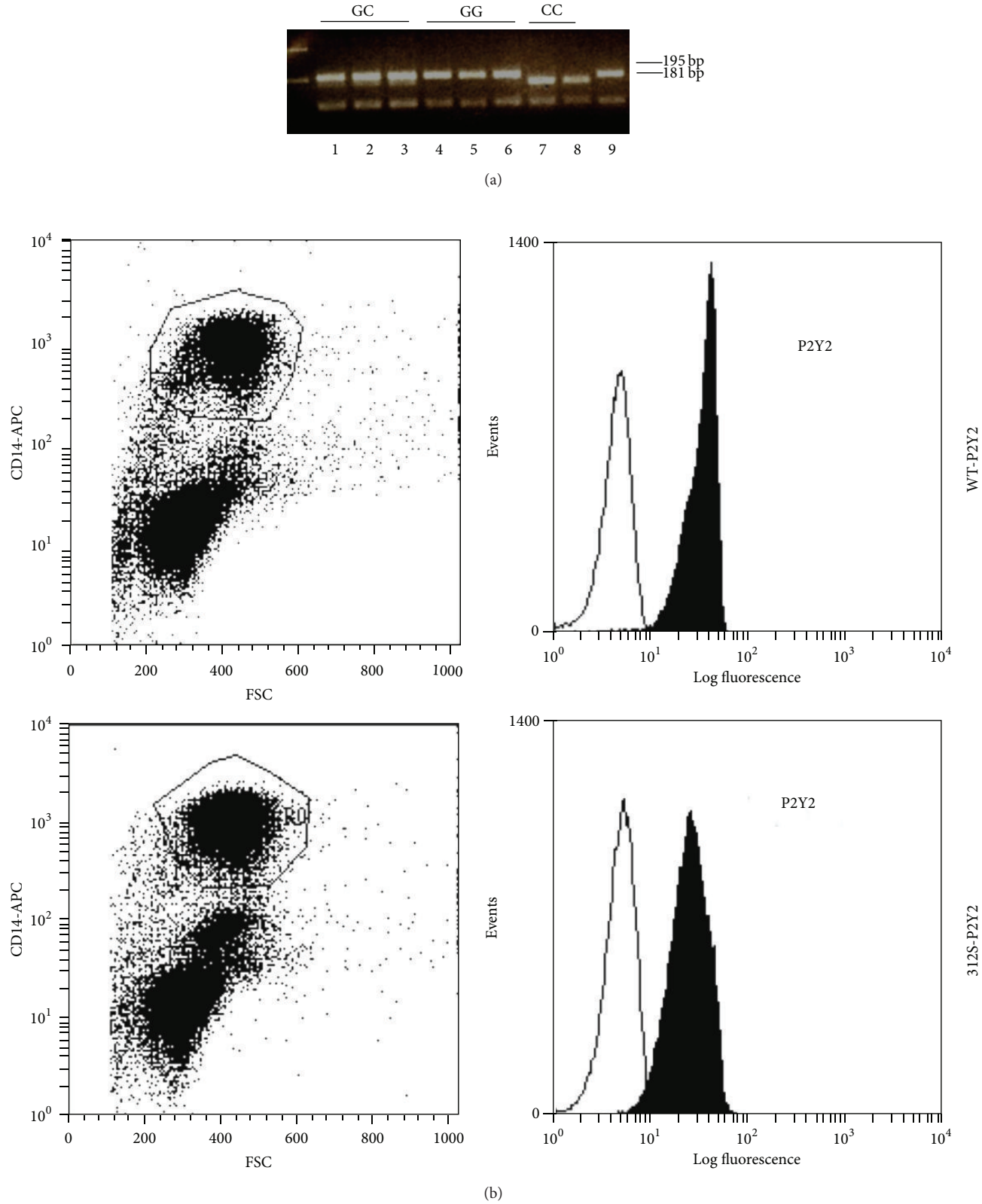

Figure 8: P2Y2 expression is similar in macrophages expressing 312Arg-P2Y2 and 312Ser-P2Y2 variants. (a) An in-house genotyping assay was designed using PCR and restriction enzyme analysis with MwoI. Individuals with GG (wild-type, lanes 4, 5, and 6) have a single upper band of 195 bp. Individuals with CC (homozygous for 312Ser, lanes 7 and 8) show a single upper band of 181 bp. Individuals with GC genotype (heterozygous for 312Ser, lanes 1, 2, and 3) show two upper bands at 195 and 181 bp. (b) Flow cytometry dot plots showing gating of CD14 positive PBMCs and representative histograms showing P2Y2 expression on $CD14^+$ gated population.

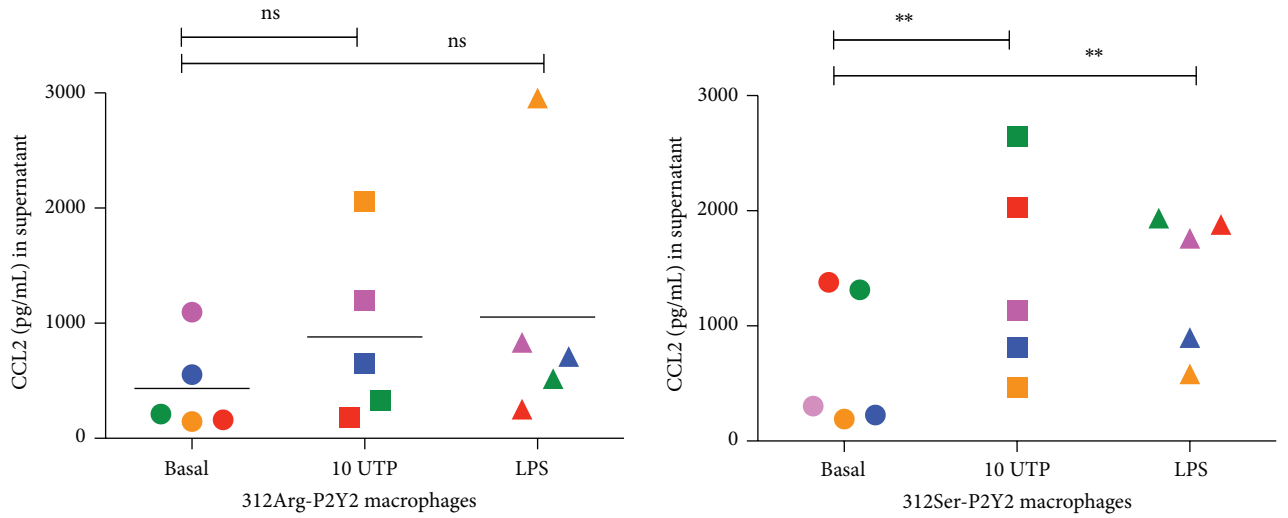

FIGURE 9: Macrophages expressing 312Ser-P2Y2 secrete higher levels of CCL2 in response to UTP and LPS. Human adherent PBMCs were plated at 2.5×10^6 cells/well and cultured for 7 days. Media were replaced and cells were stimulated with media, 10 μM UTP, or 100 μg/mL LPS for 4 hours. Each symbol represents a different donor ($n = 5$ donors per genotype group) and each donor has a different colour. Bars represent the mean data for each condition. ** denotes $P < 0.05$ using a one-way ANOVA with Dunnett's post hoc test; ns denotes not significant.

References

[1] N. V. Serbina, T. Jia, T. M. Hohl, and E. G. Pamer, "Monocyte-mediated defense against microbial pathogens," *Annual Review of Immunology*, vol. 26, pp. 421–452, 2008.

[2] A. Viola and A. D. Luster, "Chemokines and their receptors: drug targets in immunity and inflammation," *Annual Review of Pharmacology and Toxicology*, vol. 48, pp. 171–197, 2008.

[3] M. P. Abbracchio, G. Burnstock, J. Boeynaems et al., "International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy," *Pharmacological Reviews*, vol. 58, no. 3, pp. 281–341, 2006.

[4] I. von Kügelgen, "Pharmacological profiles of cloned mammalian P2Y-receptor subtypes," *Pharmacology and Therapeutics*, vol. 110, no. 3, pp. 415–432, 2006.

[5] L. Ding, W. Ma, T. Littmann, R. Camp, and J. Shen, "The P2Y 2 nucleotide receptor mediates tissue factor expression in human coronary artery endothelial cells," *Journal of Biological Chemistry*, vol. 286, no. 30, pp. 27027–27038, 2011.

[6] J. Jin, V. R. Dasari, F. D. Sistare, and S. P. Kunapuli, "Distribution of P2Y receptor subtypes on haematopoietic cells," *British Journal of Pharmacology*, vol. 123, no. 5, pp. 789–794, 1998.

[7] B. Kim, H. Jeong, J. Kim, S. Y. Lee, I. Jou, and E. Joe, "Uridine 5′-diphosphate induces chemokine expression in microglia and astrocytes through activation of the P2Y6 receptor," *Journal of Immunology*, vol. 186, no. 6, pp. 3701–3709, 2011.

[8] F. Kukulski, F. B. Yebdri, F. Bahrami, M. Fausther, A. Tremblay, and J. Sévigny, "Endothelial P2Y2 receptor regulates LPS-induced neutrophil transendothelial migration in vitro," *Molecular Immunology*, vol. 47, no. 5, pp. 991–999, 2010.

[9] B. Marcet, M. Horckmans, F. Libert, S. Hassid, J. Boeynaems, and D. Communi, "Extracellular nucleotides regulate CCL20 release from human primary airway epithelial cells, monocytes and monocyte-derived dendritic cells," *Journal of Cellular Physiology*, vol. 211, no. 3, pp. 716–727, 2007.

[10] L. Stokes and A. Surprenant, "Purinergic P2Y2 receptors induce increased MCP-1/CCL2 synthesis and release from rat alveolar and peritoneal macrophages," *Journal of Immunology*, vol. 179, no. 9, pp. 6016–6023, 2007.

[11] J. W. Bowler, R. J. Bailey, R. A. North, and A. Surprenant, "P2X4, P2Y1 and P2Y2 receptors on rat alveolar macrophages," *British Journal of Pharmacology*, vol. 140, no. 3, pp. 567–575, 2003.

[12] A. Raqeeb, J. Sheng, N. Ao, and A. P. Braun, "Purinergic P2Y2 receptors mediate rapid Ca^{2+} mobilization, membrane hyperpolarization and nitric oxide production in human vascular endothelial cells," *Cell Calcium*, vol. 49, no. 4, pp. 240–248, 2011.

[13] F. B. Yebdri, F. Kukulski, A. Tremblay, and J. Sévigny, "Concomitant activation of P2Y2 and P2Y6 receptors on monocytes is required for TLR1/2-induced neutrophil migration by regulating IL-8 secretion," *European Journal of Immunology*, vol. 39, no. 10, pp. 2885–2894, 2009.

[14] M. Warny, S. Aboudola, S. C. Robson et al., "P2Y(6) nucleotide receptor mediates monocyte interleukin-8 production in response to UDP or lipopolysaccharide," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 26051–26056, 2001.

[15] C. Geary, H. Akinbi, T. Korfhagen, J. Fahre, R. Boucher, and W. Rice, "Increased susceptibility of purinergic receptor-deficient mice to lung infection with *Pseudomonas aeruginosa*," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 289, no. 5, pp. L890–L895, 2005.

[16] H. Kouzaki, K. Iijima, T. Kobayashi, S. M. O'Grady, and H. Kita, "The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses," *Journal of Immunology*, vol. 186, no. 7, pp. 4375–4387, 2011.

[17] T. Müller, B. Robaye, R. P. Vieira et al., "The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation," *Allergy*, vol. 65, no. 12, pp. 1545–1553, 2010.

[18] C. J. Ciesielski, E. Andreakos, B. M. Foxwell, and M. Feldmann, "TNFalpha-induced macrophage chemokine secretion is more

dependent on NF-kappaB expression than lipopolysaccharides-induced macrophage chemokine secretion," *European Journal of Immunology*, vol. 32, no. 7, pp. 2037–2045, 2002.

[19] L. Stokes, S. J. Fuller, R. Sluyter, K. K. Skarratt, B. J. Gu, and J. S. Wiley, "Two haplotypes of the P2X7 receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1β secretion," *The FASEB Journal*, vol. 24, no. 8, pp. 2916–2927, 2010.

[20] B. D. Humphreys and G. R. Dubyak, "Induction of the P2z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human HP-1 monocytic cell line," *Journal of Immunology*, vol. 157, no. 12, pp. 5627–5637, 1996.

[21] R. Büscher, A. Hoerning, H. H. Patel et al., "P2Y2 receptor polymorphisms and haplotypes in cystic fibrosis and their impact on Ca^{2+} influx," *Pharmacogenetics and Genomics*, vol. 16, no. 3, pp. 199–205, 2006.

[22] R. Janssens, P. Paindavoine, M. Parmentier, and J. Boeynaems, "Human P2Y2 receptor polymorphism: Identification and pharmacological characterization of two allelic variants," *British Journal of Pharmacology*, vol. 127, no. 3, pp. 709–716, 1999.

[23] K. A. Jacobson, A. A. Ivanov, S. de Castro, T. K. Harden, and H. Ko, "Development of selective agonists and antagonists of P2Y receptors," *Purinergic Signalling*, vol. 5, no. 1, pp. 75–89, 2009.

[24] K. G. Steube, C. Meyer, and H. G. Drexler, "Constitutive protein expression of monocyte chemotactic protein-1 (MCP-1) by myelomonocytic cell lines and regulation of the secretion by anti- and proinflammatory stimuli," *Leukemia Research*, vol. 23, no. 9, pp. 843–849, 1999.

[25] E. Toyomitsu, M. Tsuda, T. Yamashita, H. Tozaki-Saitoh, Y. Tanaka, and K. Inoue, "CCL2 promotes P2X4 receptor trafficking to the cell surface of microglia," *Purinergic Signalling*, vol. 8, no. 2, pp. 301–310, 2012.

[26] M. M. Wurfel, W. Y. Park, F. Radella et al., "Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity," *Journal of Immunology*, vol. 175, no. 4, pp. 2570–2578, 2005.

[27] D. G. Perregaux, P. McNiff, R. Laliberte, M. Conklyn, and C. A. Gabel, "ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood," *Journal of Immunology*, vol. 165, no. 8, pp. 4615–4623, 2000.

[28] R. Sluyter, A. N. Shemon, and J. S. Wiley, "Glu496 to Ala polymorphism in the P2X7 receptor impairs ATP-induced IL-1 beta release from human monocytes," *Journal of Immunology*, vol. 172, no. 6, pp. 3399–3405, 2004.

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Research Article

Ecto-nucleotidases Activities in the Contents of Ovarian Endometriomas: Potential Biomarkers of Endometriosis

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Endometriosis, defined as the growth of endometrial tissue outside the uterus, is a common gynecologic condition affecting millions of women worldwide. It is an inflammatory, estrogen-dependent complex disorder, with broad symptomatic variability, pelvic pain, and infertility being the main characteristics. Ovarian endometriomas are frequently developed in women with endometriosis. Late diagnosis is one of the main problems of endometriosis; thus, it is important to identify biomarkers for early diagnosis. The aim of the present work is to evaluate the ecto-nucleotidases activities in the contents of endometriomas. These enzymes, through the regulation of extracellular ATP and adenosine levels, are key enzymes in inflammatory processes, and their expression has been previously characterized in human endometrium. To achieve our objective, the echo-guided aspirated fluids of endometriomas were analyzed by evaluating the ecto-nucleotidases activities and compared with simple cysts. Our results show that enzyme activities are quantifiable in the ovarian cysts aspirates and that endometriomas show significantly higher ecto-nucleotidases activities than simple cysts (5.5-fold increase for ATPase and 20-fold for ADPase), thus being possible candidates for new endometriosis biomarkers. Moreover, we demonstrate the presence of ecto-nucleotidases bearing exosomes in these fluids. These results add up to the knowledge of the physiopathologic mechanisms underlying endometriosis and, open up a promising new field of study.

1. Introduction

Endometriosis is a chronic condition characterized by the presence of endometrial tissue (stroma and glands) outside the uterine cavity, mostly on pelvic peritoneum and ovaries. It affects up to 15% of women of reproductive age and its prevalence is rising. It is an inflammatory, estrogen-dependent complex disorder, with broad symptomatic variability, pelvic pain, and infertility being the main characteristics. Both symptoms are thought to be the result of an excessive inflammatory environment not only within the pelvis but also in the eutopic endometrium, affecting implantation [1, 2].

It is one of the principal causes of infertility in women. Late diagnosis is one of the main problems of this pathology, taking between five and ten years from detection of the first symptoms. Therefore, there is a need to identify biomarkers for early diagnosis of endometriosis [3].

Ovarian endometriomas occur in 17–44% of patients affected by endometriosis [4] and account for 35% of benign ovarian cysts. Endometriomas are also called endometriotic or "chocolate cysts" because of the internal fluid blood derived dark color [5]. Although the risk of malignant transformation is low, 0.6–0.8% [6], surgery has become the gold standard therapy. Nowadays, laparoscopic cystectomy is

the most extended treatment for endometriomas, although fertility could be afterwards compromised. Some studies have reported a lower ovarian reserve after excision of ovarian endometriomas due to incidental excision of normal ovarian tissue together with the endometrioma wall [7, 8]. There is also evidence of a significant postoperative decrease in circulating Anti-Müllerian hormone, a commonly used biomarker for ovarian reserve quantification, suggesting a negative impact on ovarian reserve after the excision of endometriomas [8]. Therefore, a new minimally invasive interventional technique was implemented to treat low risk malignancy ovarian cysts, including endometriomas, the ultrasound-guided aspiration followed by sclerosis, which seems to have even a low recurrence risk (0–15%) [9–13]. The sclerosis does not seem to have consequences on the number of pregnancies, term pregnancies, abortions, extracted oocytes, embryo quality, and hormonal levels if compared with infertile women without ovarian cysts [11, 14].

The analysis of the contents of endometriomas can help identify new biomarkers for this pathology. For example, endometriotic cysts contain significantly more iron than other ovarian cysts; this has been suggested as a possible cause of carcinogenesis in endometriomas through iron-induced persistent oxidative stress [15]. Proteomic analysis of follicular fluid of women with endometriosis has also pointed to an altered immunologic function possibly related to infertility [16].

Purinergic signalling, the group of biologic effects exerted by extracellular nucleotides such as ATP and nucleosides such as adenosine through specific receptors, is an important regulatory mechanism in a wide range of inflammatory conditions [17, 18]. ATP is a "danger signal" released under tissue stress conditions, such as necrosis, apoptosis, hypoxia, and inflammation and is rapidly converted into adenosine, which protects cells and tissues from excessive inflammation and immune-mediated damage. The effects transduced by both molecules are thus often opposite, and the resulting cellular responses are the consequence of the ratio of ATP (and ADP) to adenosine concentrations, which is mainly controlled by ecto-nucleotidases, key enzymes in the purinergic signalling [19]. The most well-characterized ectonucleotidase pathway involves the sequential action of CD39 (ectonucleoside triphosphate diphosphohydrolase-1, NTPDase1), which converts extracellular ATP (or ADP) to AMP, and CD73 (ecto-5′-nucleotidase), which generates adenosine from AMP [20–23]. We have previously characterized the expression of endometrial ecto-nucleotidases, demonstrating changes along the cycle and in menopause [24, 25]. We have also demonstrated that CD39 and CD73 are highly expressed in endometrial cancer [26], contributing to the increased immunosuppressive levels of extracellular adenosine found in tumor microenvironment.

Exosomes are small, secreted membrane vesicles (30–100 nm in diameter) of endocytic origin, produced by many different cell types that are formed by the fusion of multivesicular endosomes with the plasma membrane. They are thought to play an important role in intercellular communication, for example, during immune cell-cell interaction, and have been identified in biological fluids, such as plasma and urine, as well as in cell culture supernatants [27, 28]. The presence of the ecto-nucleotidases CD39 and CD73 has been previously demonstrated in cancer exosomes [29–31], contributing to the adenosine production that suppresses T-cell function.

In the present work, we aimed to determine whether ecto-nucleotidases activities (ATPase, ADPase, and AMPase) are present and measurable in the ultrasound-guided aspirated contents of ovarian cysts and whether these activities are altered in endometriotic cysts compared with simple cysts, affecting the inflammatory condition of endometriosis. Our goal is to evaluate their potential as biomarkers for endometriosis and to contribute to the knowledge of the physiopathologic mechanisms underlying endometriosis.

2. Materials and Methods

2.1. Samples. The ethical principles of this study adhere to the Declaration of Helsinki, and all the procedures were approved by the ethics committee for clinical investigation of Bellvitge Hospital. A prospective cohort of 27 adnexal cysts with a low risk of malignancy, including 14 endometriomas and 13 ovarian simple cysts, was studied at the Gynecology Service of Bellvitge Hospital (Barcelona, Spain) between March 2013 and April 2014.

Inclusion criteria in the study for patients were (1) aged >18 years old; (2) ultrasound features predictive of a low risk of cyst malignancy: smooth walls, no solid component, maximum diameter between 40 and 100 mm, no more than one thin septation (<2-3 mm), and no internal flow on color Doppler imaging in the case of bilocular cysts, according to IOTA criteria [32]; (3) cyst persistence from diagnosis ≥6 months; (4) tumor marker Ca 125 < 200 IU/mL and Ca 19.9 < 35 IU/mL; and (5) written informed consent from the patients.

For ultrasound-guided aspiration, following the abdomen, or the vagina, disinfection, a sterilized 17 G spinal needle (BD Medical Franklin Lakes, NJ Becton, Dickinson and Company) was aimed at the center of the cyst under direct ultrasound guidance and the contents was aspirated. When thick highly dense intracystic fluid was present, in order to facilitate the aspiration, saline dilutions were performed. Dilutions made were then considered in the final calculations. Then, several intracyst saline washes were performed until clearance, followed by the instillation of ethanol as sclerosing agent. The volume of instilled ethanol was 2/3 of the aspirated volume of the endometrioma and always less than 100 mL. Ethanol was left inside the cyst for 15 min and then removed and washed out again with a saline solution. The intracystic fluids were analyzed cytologically at the Pathology Service of Bellvitge Hospital to confirm the absence of atypical cells.

Patients were divided into two groups following the ultrasound features and internal color fluid: (a) *endometriomas group* that had homogeneous appearance involving diffuse internal echoes on a hypoechoic background and contained blood color fluid and (b) *simple cysts group* that had an anechoic structure and contained clear serous fluid. Table 1 includes the descriptive statistics of the two groups of

Table 1: Descriptive statistics of the two groups of patients and samples.

	Age (years)	Size (mm)	Volume (mL)
Simple cyst ($N = 13$)			
Mean (SD)	39,8 (13,5)	69,54 (14,02)	69,85 (59,36)
Range	20–64	49–98	20–248
Endometrioma ($N = 14$)			
Mean (SD)	38,6 (14,1)	73,71 (33,26)	112,50 (111,77)
Range	28–84	42–170	20–410

The size represents the largest cystic diameter measured on ultrasound. The volume represents the total aspirated volume of intracystic fluid (SD: standard deviation).

patients, including the age and the size and the volume of the cysts.

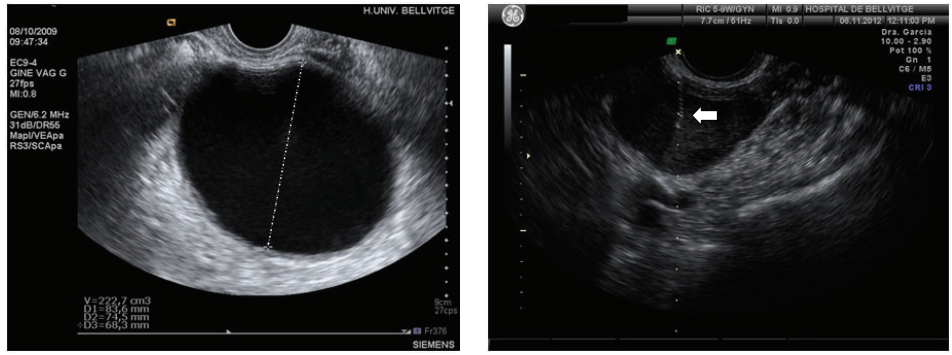
An aliquot of each sample was maintained on ice until usage for exosome enrichment. The rest was aliquoted and stored at −80°C.

2.2. Exosome Enrichment. Endometriomas and simple cysts fluid samples were centrifuged at 3000 ×g for 15 min to remove cells and cell debris, and supernatants were transferred to sterile tubes. 250 μL of each supernatant was mixed with 63 μL of ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) by inversion, and the mixture was refrigerated overnight at 4°C, following the supplier's instructions. Samples were then centrifuged at 1500 ×g for 30 min. After centrifugation, the exosomes appeared as a beige or white pellet at the bottom of the tube. Supernatant was aspirated and the residual ExoQuick solution was spun down by centrifugation at 1500 ×g for 5 min. All traces of fluid were removed by aspiration and exosome pellets were resuspended in two different buffers. For Western blot analysis, exosome pellets were resuspended in 75 μL of a buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1 : 100 proteases inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). Alternatively, for ecto-nucleotidases activity assays, exosome pellets were resuspended in 75 μL of enzyme assay buffer (160 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$). Protein concentration in exosome samples was determined by the method of Bradford [33]. Samples were stored at −20°C until usage.

2.3. Western Blotting Analysis. 15 μg of protein was heated at 95°C for 3 min, loaded onto any kD Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA, USA), and transferred to polyvinylidene fluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Blots were blocked with Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% nonfat dry milk for 1 hour at room temperature (RT). Membranes were then incubated overnight at 4°C with the following antibodies: exosomal marker antibodies CD9, CD63, CD81, HSP70 (diluted 1 : 1000) from the ExoAB-KIT-1 Western blot antibody detection kit (System Biosciences), anti-CD39 (clone BU61, diluted 1 : 500), and anti-CD73 (clone 4G4 from Abcam, diluted 1 : 50). Blots were then washed with TBS-T and incubated with the appropriate HRP-conjugated secondary antibody (System Biosciences, dilution 1 : 20,000) for 1 hour at RT. Membranes were then incubated with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Pittsburgh, PA, USA) for 1 min and exposed on X-ray films.

2.4. Nucleotidase Activity Assays. Nucleotidase activities (ATPase, ADPase, and AMPase) were determined in flat-bottom 96-well plates by measuring the amount of liberated inorganic phosphate (Pi) using the malachite green colorimetric assay [34]. All the samples were used for these assays. The incubation mixture contained 160 mM Tris–HCl pH 7.4, 10 mM $CaCl_2$, 5 mM levamisole, as alkaline phosphatases inhibitor, and 1 mM ATP, ADP, or AMP as substrates in a final volume of 150 μL. The assay was initiated by adding the sample, either the fluid directly (5 μL for ATPase and ADPase assays and 10 μL for AMPase assay) or 50 μg of the isolated exosome fraction. Alternatively, increasing amounts of exosomes were added to the well to assess if ectonucleotidase activity depended on the exosome dose (5, 10, 15, 20, 30, and 50 μg). After 20 minutes of incubation at 37°C, 50 μL of malachite green solution was added to each well and 10 and 20 minutes later, the OD_{620} was measured and recorded. Incubation times and protein concentrations were chosen to ensure the linearity of the enzymatic reaction. KH_2PO_4 was used as a Pi standard. Controls to determine nonenzymatic Pi accumulation were performed by incubating either the samples in the absence of the substrate or the substrate alone. Each experiment was performed three times and in each experiment samples were analyzed in triplicate. Enzyme activity was expressed as pmols Pi/min/μL in the case of the whole fluids and as pmol Pi/min/μg for exosomes.

2.5. Electron Microscopy Studies. For morphological analysis, whole-mount electron microscopy preparations of exosomes were obtained as previously described by Théry et al. [35]. In brief, exosome-containing pellets were resuspended in 2% paraformaldehyde in 100 mM phosphate-buffered saline (PBS). 5 μL of resuspended pellets was deposited on formvar-coated grids and incubated at RT for 20 min. After thorough rinses, the samples were postfixed in 1% glutaraldehyde for 5 min and then contrasted in a solution of 2% uranyl acetate, 150 mM oxalic acid pH 7. Then the grids were transferred to a drop of 1.8% methyl-cellulose, 0.4% uranyl acetate for 10 min on ice. Finally, the grids were removed with stainless steel loops and air-dried for additional 10 min.



(a) (b)

Figure 1: Transvaginal ultrasound-guided aspiration of an endometrioma. (a) Endometrioma measured before aspiration. Dotted line indicates one of the diameter measurements. (b) Endometrioma being aspirated. The arrow indicates the end of the needle inside the cyst. Note the reduction in the volume after aspiration.

Alternatively, and in order to obtain thin sections, the paraformaldehyde-fixed exosome-containing pellets were postfixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM PBS, dehydrated in a graded ethanol series, and embedded in the Spurr low viscosity resin. Ultrathin sections (50–60 nm) from exosome-enriched pellets were cut with a diamond knife in an ultramicrotome UltracutE (Leica Microsystems). After being picked up on copper grids, the ultrathin sections were stained with uranyl acetate and lead citrate. Finally, exosomes were observed with a JEM-1010 transmission electron microscope (JEOL, Japan), and the images were recorded with a Bio Scan 792 camera (GATAN, Germany).

2.6. Statistical Analysis. Statistical analysis was performed using SigmaStat 3.2 software (SPSS Inc., Chicago, IL, USA). Values are reported as the mean ± S.E.M. Student's t-test was used to compare the means of two independent groups of normally distributed data.

3. Results and Discussion

Most of the attempts to identify peripheral biomarkers of endometriosis have been focused on their presence in plasma, serum, or urine [3]. However, the study of endometriomas contents has been proven to be very informative [15, 16]. In the present work, we have evaluated the ectonucleotidase enzyme activities present in endometriomas in comparison with ovarian simple cysts.

Echo-guided aspiration was the technique used to remove the fluid contained in endometriomas and simple cysts. Immediate ethanol sclerosis was performed to chemically destroy the cyst lining and to prevent its regrowth. Figure 1 shows the ultrasound picture of an endometrioma before and during the aspiration of its contents.

Aspirated fluids were then used for enzyme activity studies. Ecto-nucleotidases activities (ATPase, ADPase, and, to a lesser extent, AMPase) were present and quantifiable in the aspirated fluid of both endometriomas and ovarian simple cysts (Figure 2). Endometriomas aspirates

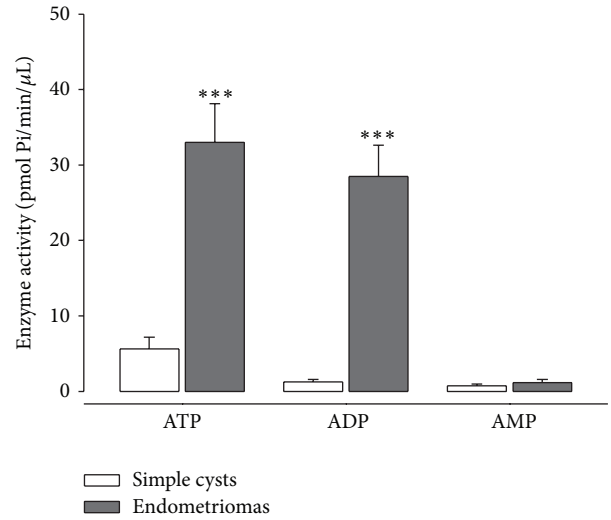


Figure 2: Enzyme activity in fluids from simple cysts and from endometriomas. Ectonucleotidase activity, represented as pmoles of generated Pi, in fluids from simple cysts (white columns) and from endometriomas (gray columns) in the presence of ATP, ADP, or AMP as substrate. ATPase and ADPase activities are significantly higher in endometriomas than in simple cysts ($^{***}P < 0,001$), while AMPase activity was barely detectable and differences could not be determined.

showed significantly higher ($P < 0,001$) ATPase and ADPase activities (mean ± SEM, 38,96 ± 5,11 and 33,68 ± 3,95 pmol/min/μL, resp.) than simple cysts (7,19 ± 1,88 and 1,55 ± 0,4 pmol/min/μL, resp.). Low activity rates were detected with AMP as substrate in the aspirated fluid and although endometriomas displayed higher AMPase activity (1,28 ± 0,46 pmol/min/μL) than simple cysts (0,87 ± 0,25 pmol/min/μL), differences were not significant. The same results were also obtained when the activities were calculated per μg of protein in the fluid, with even higher activities in endometriomas (data not shown); however, since it is known that endometriotic cysts differ from simple cysts in protein contents [16], results are shown here as Pi

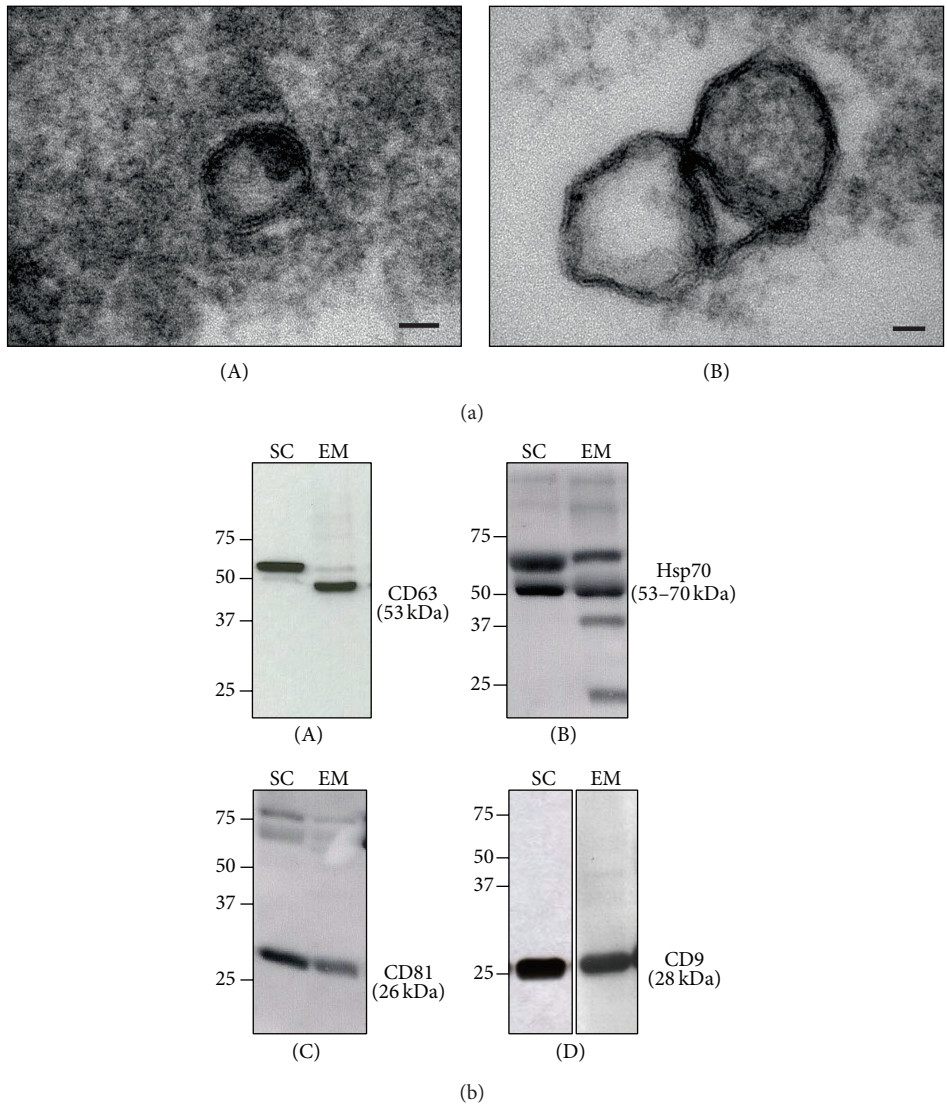


Figure 3: Exosome characterization. (a) Representative TEM images of exosomes isolated from endometriomas (bar = 25 nm). (b) Western blot with four different exosome markers: CD63 (A), Hsp70 (B), CD81 (C), and CD9 (D) of exosome-enriched fractions from simple cysts (SC) and from endometriomas (EM). The figure shows one representative experiment of each type of the sample. Molecular weight markers in kDa are indicated at the left of each image.

generation per unit of volume. This is the first time that ecto-nucleotidases have been studied in ovarian cysts, although these enzyme activities have been previously characterized in other fluids such as pancreatic juice [36] and plasma [37], where there is a correlation between their activity and different inflammatory pathologies [38].

Since to perform the enzyme activity assays, aspirated fluids were previously centrifuged to remove cells and cell debris, the resulting activities could be attributed either to soluble enzymes or to membrane bound ecto-nucleotidases present in extracellular membrane vesicles such as microvesicles and exosomes that were not removed by the initial centrifugation. Therefore, we decided to perform exosome enrichment to assess their presence and to determine if they had ectonucleotidase activities. To our knowledge, this is the first study demonstrating the presence of exosomes in the fluids contained in ovarian cysts. Electron microscopy analysis confirmed the size and morphology of exosomes (Figure 3(a)). Western blots using antibodies against four exosome markers (CD9, CD63, CD81, and Hsp70) demonstrated the presence of these structures in 100% of endometriomas and in 46% of simple cysts aspirates (Figure 3(b)). From these experiments, we conclude that, among the markers used, CD9 is the most suitable for exosome demonstration in the aspirated fluids of ovarian cysts, since it was present in all exosome samples analyzed. CD9 has been already demonstrated to be a good exosome marker for epithelial endometrial cells [39, 40]. All the samples expressed, together with CD9, at least one more of the other exosome markers assayed. Interestingly, using the anti-CD63 antibody, the predominant band in endometriomas was of a lower molecular weight than in simple cysts. A possible explanation is the

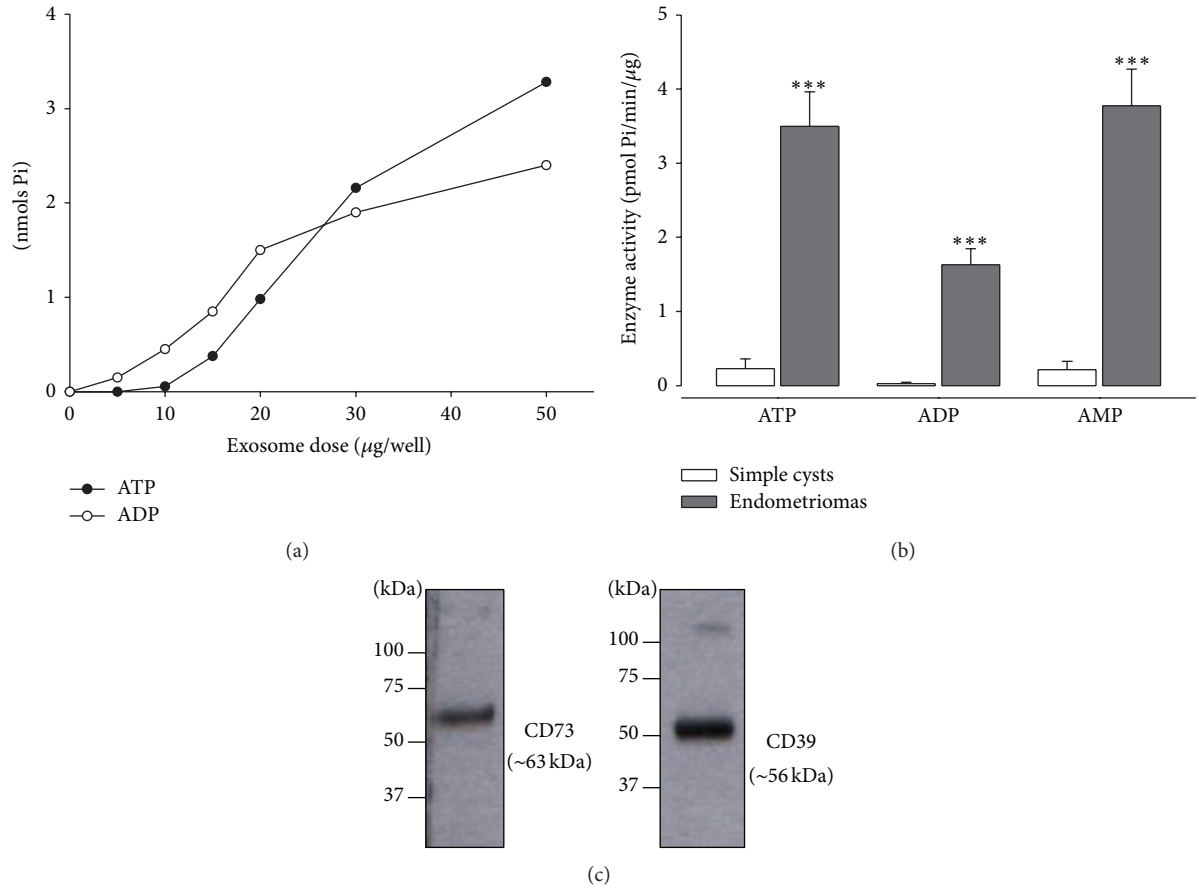


Figure 4: Enzyme activity in exosome-enriched fractions from simple cysts and from endometriomas. (a) ATPase (black circles) and ADPase (white circles) activities of increasing amounts of exosomes from endometriomas (0, 5, 10, 20, 30, and 50 μg). (b) Ectonucleotidase activity, represented as pmoles of generated Pi, in exosome-enriched fractions from simple cysts (white columns) and from endometriomas (gray columns) in the presence of ATP, ADP, or AMP as substrate. All the activities are significantly higher in endometriomas than in simple cysts ($^{***}P < 0,001$). (c) Western blot with anti-CD39 and anti-CD73 antibodies of exosome-enriched fractions from an endometrioma. Molecular weight markers in kDa are indicated at the left of each image.

alternative splicing already described for the gene encoding CD63 that results in multiple transcript variants encoding different proteins. Since this result was consistent in all the samples studied, we propose to further study its presence in exosomes from other origins in endometriosis patients, to establish if it could be used as biomarker for the pathology.

Enzyme experiments performed with exosome-enriched fractions demonstrated that these samples retain the ectonucleotidase activity (ATPase, ADPase, and AMPase) displayed by the whole fluid. Moreover, these activities are dependent on the sample dose, as shown in Figure 4(a), in line with previous results obtained with exosomes from other origins that express active CD39 and CD73 [29]. For ATPase and ADPase, maximal activity was found with 50 μg of exosomes. AMPase activity, which was barely detectable in the whole fluid, was present in purified exosomes, though only at high concentrations (50 μg), indicating that this activity in the whole fluid is mainly due to the enzymes bearing exosomes. On the contrary, ATPase and ADPase activities would be related to both exosome membrane bound and soluble enzymes. Figure 4(b) shows the enzyme activity using ATP, ADP, and AMP as substrates of exosomes from simple cysts and from endometriomas. These activities were significantly higher in endometriomas than in simple cysts. Western blot analysis demonstrated the presence of CD39 and CD73 in these samples (Figure 4(c)).

Our results reinforce the emerging evidence linking inflammation to endometriosis pathophysiology, where evasion of the immune surveillance seems to be crucial in the initial establishment and subsequent development of displaced endometrial tissues [41]. In this sense, increased sequentially acting ectonucleotidase activities would contribute to the maintenance of the suppression of local immune responses necessary for the disease progression, by regulating extracellular ATP and rising extracellular adenosine levels. We propose to detect their presence in other more accessible biological fluids, a fact that, together with the simplicity of their detection, would make them promising endometriosis biomarkers.

4. Conclusions

Our work contributes to the knowledge of endometriosis by providing new data on the fluid composition of endometriomas. Ecto-nucleotidases activities are quantifiable in the ovarian cysts aspirates, and they are significantly increased in the contents of endometriomas compared with simple cysts. Moreover, the identification of ecto-nucleotidases bearing exosomes in ovarian cyst contents opens the field for a broad range of proteomics and genomics studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Laura Texidó, Claudia Romero, and August Vidal contributed equally to this work.

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References

[1] S. E. Bulun, "Endometriosis," *New England Journal of Medicine*, vol. 360, no. 3, pp. 268–279, 2009.

[2] J. A. Maybin, H. O. D. Critchley, and H. N. Jabbour, "Inflammatory pathways in endometrial disorders," *Molecular and Cellular Endocrinology*, vol. 335, no. 1, pp. 42–51, 2011.

[3] K. E. May, S. A. Conduit-Hulbert, J. Villar, S. Kirtley, S. H. Kennedy, and C. M. Becker, "Peripheral biomarkers of endometriosis: a systematic review," *Human Reproduction Update*, vol. 16, no. 6, pp. 651–674, 2010.

[4] D. B. Redwine, "Ovarian endometriosis: a marker for more extensive pelvic and intestinal disease," *Fertility and Sterility*, vol. 72, no. 2, pp. 310–315, 1999.

[5] J. A. Sampson, "Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation," *American Journal of Pathology*, vol. 3, no. 2, pp. 93–110.43, 1927.

[6] M. Nishida, K. Watanabe, N. Sato, and Y. Ichikawa, "Malignant transformation of ovarian endometriosis," *Gynecologic and Obstetric Investigation*, vol. 50, no. 1, pp. 18–25, 2000.

[7] L. Muzii, A. Bianchi, C. Crocè, N. Manci, and P. B. Panici, "Laparoscopic excision of ovarian cysts: is the stripping technique a tissue-sparing procedure?" *Fertility and Sterility*, vol. 77, no. 3, pp. 609–614, 2002.

[8] F. Raffi, M. Metwally, and S. Amer, "The impact of excision of ovarian endometrioma on ovarian reserve: a systematic review and meta-analysis," *Journal of Clinical Endocrinology and Metabolism*, vol. 97, no. 9, pp. 3146–3154, 2012.

[9] N. Akamatsu, T. Hirai, H. Masaoka, K. Sekiba, and T. Fujita, "Ultrasonically guided puncture of endometrial cyst - Aspiration of contents and infusion of ethanol," *Nihon Sanka Fujinka Gakkai Zasshi*, vol. 40, no. 2, pp. 187–191, 1988.

[10] E. M. Messalli, G. Cobellis, E. Pecori et al., "Alcohol sclerosis of endometriomas after ultrasound-guided aspiration," *Minerva Ginecologica*, vol. 55, no. 4, pp. 359–362, 2003.

[11] J. D. Fisch and G. Sher, "Sclerotherapy with 5% tetracycline is a simple alternative to potentially complex surgical treatment of ovarian endometriomas before in vitro fertilization," *Fertility and Sterility*, vol. 82, no. 2, pp. 437–441, 2004.

[12] C. Yazbeck, P. Madelenat, J. P. Ayel et al., "Ethanol sclerotherapy: a treatment option for ovarian endometriomas before ovarian stimulation," *Reproductive BioMedicine Online*, vol. 19, no. 1, pp. 121–125, 2009.

[13] G. Gatta, V. Parlato, G. Di Grezia et al., "Ultrasound-guided aspiration and ethanol sclerotherapy for treating endometrial cysts," *Radiologia Medica*, vol. 115, no. 8, pp. 1330–1339, 2010.

[14] T. Koike, H. Minakami, M. Motoyama, S. Ogawa, H. Fujiwara, and I. Sato, "Reproductive performance after ultrasound-guided transvaginal ethanol sclerotherapy for ovarian endometriotic cysts," *European Journal of Obstetrics Gynecology and Reproductive Biology*, vol. 105, no. 1, pp. 39–43, 2002.

[15] K. Yamaguchi, M. Mandai, S. Toyokuni et al., "Contents of endometriotic cysts, especially the high concentration of free iron, are a possible cause of carcinogenesis in the cysts through the iron-induced persistent oxidative stress," *Clinical Cancer Research*, vol. 14, no. 1, pp. 32–40, 2008.

[16] E. G. Lo Turco, F. B. Cordeiro, P. H. de Carvalho Lopes et al., "Proteomic analysis of follicular fluid from women with and without endometriosis: new therapeutic targets and biomarkers," *Molecular Reproduction and Development*, vol. 80, no. 6, pp. 441–450, 2013.

[17] H. K. Eltzschig, M. V. Sitkovsky, and S. C. Robson, "Purinergic signaling during inflammation," *The New England Journal of Medicine*, vol. 367, no. 24, pp. 2322–2333, 2012.

[18] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[19] F. Kukulski, S. A. Lévesque, and J. Sévigny, "Impact of ectoenzymes on p2 and p1 receptor signaling," *Advances in Pharmacology*, vol. 61, pp. 263–299, 2011.

[20] G. G. Yegutkin, "Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade," *Biochimica et Biophysica Acta: Molecular Cell Research*, vol. 1783, no. 5, pp. 673–694, 2008.

[21] M. Martín-Satué, E. G. Lavoie, M. Fausther et al., "High expression and activity of ecto-5'-nucleotidase/CD73 in the male murine reproductive tract," *Histochemistry and Cell Biology*, vol. 133, no. 6, pp. 659–668, 2010.

[22] M. Fausther, J. Lecka, E. Soliman et al., "Coexpression of ecto-5˚-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 302, no. 4, pp. 447–459, 2012.

[23] L. Antonioli, P. Pacher, E. S. Vizi, and G. Haskó, "CD39 and CD73 in immunity and inflammation," *Trends in Molecular Medicine*, vol. 19, no. 6, pp. 355–367, 2013.

[24] E. Aliagas, B. Torrejón-Escribano, E. G. Lavoie et al., "Changes in expression and activity levels of ecto-5′-nucleotidase/ CD73 along the mouse female estrous cycle," *Acta Physiologica*, vol. 199, no. 2, pp. 191–197, 2010.

[25] E. Aliagas, A. Vidal, B. Torrejón-Escribano et al., "Ecto-nucleotidases distribution in human cyclic and postmenopausic endometrium," *Purinergic Signalling*, vol. 9, no. 2, pp. 227–237, 2013.

[26] E. Aliagas, A. Vidal, L. Texido, J. Ponce, E. Condom, and M. Martin-Satue, "High expression of ecto-nucleotidases CD39 and CD73 in human endometrial tumors," *Mediators of Inflammation*, vol. 2014, Article ID 509027, 8 pages, 2014.

[27] C. Théry, L. Zitvogel, and S. Amigorena, "Exosomes: composition, biogenesis and function," *Nature Reviews Immunology*, vol. 2, no. 8, pp. 569–579, 2002.

[28] C. Gutiérrez-Vázquez, C. Villarroya-Beltri, M. Mittelbrunn, and F. Sánchez-Madrid, "Transfer of extracellular vesicles during immune cell-cell interactions," *Immunological Reviews*, vol. 251, no. 1, pp. 125–142, 2013.

[29] A. Clayton, S. Al-Taei, J. Webber, M. D. Mason, and Z. Tabi, "Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production," *Journal of Immunology*, vol. 187, no. 2, pp. 676–683, 2011.

[30] L. A. Smyth, K. Ratnasothy, J. Y. Tsang et al., "D73 expression on extracellular vesicles derived from $CD4^+$ $CD25^+$ $Foxp3^+$ T cells contributes to their regulatory function," *European Journal of Immunology*, vol. 43, no. 9, pp. 2430–2440, 2013.

[31] P. J. Schuler, Z. Saze, C. S. Hong et al., "Human CD4(+) CD39(+) regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73(+) exosomes or CD73(+) cells," *Clinical & Experimental Immunology*, vol. 177, no. 2, pp. 531–543, 2014.

[32] D. Timmerman, L. Valentin, T. H. Bourne, W. P. Collins, H. Verrelst, and I. Vergote, "Terms, definitions and measurements to describe the sonographic features of adnexal tumors: a consensus opinion from the International Ovarian Tumor Analysis (IOTA) group," *Ultrasound in Obstetrics & Gynecology*, vol. 16, no. 5, pp. 500–505, 2000.

[33] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[34] P. A. Lanzetta, L. J. Alvarez, P. S. Reinach, and O. A. Candia, "An improved assay for nanomole amounts of inorganic phosphate," *Analytical Biochemistry*, vol. 100, no. 1, pp. 95–97, 1979.

[35] C. Théry, S. Amigorena, G. Raposo, and A. Clayton, "Isolation and characterization of exosomes from cell culture supernatants and biological fluids," *Current Protocols in Cell Biology*, vol. 3, 2006.

[36] G. G. Yegutkin, S. S. Samburski, S. Jalkanen, and I. Novak, "ATP-consuming and ATP-generating enzymes secreted by pancreas," *The Journal of Biological Chemistry*, vol. 281, no. 40, pp. 29441–29447, 2006.

[37] G. G. Yegutkin, B. Wieringa, S. C. Robson, and S. Jalkanen, "Metabolism of circulating ADP in the bloodstream is mediated via integrated actions of soluble adenylate kinase-1 and NTPDase1/CD39 activities," *The FASEB Journal*, vol. 26, no. 9, pp. 3875–3883, 2012.

[38] M. R. Schetinger, V. M. Morsch, C. D. Bonan, and A. T. S. Wyse, "NTPDase and 5°-nucleotidase activities in physiological and disease conditions: new perspectives for human health," *Biofactors*, vol. 31, no. 2, pp. 77–98, 2007.

[39] K. R. Park, T. Inoue, M. Ueda et al., "CD9 is expressed on human endometrial epithelial cells in association with integrins $\alpha 6$, $\alpha 3$ and $\beta 1$," *Molecular Human Reproduction*, vol. 6, no. 3, pp. 252–257, 2000.

[40] Y. H. Ng, S. Rome, A. Jalabert et al., "Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation," *PLoS ONE*, vol. 8, no. 3, Article ID e58502, 2013.

[41] K. L. Bruner-Tran, J. L. Herington, A. J. Duleba, H. S. Taylor, and K. G. Osteen, "Medical management of endometriosis: emerging evidence linking inflammation to disease pathophysiology," *Minerva Ginecologica*, vol. 65, no. 2, pp. 199–213, 2013.

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Research Article

Consequences of the Lack of CD73 and Prostatic Acid Phosphatase in the Lymphoid Organs

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CD73, ecto-5′-nucleotidase, is the key enzyme catalyzing the conversion of extracellular AMP to adenosine that controls vascular permeability and immunosuppression. Also prostatic acid phosphatase (PAP) possesses ecto-5′-nucleotidase/AMPase activity and is present in leukocytes. However, its role related to immune system is unknown. Therefore, we analyzed enzymatic activities and leukocyte subtypes of CD73 and PAP knockouts and generated CD73/PAP double knockout mice to elucidate the contribution of CD73 and PAP to immunological parameters. Enzymatic assays confirmed the ability of recombinant human PAP to hydrolyze [^{3}H]AMP, although at much lower rate than human CD73. Nevertheless, 5′-nucleotidase/AMPase activity in splenocytes and lymphocytes from PAP$^{-/-}$ mice tended to be lower than in wild-type controls, suggesting potential contribution of PAP, along with CD73, into lymphoid AMP metabolism ex vivo. Single knockouts had decreased number of CD4$^+$/CD25$^+$/FoxP$_3^+$ regulatory T cells in thymus and CD73/PAP double knockouts exhibited reduced percentages of CD4$^+$ cells in spleen, regulatory T cells in lymph nodes and thymus, and CD4$^+$ and CD8$^+$ cells in blood. These findings suggest that PAP has a synergistic role together with CD73 in the immune system by contributing to the balance of leukocyte subpopulations and especially to the number of regulatory T cells in lymph nodes and thymus.

1. Introduction

Balance between extracellular ATP, ADP, AMP, and adenosine controls the inflammatory status of the microenvironment, because ATP is proinflammatory while adenosine is highly anti-inflammatory. CD73 ectonucleotidase is a key enzyme in the purinergic signaling cascade as it dephosphorylates AMP and produces adenosine. Both endothelial cells and certain subsets of leukocytes express CD73. On endothelium [1–4] CD73-dependent adenosine production controls vascular permeability as clearly demonstrated by leaky vasculature and aggravated inflammations in CD73 deficient mice [5–8]. Regulatory T cells (Tregs) express CD73 and exert their immunosuppressive effects via adenosine. On the other hand, adenosine promotes Treg expansion and triggers A_{2A} adenosine receptor on effector T cells inducing NF-kB-mediated suppression of cytokine production and other anti-inflammatory effects [9, 10].

Human prostate expresses high amounts of PAP and the levels of the enzyme activity in serum has been connected to prostate cancer already decades ago [11], and in the recent years PAP has become the target for prostate cancer immunotherapy [12]. Indeed, there are two isoforms of prostatic acid phosphatase, a secretory (sPAP) and

a type 1 transmembrane (TMPAP) isoform, which are splice variants encoded by the same gene (*ACPP*) [13]. PAP presents ecto-5′-nucleotidase activity, and subsequent studies have unambiguously demonstrated its wide expression beyond the prostate including leukocytes [14], and the TMPAP isoform has been found in various tissues, including spleen, thymus, and neurons [13, 15]. Besides substrates such as β-glycerophosphate, lysophosphatidic acid, and phosphoamino acids, PAP can also catalyze AMP [16] and, in fact, PAP-mediated adenosine formation has been shown to be an important regulator of pain by inhibiting nociceptive neurotransmission [17–19]. Thus, the studies regarding PAP have concentrated on its function in the prostate and the neural system and, unlike CD73, its role in the immune system has remained unknown. In this work, we analyzed the nucleotidase activities of purified hPAP in comparison to recombinant CD73 in vitro and, further, ex vivo by using lymphoid cell suspensions isolated from wild type, CD73 and PAP knockout, and CD73/PAP double knockout mice, as well as mouse lymph nodes and human tonsils cryosections as enzyme sources. Moreover, we show the consequences of PAP deficiency alone and combined with the absence of CD73 to the composition of immune cells in main lymphoid organs. To the best of our knowledge, this is the first demonstration of a role for PAP in the immune system.

2. Materials and Methods

2.1. Mice. $Pap^{-/-}$ (mPAP KO) and $Nt5e^{-/-}$ (CD73 KO) were created by targeted gene disruption as previously described [20, 21]. Mice were backcrossed to C57BL/6J mice for >10 generations. dKO (mPAP KO/CD73 KO) mice were generated by breeding backcrossed $Nt5e^{-/-}$ and $Pap^{-/-}$ mice and the double mutant mice were selected by PCR-based genotyping. They were analyzed alongside age-matched C57BL/6J wild type mice as controls. Two- to 4-month-old mice were used. Mice were raised under a 12 : 12 light : dark cycle and used during the light phase. The mice were maintained in a specific pathogen-free stage at Central Animal Laboratory at the University of Turku, complying with international guidelines on the care and use of laboratory animals and performed in compliance with the 3Rs principle. All animal experiments were approved by the Finnish Animal Ethics Committee, project license number 3791/04.10.03/2011.

2.2. Purification of Human CD73 and PAP. Recombinant human CD73 was purified from transfected CHO cells. The harvest was purified using immobilized N6-(6-amino)hexyl-AMP (Jena Bioscience Gmbh, Jena, Germany). The bound rhCD73 was eluted using 0.25 M AMP in 50 mM Tris-HCl, 0.1 M NaCl, and pH 7.4. After the purification, the buffer was changed to 20 mM Tris-HCl, pH 8.0, using Amicon Ultra 30 device and the final concentration of the CD73 was measured using specific in-house ELISA.

hPAP was purified from human seminal plasma as described previously [22, 23]. Enzyme specific activity for the dephosphorylation of p-nitrophenylphosphate at pH 4.8 (50 mM Na citrate buffer) was 5500 μmol/min$*$mg.

2.3. Cell Isolation, Flow Cytometric Analyses, and Immunohistochemistry. Single cell suspensions were prepared from minced pieces of peripheral lymph nodes (pooled axial and inguinal nodes), thymus, and spleen by mechanical teasing through a metal meshwork and bone marrow cells by flushing the femurs. Blood was collected by cardiac puncture. Erythrocytes were removed from spleen and blood by hypotonic lysis. Leukocyte phenotyping was done using mAbs against CD4 (clone RM4-5, Alexa Fluor 647 conjugated), CD8 (clone 53-6.7, PerCP-Cy5.5 conjugated), and anti-B220 (clone RA3-6B2, Pacific blue conjugated), all obtained from BD Biosciences. Regulatory T cells were identified using mouse regulatory T cell staining kit (eBioscience; CD4-FITC, CD25-allophycocyanin, and FoxP3-PE). Cells were analyzed using LSRII using BDFACS-Diva software. Representative dot blots and histograms were made with FlowJo software. Frozen sections of thymuses were also stained for CD8, CD4, and CD25.

2.4. Thin-Layer Chromatographic (TLC) Analysis of Enzymatic Activities. Purinergic activities of isolated single cells from bone marrow, spleen, and peripheral lymph nodes were determined by using [2,8-^{3}H]ATP, [2,8-^{3}H]ADP (Perkin Elmer), [2-^{3}H]AMP (Quotient Bioresearch, GE Healthcare, Rushden, UK), and [2-^{3}H]adenosine (Amersham, Little Chalfont, UK) as described previously [24]. Briefly, the single cell suspensions (5–10 $\times$ 10^4 cells) were incubated for 45–60 min at 37°C in a final volume of 80 μL RPMI-1640 in neutral pH supplemented with 4 mmol/L β-glycerophosphate and the following tracer substrates: 500 μM [^{3}H]ATP (ATPase), 500 μM [^{3}H]ADP (ADPase), 300 μM [^{3}H]AMP (ecto-5′-nucleotidase), 300 μM [^{3}H]adenosine (adenosine deaminase), and 400 μM [^{3}H]AMP plus 600 μM γ-phosphate-donating ATP (adenylate kinase). Likewise, AMP-hydrolyzing activity of purified human enzymes was determined by incubating PAP (1 μg of protein) or CD73 (0.5 ng) with 40 μM [^{3}H]AMP in the absence and presence of β-glycerophosphate (4 mmol/L) or selective ecto-5′-nucleotidase/CD73 inhibitor α,β-methylene-ADP (AMPCP; 5 μM). Mixture aliquots were applied onto Alugram G/UV_{254} sheets (Macherey-Nagel) and ^{3}H-labeled substrates and their metabolites were separated by TLC and quantified by scintillation β-counting.

2.5. Enzyme Histochemistry. Localization of ecto-5′-nucleotidase/AMPase and other nucleotidase activities was determined in murine lymph node and also human tonsil cryosections by using a modification of the lead nitrate [$Pb(NO_3)_2$] method, as described previously [25, 26]. Tissue sections were also stained with hematoxylin and eosin. Slides were mounted with Aquatex (Merck) and images were acquired using an Olympus BX60 microscope.

2.6. Statistical Analyses. The results are presented as mean ± SEM, unless otherwise stated. Statistical analyses were done using two-sided Student's t-test and values of $P < 0.05$ were considered significant.

3. Results and Discussion

3.1. Activity of CD73 Dominates over PAP in AMP Hydrolysis Both In Vitro and In Vivo. As PAP has been demonstrated to contribute, along with a predominant role of CD73, to the conversion of extracellular AMP into adenosine [16, 17], we first compared the activities of purified hPAP and purified CD73. Measurement of hPAP activity revealed efficient hydrolysis of artificial substrate pNPP by the purified enzyme (Figure 1(a)) and further demonstrated its ability to metabolize [^{3}H]AMP (Figure 1(b)), with highest catalytic activities being detected at acidic pH. Interestingly, AMP-hydrolyzing capability of hPAP was ~400- and ~50-fold less in comparison with purified CD73 when assayed at pH 7.2 and 5.5, respectively, and, in addition, it was not inhibited by selective ecto-5′-nucleotidase inhibitor AMPCP (Figure 1). Noteworthy, PAP could also potentially hydrolyze β-glycerophosphate as an alternative phosphorylated substrate [16]. However, no differences in the rates of [^{3}H]AMP hydrolysis by purified hPAP or hCD73 were detected in our TLC assays performed in the absence and presence of 4 mM β-glycerophosphate (data not shown).

To further evaluate the potential role of PAP in lymphoid nucleotide homeostasis, we measured the activities of ecto-5′-nucleotidase (Figure 2(a)) and other related purinergic enzymes, ATPase (Figure 2(b)), ADPase (Figure 2(c)), and adenylate kinase (Figure 2(d)), in lymphocytes isolated from spleen and bone marrow of PAP knockout and wild type mice. No statistical differences were found in these activities, although there was a tendency towards reduced ecto-5′-nucleotidase activity in the spleen of the PAP knockout mice. Next, we concentrated on lymphocytes isolated from peripheral lymph nodes, which drain the periphery and are the primary sites for immune response against antigens arriving from the periphery via afferent lymphatics, and analyzed the enzymatic activities in wild type, CD73, PAP, and CD73/PAP double knockouts. The double knockouts generated for this work did not show any obvious phenotype, when housed in specific pathogen-free conditions.

As expected, practically no AMPase activity was detected in the lymphocytes isolated from CD73 knockout mice, thus confirming that lymphoid ecto-5′-nucleotidase/CD73 indeed represents the major enzyme responsible for conversion of extracellular AMP into adenosine (Figure 3(a)). Nonetheless, as in the case of spleen studies (see Figure 2(a)), a clear trend for diminished [^{3}H]AMP hydrolysis was observed in PAP$^{-/-}$ lymphocytes (Figure 3(a)). These data suggest that PAP may contribute, along with CD73, to lymphoid AMP metabolism, presumably in certain synergistic fashion. Consistent with our previous observations [27], we also found significantly increased ATPase and ADPase activities in lymphocytes from CD73 deficient animals, with the latter nucleotidase activity remaining significantly upregulated also in CD73/PAP double knockout mice. No significant shifts in other purine-converting enzymes, adenosine deaminase and adenylate kinase, were detected among the studied genotypes (Figure 3).

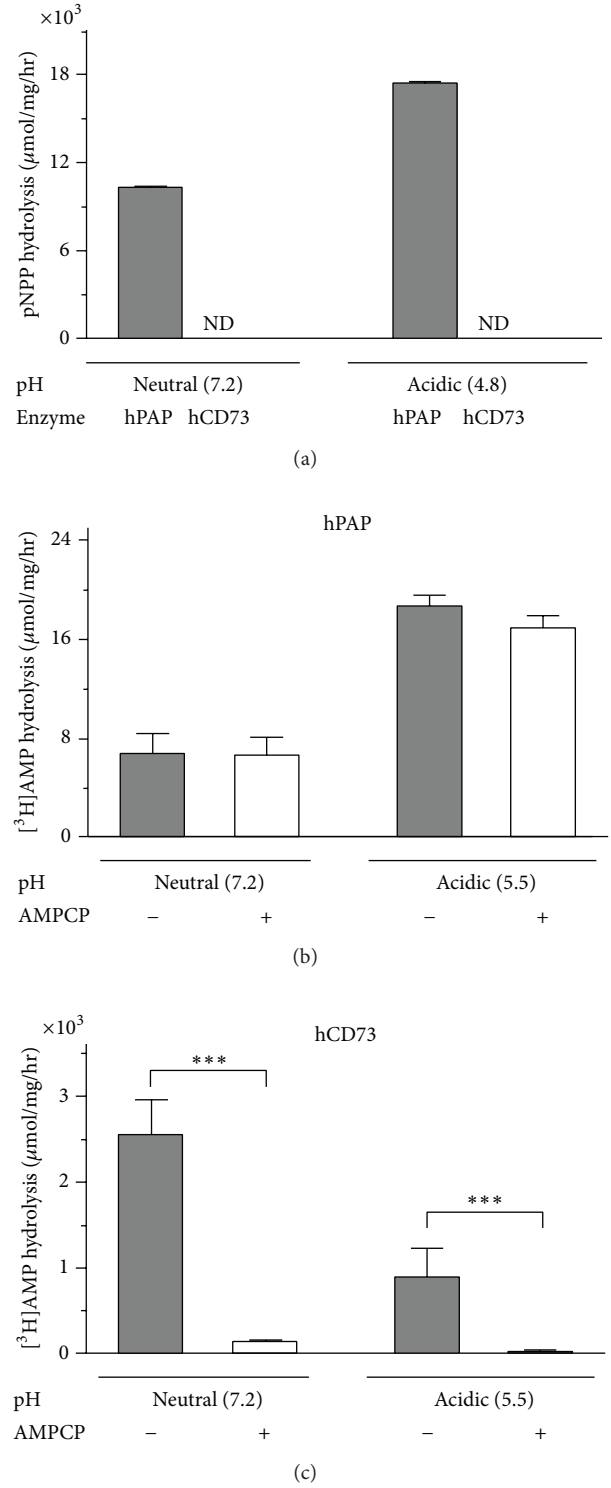

Figure 1: Nucleotidase activities of purified human PAP and recombinant human CD73. (a) The activities of hPAP and hCD73 were determined spectrophotometrically at neutral or acidic pH by using artificial substrate p-nitrophenylphosphate (pNPP). *ND*: nondetectable. Radio-TLC analysis of nucleotidase activities was also performed by incubating purified hPAP (b) and hCD73 (c) with 40 μM [^{3}H]AMP at different pH without and with 5 μM AMPCP, as indicated. Enzymatic activities are expressed as μmoles of pNPP or [^{3}H]AMP hydrolyzed per hour by one milligram of enzyme protein. $^*P < 0.05$.

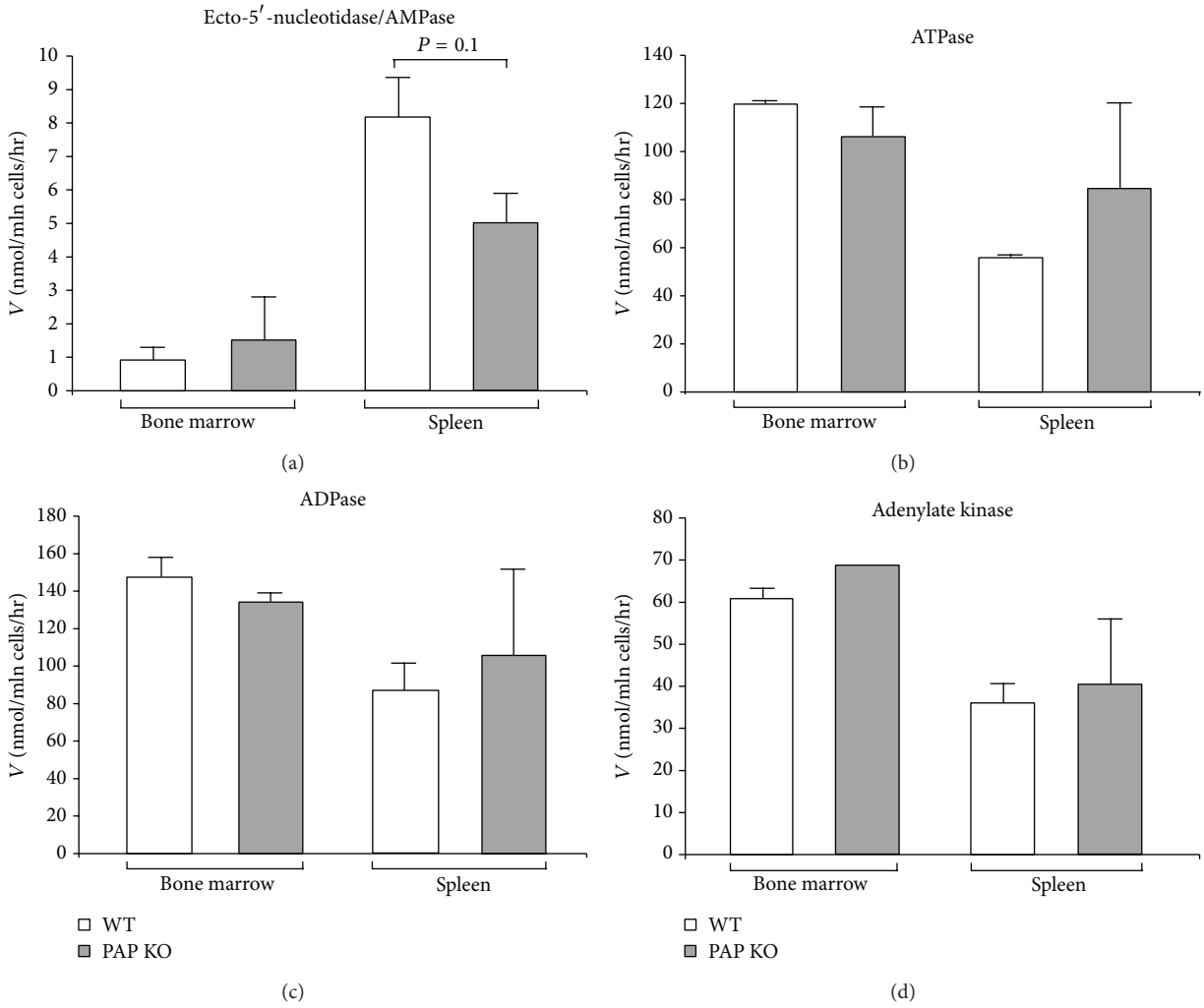

Figure 2: PAP deficient mice do not have significant alterations in extracellular nucleotide metabolism. The activities of ecto-5′-nucleotidase/AMPase (a), ATPase (b), ADPase (c), and adenylate kinase (d) were determined radiochemically in bone marrow and spleen leukocytes isolated from wild type and PAP knockout mice (mean ± SEM; n = 3–5).

3.2. PAP and CD73 Are Differently Expressed in Mouse and Human Lymphoid Tissues and Contribute to Nucleotide Metabolism. The distribution of different nucleotidases in the lymphoid tissues was then evaluated in situ by a lead precipitation method. Short-time incubation of mouse lymph node and human tonsil cryosections with different nucleotide substrates revealed the presence of high AMPase and especially ATPase activities (Figure 4). The employment of TMP as a substrate and increasing the incubation time also allowed us to detect relatively moderate but clearly visible staining, which was specifically restricted to the subcapsular area of mouse lymph nodes, the epithelial layer of human tonsils, and also individual lymphoid cells scattered throughout the lymphoid tissues (Figure 4). Strikingly, the most intense TMPase staining was observed at acidic pH, suggesting the predominant contribution of PAP enzyme to the measured nucleotidase activity. Noteworthy, it has been proposed recently that selective expression of tissue-nonspecific alkaline phosphatase and another pH-dependent enzyme tartrate-resistant acid phosphatase (TRAP) in corresponding alkaline and acidic lacunas of bone might function as specific pH sensors directionally regulating nucleotide receptor-mediated osteoclast function and bone resorption [28]. It is also pertinent to mention that, under certain circumstances, like ischemia, intracellular pH is decreased to ~6.4, accompanied by marked inhibition of 5′-nucleotidase activity and changes in purine homeostasis in the heart [29]. It may be reasonably speculated that different localization of CD73 and PAP and their inverse pH dependence provide efficient means for tuned regulation of purine homeostasis in lymphoid tissues during inflammation, hypoxia, and other (patho)physiological states. Further studies are required to validate this hypothesis.

3.3. PAP Deficiency Has Only Minor Effect on Lymphocyte Populations. As there is no reliable antibody to analyze PAP expression at the protein level in mouse, we were mining the ImmGen consortium data bank that contains detailed

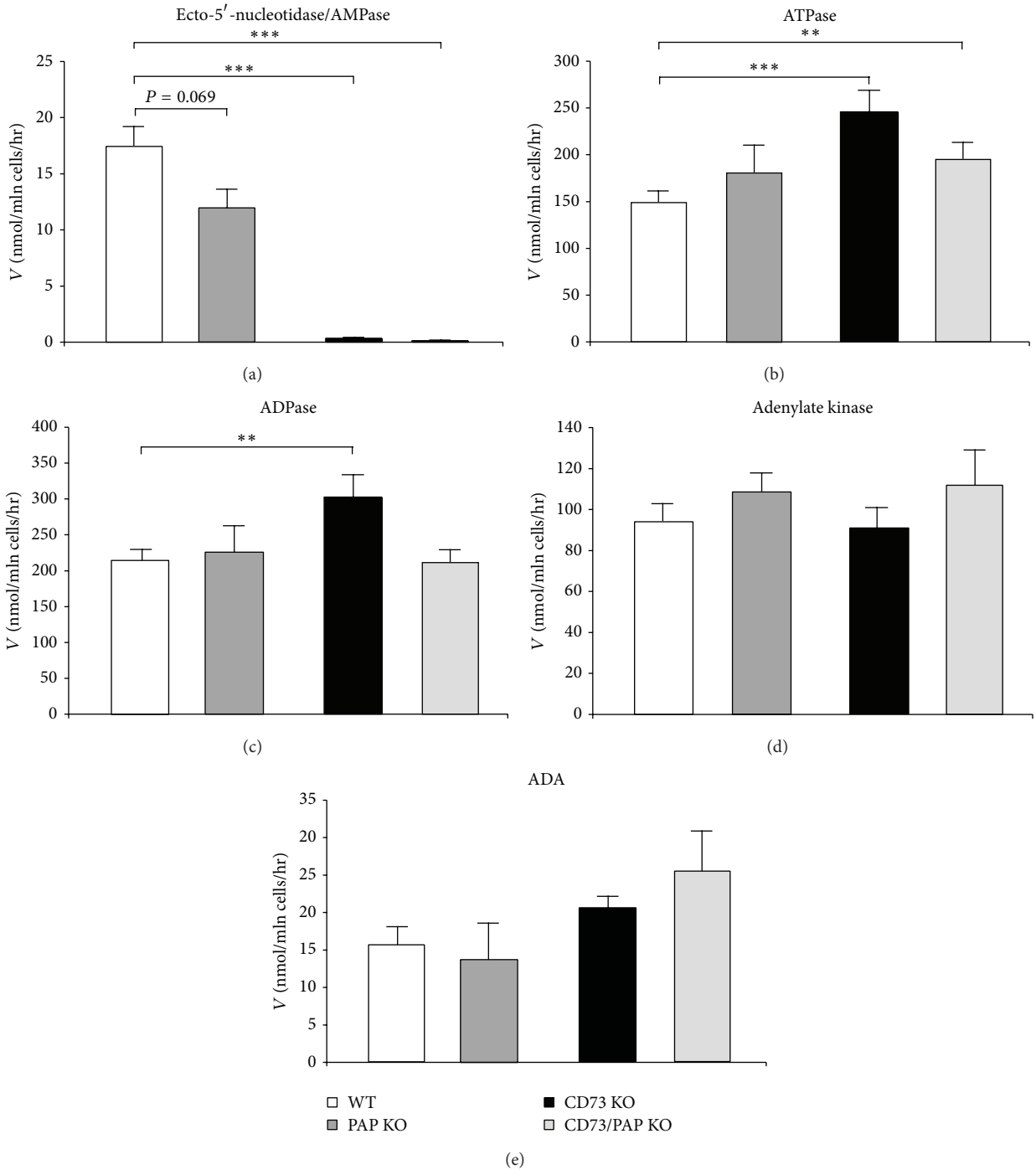

Figure 3: Purine-converting activities in CD73 and PAP deficient mice. The activities of ecto-5′-nucleotidase/AMPase (a), ATPase (b), ADPase (c), adenylate kinase (d), and adenosine deaminase (e) were determined by TLC in lymph node lymphocytes from wild type mice ($n = 14$), CD73 single ($n = 7$), PAP single ($n = 9$), and CD73/PAP double knockouts ($n = 7$). Enzymatic activities were expressed as nanomoles of ^{3}H-substrate metabolized per hour by 10^6 cells. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

analyses of different murine leukocyte and endothelial cell populations in respect to their gene expression profiles. PAP is expressed mainly in subsets of $\gamma\delta$ T cells and invariant natural killer T cells in thymus and both in CD4 and CD8 positive T cells in secondary lymphoid organs although generally in lower levels than CD73. A simplified comparison to CD73 expression is shown in Table 1. PAP deficiency alone did not change B cell, CD8, or CD4 populations in mice (Figures 5(a)–5(d)). The only statistically significant difference was seen in $CD8^+$ cells in the blood, although in percentage-wise the increase is most likely biologically meaningless. The same held true when the lymphocyte populations were analyzed

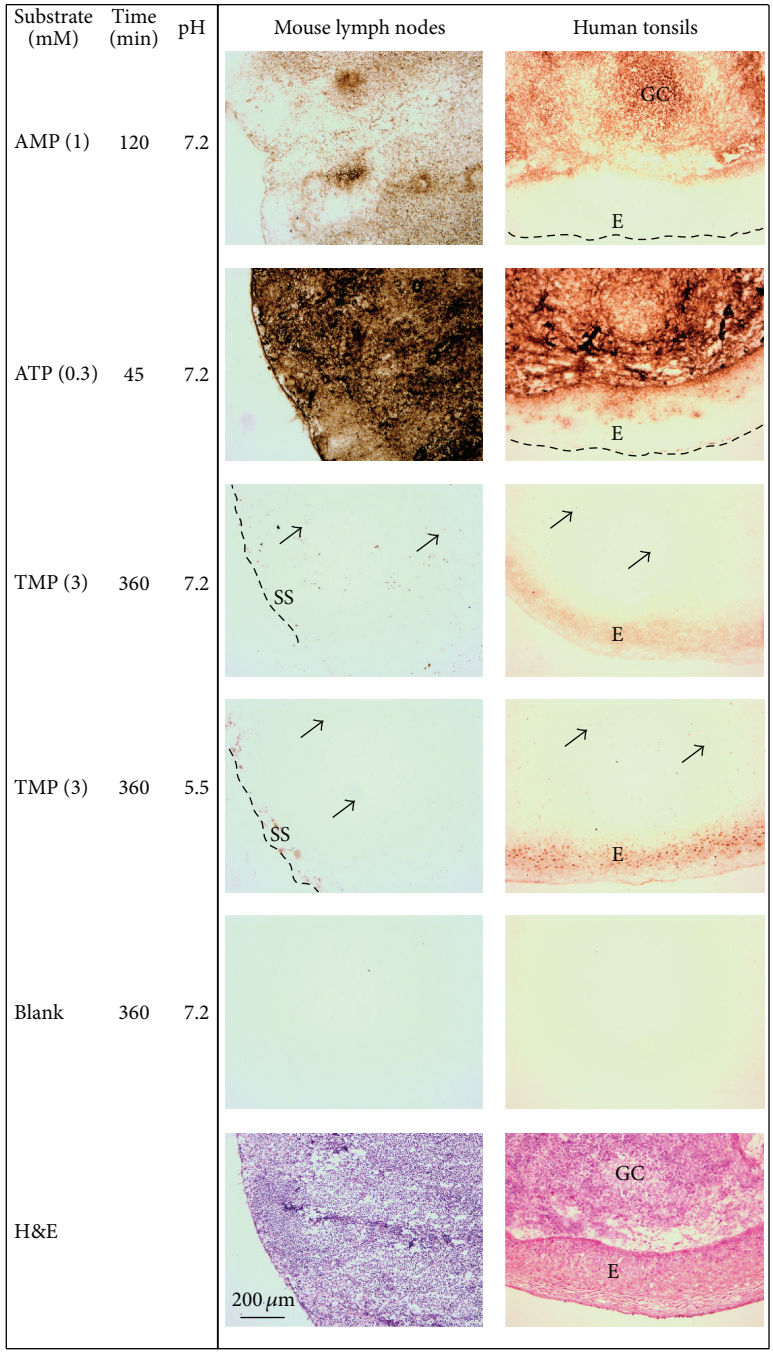

Figure 4: Distribution of nucleotidase activities in mouse lymph nodes and human tonsils. Enzyme histochemical staining was performed by incubating tissue sections at different pH for the indicated time without (Blank) and with different nucleotide substrates. Tissue sections were also stained with hematoxylin and eosin (H&E). The capsules are indicated by dashed lines, the arrows point at some positive scattered cells, GC: germinal center, SS: subcapsular sinus, and E: epithelium. Original magnification: ×100. Scale bar: 200 μm.

in blood and bone marrow of 8-month-old PAP mice (data not shown). We chose this time point as PAP knockout mice develop nonmetastatic prostate carcinoma at an old age [30]. The knockout prostates at this age contained more B cells than wild type prostates ($6.6 \pm 0.2\%$ versus $1.4 \pm 0.9\%$, $P = 0.004$). Also salivary glands contain more immune cells in these male mice, when they are young, but the difference disappears with aging [31]. The practically nonexisting alterations of major immune cell subpopulations in absence of PAP suggest that PAP does not seem to have any marked role in the immune system or its absence is compensated by CD73. Likewise, the changes in CD73 knockout mice were minor (Figures 5(a)–5(d)). Fewer B cells and slightly increased number of regulatory T cells were detected in peripheral lymph nodes.

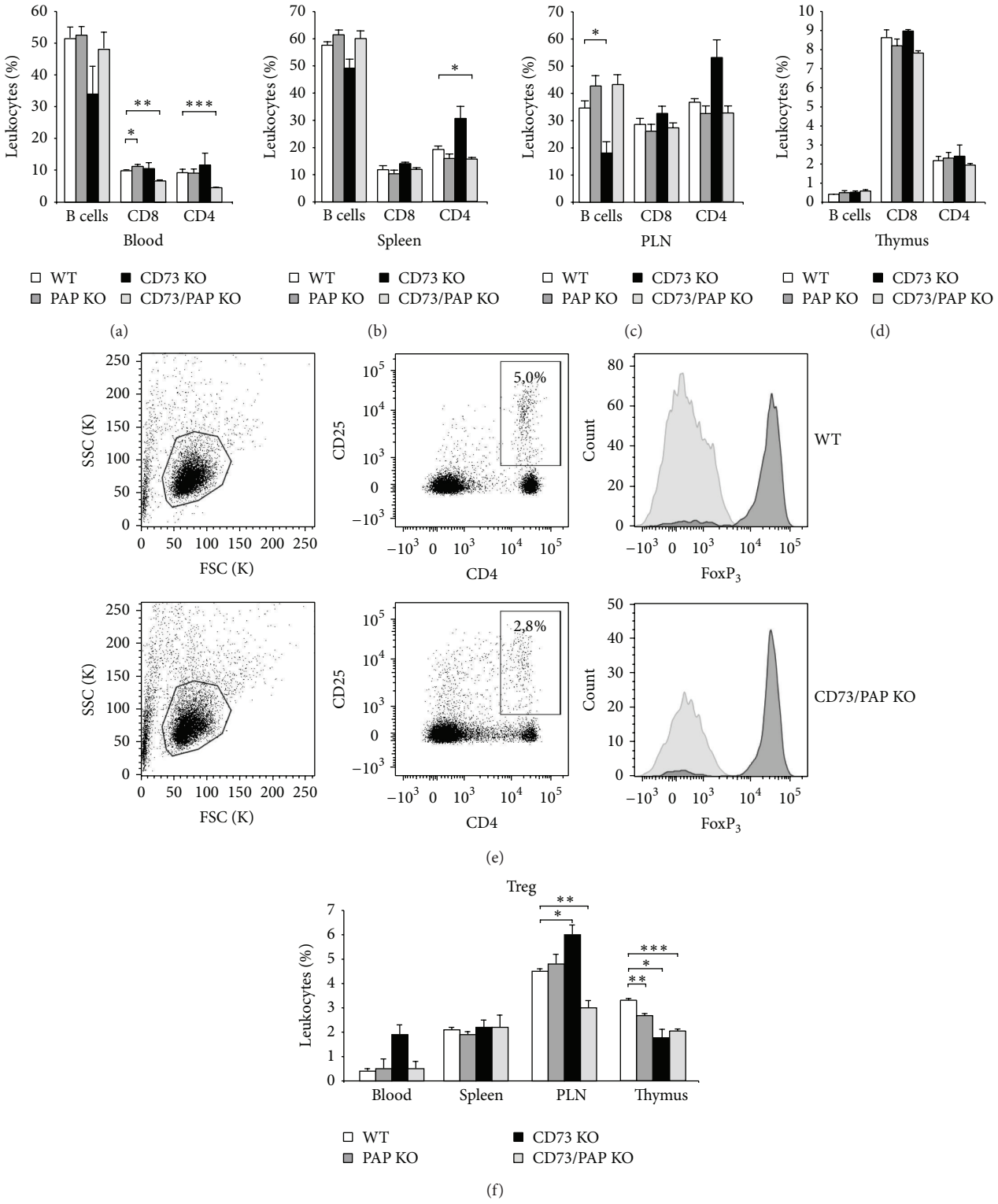

Figure 5: Synergistic effect of PAP and CD73 deficiency on the leukocyte subpopulations. Lymphocyte phenotypes in blood (a), spleen (b), lymph nodes (c), and thymus (d) of wild type mice ($n = 7$), CD73 single ($n = 3$), PAP single ($n = 6$), and CD73/PAP double knockouts ($n = 4$) (thymus: $n = 3$ for all genotypes) were analyzed using flow cytometry. (e) Regulatory T cells in lymph nodes. Examples of the FACS analyses of regulatory T cells in wild type and CD73/PAP KO mice are shown. In the left panels the gating strategy is shown and in the middle panels percentages of $CD4^+/CD25^+$ cells are indicated. Intensities of FoxP3 stainings of $CD4^+/CD25^+$ cells are shown as histograms in the right panels. Logarithmic fluorescence is on x-axis and the number of cells on y-axis. (f) Combined results of regulatory T cell analyses of different organs are shown as indicated. $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

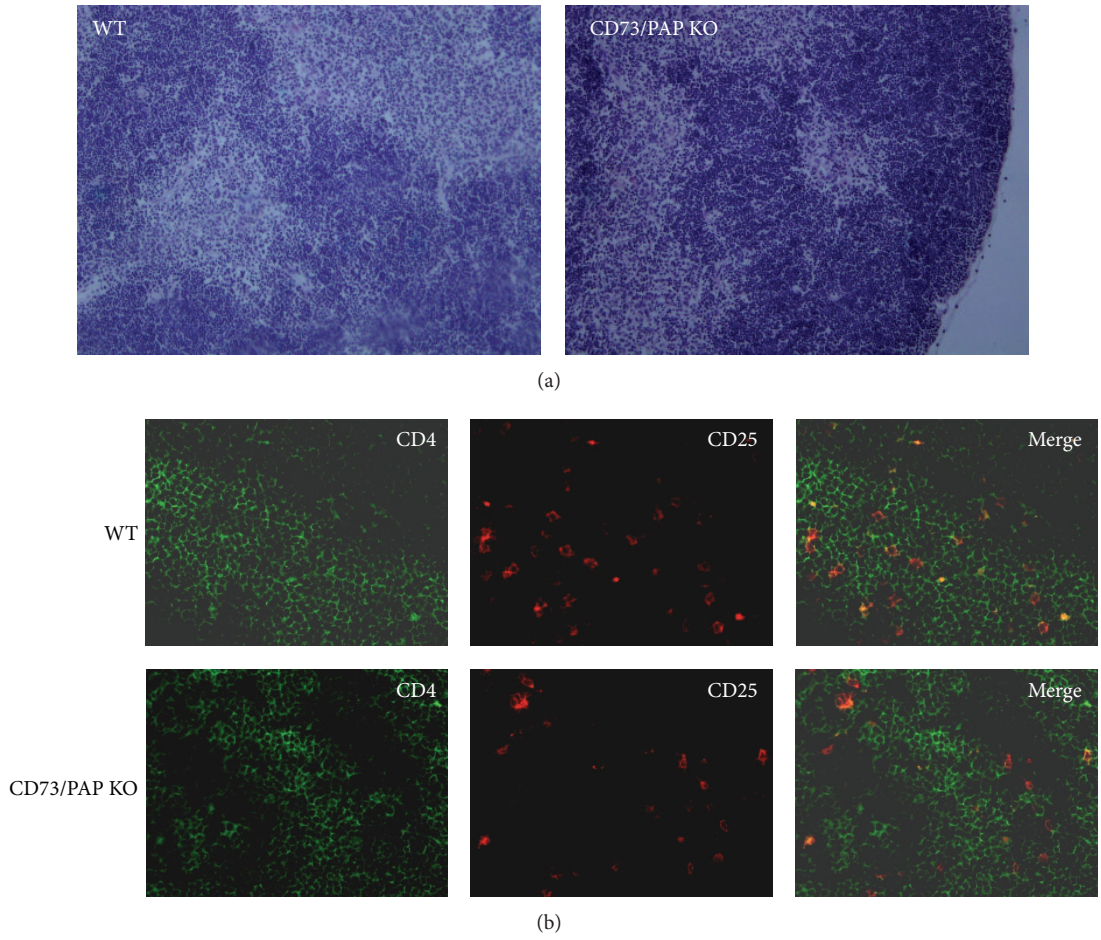

Figure 6: Thymuses of CD73/PAP double knockouts show comparable morphology and cell distribution to wild type mice. (a) Hematoxylin and eosin stainings of wild type and CD73/PAP knockout thymuses. (b) Immunofluorescence stainings of wild type and CD73/PAP knockout thymuses using anti-CD4 (green) and anti-CD25 (red) antibodies.

3.4. CD73 and PAP Together Regulate the Number of CD4 and CD8 Cells in Blood and Number of Regulatory T Cells in Lymph Nodes and Thymus. In single knockouts, the number of regulatory T cells was significantly decreased in thymus and in the double knockouts both in PLN and thymus (Figures 5(e) and 5(f)). Still the intensity of FoxP3 expression, a marker for regulatory T cells, was at the same level in both double knockouts and wild type mice (Figure 5(e)). Thus, we may conclude that in thymus both CD73 and PAP contribute to number of regulatory T cells alone, but absence of both is needed to have an effect in PLN.

Although adenosine produced by CD73 on regulatory T cells propagates generation of more regulatory T cells via A_{2A} adenosine receptor [32], the number of regulatory T cells is very low and the main adenosine may come from peripheral lymph node stromal cells such as from endothelial cells. The stromal cells were not included in our enzymatic analyses showing nearly complete absence of ecto-5′-nucleotidase activity on isolated lymph node lymphocytes in CD73 KO mice.

Our finding of normal levels of regulatory T cells in organs other than in thymus, which had decreased number of regulatory T cells, and in lymph nodes, which had slightly increased number of regulatory T cells, is in agreement with the observation of Ehrentraut et al. [33], who did not find differences in the number of regulatory T cells between wild type and CD73 deficient mice in the lungs. This demonstrates that expression of CD73 overall is not needed for generation of regulatory T cells as such, although it is fundamental for the immunosuppressive function of these cells [9, 33].

The double knockout mice also have significantly lower percentages of $CD8^+$ (32% reduction, $P < 0.0001$) and $CD4^+$ cells (51% reduction, $P < 0.006$) in their blood and reduced number of $CD4^+$ cells in the spleen (Figures 5(a) and 5(b)) further suggesting the synergistic effects of these two enzymes in controlling the lymphocyte pools.

As thymus is the central organ in T cell development, we also analyzed thymic morphology and T cell subtypes of CD73, PAP, and double knockouts and compared them to those of wild type mice. The double knockout mice had normal thymic morphology (Figure 6(a)) and tissue location of different cell types was comparable between the genotypes. Double stainings of CD4 and CD25 are shown as examples in Figure 6(b).

Table 1: Expression profiles of CD73 and PAP at the mRNA level in different lymphoid tissues*.

	CD73 (*Nt5e*)	PAP (*Acpp*)
Thymus	$\gamma\delta$ T cells**	$\gamma\delta$ T cells**
	CD4 single positive	iNKT precursors**
	Double negative thymocytes	
	CD8 single positive, mature	
	iNKT*** precursors**	
Lymph nodes	CD4 memory	CD8 naïve
	CD8 naïve	
	CD8 memory	
	Treg	
	Blood endothelial cells	
Spleen	Germinal center B cells	CD4 naïve
	Memory B cells	CD4 memory
	$\gamma\delta$ T cells**	$\gamma\delta$ T cells**
	CD4 naïve	CD8 naïve
	CD4 memory	CD8 memory
	CD8 naïve	iNKT cells
	CD8 memory	
	Treg	
	iNKT cells	
	NK cells**	

*The data has been collected from the Immunological Genome Project (http://www.immgen.org/databrowser). The cell types with an expression value (EV) >120 for a given gene are listed (95% confidence of positive expression).
**Only in selected subpopulations.
***iNKT = invariant natural killer T cells.

Lymph nodes, spleen, and blood represent anatomically and functionally different environments for leukocytes providing unique cellular networks and extracellular milieu. This can also be realized by the differences in the expression of PAP and CD73 (Table 1). Therefore, our results showing organ-specific changes in lymphocyte subpopulations of double knockouts are understandable and emphasize the fact that, for example, in lymph nodes and spleen the factors promoting activation and differentiation of regulatory T cells are at least partially different and in spleen not markedly regulated by CD73 and PAP, although both enzymes are present in these organs. Regulatory T cells are produced in thymus and from there they disperse throughout the body to various organs in the hematolymphoid system or differentiated in the periphery. Regulatory T cells are heterogeneous regarding their phenotype, function, and epigenetic status and also the homing-associated molecules on regulatory T cells vary [34, 35]. This allows them to preferentially migrate to different organs such as peripheral lymph nodes, gut-associated lymphatic tissues, spleen, or nonlymphoid organs depending on the pattern of homing-receptors on their surface [35]. For example, CXCL12 highly expressed in bone marrow and splenic red pulp attracts regulatory T cells, while CCL25 recruits them to the small intestine. Thus, the regulatory T cells in different organs have phenotypically unique characteristics.

4. Conclusions

Generation and maintenance of the balance between different leukocyte subpopulations is a key element for proper functioning of the immune system in health and diseases. Anti-inflammatory adenosine produced by the AMPase activity of CD73 is central in immunosuppression. Although adenosine is important propagating regulatory T cells, lack of CD73 has surprisingly little effect on the number of regulatory T cells. This is the first report demonstrating the distribution of PAP in various mouse and human lymphoid tissues and its contribution, in conjunction with CD73, to the lymphoid purine homeostasis and the balance of leukocyte subpopulations in various organs. However, alone absence of PAP does not have any significant effect outside the thymus, but, combined to the deficiency with CD73, the consequences become significant. Further work is needed to elucidate the exact mechanism of the mode of action of PAP in the immune system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Dr. Gennady G. Yegutkin, Kaisa Auvinen, and Marika Karikoski contributed equally to the paper.

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References

[1] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[2] J. Linden and C. Cekic, "Regulation of lymphocyte function by adenosine," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 9, pp. 2097–2103, 2012.

[3] H. K. Eltzschig, M. V. Sitkovsky, and S. C. Robson, "Purinergic signaling during inflammation," *New England Journal of Medicine*, vol. 367, no. 24, pp. 2322–2333, 2012.

[4] M. Salmi and S. Jalkanen, "Ectoenzymes in leukocyte migration and their therapeutic potential," *Seminars in Immunopathology*, vol. 36, no. 2, pp. 163–176, 2014.

[5] P. Koszalka, B. Özüyaman, Y. Huo et al., "Targeted disruption of cd73/ecto-5′-nucleotidase alters thromboregulation and augments vascular inflammatory response," *Circulation Research*, vol. 95, no. 8, pp. 814–821, 2004.

[6] A. Zernecke, K. Bidzhekov, B. Özüyaman et al., "CD73/Ecto-5′-nucleotidase protects against vascular inflammation and neointima formation," *Circulation*, vol. 113, no. 17, pp. 2120–2127, 2006.

[7] M. Takedachi, D. Qu, Y. Ebisuno et al., "CD73-generated adenosine restricts lymphocyte migration into draining lymph nodes," *The Journal of Immunology*, vol. 180, no. 9, pp. 6288–6296, 2008.

[8] J. Kiss, G. G. Yegutkin, K. Koskinen, T. Savunen, S. Jalkanen, and M. Salmi, "IFN-β protects from vascular leakage via up-regulation of CD73," *European Journal of Immunology*, vol. 37, no. 12, pp. 3334–3338, 2007.

[9] S. Deaglio, K. M. Dwyer, W. Gao et al., "Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression," *Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1257–1265, 2007.

[10] M. Mandapathil, S. Lang, E. Gorelik, and T. L. Whiteside, "Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression," *Journal of Immunological Methods*, vol. 346, no. 1-2, pp. 55–63, 2009.

[11] A. B. Gutman and E. B. Gutman, "An Acid Phosphatase occurring in the serum of patients with metastasizing carcinoma of the prostate gland," *The Journal of Clinical Investigation*, vol. 17, no. 4, pp. 473–478, 1938.

[12] M. A. Cheever and C. S. Higano, "PROVENGE (sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine," *Clinical Cancer Research*, vol. 17, no. 11, pp. 3520–3526, 2011.

[13] I. B. Quintero, C. L. Araujo, A. E. Pulkka et al., "Prostatic acid phosphatase is not a prostate specific target," *Cancer Research*, vol. 67, no. 14, pp. 6549–6554, 2007.

[14] A. C. Cunha, B. Weigle, A. Kiessling, M. Bachmann, and E. P. Rieber, "Tissue-specificity of prostate specific antigens: comparative analysis of transcript levels in prostate and non-prostatic tissues," *Cancer Letters*, vol. 236, no. 2, pp. 229–238, 2006.

[15] H. Nousiainen, I. Quintero, T. Myöhänen et al., "Mice deficient in transmembrane prostatic acid phosphatase display increased gabaergic transmission and neurological alterations," *PloS ONE*, vol. 9, no. 5, Article ID e97851, 2014.

[16] P. Vihko, "Characterization of the principal human prostatic acid phosphatase isoenzyme, purified by affinity chromatography and isoelectric focusing, part II," *Clinical Chemistry*, vol. 24, no. 10, pp. 1783–1787, 1978.

[17] M. J. Zylka, N. A. Sowa, B. Taylor-Blake et al., "Prostatic acid phosphatase is an ectonucleotidase and suppresses pain by generating adenosine," *Neuron*, vol. 60, no. 1, pp. 111–122, 2008.

[18] H. Zimmermann, "Prostatic acid phosphatase, a neglected ectonucleotidase," *Purinergic Signalling*, vol. 5, no. 3, pp. 273–275, 2009.

[19] S. E. Street, P. L. Walsh, N. A. Sowa et al., "PAP and NT5E inhibit nociceptive neurotransmission by rapidly hydrolyzing nucleotides to adenosine," *Molecular Pain*, vol. 7, article 80, 2011.

[20] L. F. Thompson, H. K. Eltzschig, J. C. Ibla et al., "Crucial role for ecto-5′-nucleotidase (CD73) in vascular leakage during hypoxia," *Journal of Experimental Medicine*, vol. 200, no. 11, pp. 1395–1405, 2004.

[21] P. Vihko, I. Quintero, A. Rönkö et al., "Prostatic acid phosphatase (PAP) is pi(3)p-phosphatase and its inactivation leads to change of cell polarity and invasive prostate cancer," in *Proceedings of the AACR Abstract 5239: 96th Annual Meeting*, Anaheim, Calif, USA, 2005.

[22] P. Vihko, M. Kontturi, and L. K. Korhonen, "Purification of human prostatic acid phosphatase by affinity chromatography and isoelectric focusing, part I," *Clinical Chemistry*, vol. 24, no. 3, pp. 466–470, 1978.

[23] A. M. Herrala, I. B. Quintero, and P. T. Vihko, "Purification of prostatic acid phosphatase (PAP) for structural and functional studies," *Methods in Molecular Biology*, vol. 1053, pp. 167–178, 2013.

[24] G. G. Yegutkin, J. Hytönen, S. S. Samburski, H. Yrjänäinen, S. Jalkanen, and M. K. Viljanen, "Disordered lymphoid purine metabolism contributes to the pathogenesis of persistent Borrelia garinii infection in mice," *Journal of Immunology*, vol. 184, no. 9, pp. 5112–5120, 2010.

[25] N. Mercier, T. O. Kiviniemi, A. Saraste et al., "Impaired ATP-induced coronary blood flow and diminished aortic NTPDase activity precede lesion formation in apolipoprotein E-deficient mice," *The American Journal of Pathology*, vol. 180, no. 1, pp. 419–428, 2012.

[26] D. Langer, K. Hammer, P. Koszalka, J. Schrader, S. Robson, and H. Zimmermann, "Distribution of ectonucleotidases in the rodent brain revisited," *Cell and Tissue Research*, vol. 334, no. 2, pp. 199–217, 2008.

[27] G. G. Yegutkin, F. Marttila-Ichihara, M. Karikoski et al., "Altered purinergic signaling in CD73-deficient mice inhibits tumor progression," *European Journal of Immunology*, vol. 41, no. 5, pp. 1231–1241, 2011.

[28] J. D. Kaunitz and D. T. Yamaguchi, "TNAP, TrAP, ecto-purinergic signaling, and bone remodeling," *Journal of Cellular Biochemistry*, vol. 105, no. 3, pp. 655–662, 2008.

[29] M. I. Bak and J. S. Ingwall, "Regulation of cardiac AMP-specific 5′-nucleotidase during ischemia mediates ATP resynthesis on reflow," *The American Journal of Physiology—Cell Physiology*, vol. 274, no. 4, pp. C992–C1001, 1998.

[30] I. B. Quintero, A. M. Herrala, C. L. Araujo et al., "Transmembrane prostatic acid phosphatase (TMPAP) interacts with snapin and deficient mice develop prostate adenocarcinoma," *PLoS ONE*, vol. 8, no. 9, Article ID e73072, 2013.

[31] C. L. Araujo, I. B. Quintero, and A. Kipar, "Prostatic acid phosphatase is the main acid phosphatase with 5′-ectonucleotidase activity in the male mouse saliva and regulates salivation," *The American Journal of Physiology—Cell Physiology*, vol. 306, no. 11, pp. C1017–C1027, 2014.

[32] A. Ohta, R. Kini, M. Subramanian et al., "The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2a adenosine receptor pathway," *Frontiers in Immunology*, vol. 3, p. 190, 2012.

[33] H. Ehrentraut, E. T. Clambey, E. N. McNamee et al., "CD73+ regulatory T cells contribute to adenosine-mediated resolution of acute lung injury," *The FASEB Journal*, vol. 27, no. 6, pp. 2207–2219, 2013.

[34] S. Sakaguchi, D. A. A. Vignali, A. Y. Rudensky, R. E. Niec, and H. Waldmann, "The plasticity and stability of regulatory T cells," *Nature Reviews Immunology*, vol. 13, no. 6, pp. 461–467, 2013.

[35] C. H. Kim, "Migration and function of FoxP3$^+$ regulatory T cells in the hematolymphoid system," *Experimental Hematology*, vol. 34, no. 8, pp. 1033–1040, 2006.

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Research Article

Macrophage P2X7 Receptor Function Is Reduced during Schistosomiasis: Putative Role of TGF-β1

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Schistosomiasis is a chronic inflammatory disease whose macrophages are involved in immunopathology modulation. Although P2X7 receptor signaling plays an important role in inflammatory responses mediated by macrophages, no reports have examined the role of P2X7 receptors in macrophage function during schistosomiasis. Thus, we evaluated P2X7 receptor function in peritoneal macrophages during schistosomiasis using an ATP-induced permeabilization assay and measurements of the intracellular Ca^{2+} concentration. ATP treatment induced significantly less permeabilization in macrophages from *S. mansoni*-infected mice than in control cells from uninfected animals. Furthermore, P2X7-mediated increases in intracellular Ca^{2+} levels were also reduced in macrophages from infected mice. TGF-β1 levels were increased in the peritoneal cavity of infected animals, and pretreatment of control macrophages with TGF-β1 reduced ATP-induced permeabilization, mimicking the effect of *S. mansoni* infection. Western blot and qRT-PCR data showed no difference in P2X7 protein and mRNA between uninfected, infected, and TGF-β1-treated groups. However, immunofluorescence analysis revealed reduced cell surface localization of P2X7 receptors in macrophages from infected and TGF-β1-treated mice compared to controls. Therefore, our data suggest that schistosomiasis reduces peritoneal macrophage P2X7 receptor signaling. This effect is likely due to the fact that infected mice have increased levels of TGF-β1, which reduces P2X7 receptor cell surface expression.

1. Introduction

Macrophages are plastic phagocytic cells that participate in innate and adaptive immunity, with important roles in the response against extra- and intracellular parasites, as well as in tissue homeostasis [1, 2]. These cells have complex functions during acute and chronic inflammation. In inflammatory foci, macrophages contribute to mounting specific immunological responses for host defense, and the plasticity of macrophage phenotypes is modulated by the cytokine profile [1].

Tissue damage during inflammation increases the extracellular levels of otherwise intracellular molecules, resulting in damage-associated molecular patterns (DAMPs) recognized by the immune system [3–5]. Extracellular nucleotides, such as the ATP released by dying cells and by activated immune cells during inflammation, are important danger signals involved in immune response [6], participating in

both paracrine [7] and autocrine [8] signaling pathways. In inflammatory processes, high extracellular ATP levels generated by tissue damage or secretion are recognized by the immune system as a danger signal, activating purinergic P2 receptors that contribute to a proinflammatory response [9].

Purinergic P2 receptors can be classified as ionotropic ATP-gated (P2X) or G-protein coupled metabotropic (P2Y) [10]. Extracellular ATP (eATP) induces macrophage-mediated immune responses mainly through the activation of P2X7 receptors [11]. These receptors have a ubiquitous distribution, although the highest levels of receptor expression are observed in immune cells of monocyte/macrophage origin [12]. P2X7 receptor activation induces a myriad of intracellular events, including the production of nitric oxide (NO) and of reactive oxygen species (ROS) and the activation of phospholipase-D (PLD). These events are important for intracellular parasite killing, for the release of proinflammatory cytokines (such as IL-1β), and for inducing apoptosis [13, 14].

The expression and function of P2X7 receptors are regulated by both pro- and anti-inflammatory stimuli [15]. Initially, P2X7 receptor activation in macrophages opens a plasma membrane cation channel that allows a substantial efflux of K^+, and influx of Ca^{2+}, with later formation of pores permeable to large molecules [16, 17]. P2X receptor activation may also induce caspase-1 activation, interleukin IL-1β release, apoptosis, phagolysosomal fusion, and the elimination of intracellular pathogens [14, 18, 19]. In murine macrophages, the mature IL-1β secretion by the activation of P2X7 receptors is reduced by the incubation in a free-Ca^{2+} buffer [20]. Thus, murine macrophage release of mature IL-1β depends on intracellular Ca^{2+}, confirming that Ca^{2+} signaling is essential for this P2X7 receptor function.

Schistosomiasis is a chronic inflammatory disease caused by *Schistosoma mansoni* and it represents the second most common tropical parasitic disease related to socioeconomical factors. While migrating through the vascular system of infected mammalian hosts, parasites evolve from the schistosomula migratory form into adult worms, causing endothelial cell activation, immunological responses, and tissue damage. The disease starts with a Th1-type immune response that gradually changes to a Th2 profile [21–24]. Previous data suggest that the Th2 "stage" begins four to six weeks after infection and is related to egg deposition by adult worms.

Macrophages play important roles in both phases of schistosomiasis [24]. In the early phase of the disease, macrophages act as immune system effectors cells killing schistosomula and promoting tissue repair [24]. After egg deposition, the immune response switches to a Th2 profile that is involved in the formation of liver and colonic granulomas and fibrosis. The eggs are laid in mesenteric microcirculation and they may reach peritoneal cavity where the granulomas formed contain mainly macrophages [25]. Although granulomas are important to limit egg-derived potential cytotoxic products, macrophages isolated, for example, from hepatic granulomas, produce lipids mediators and free radicals that are potentially destructive to host tissues [26]. Among cytokines involved in schistosomiasis, transforming growth factor-β1 (TGF-β1) is of particular interest, since high levels of this cytokine are released by peripheral blood mononuclear cells (PBMCs) from *S. mansoni*-infected mice [27]. TGF-β has an important role in immune modulation later during infection, limiting liver inflammation and favoring host survival [27, 28].

Recently, Bhardwaj and Skelly [29] showed that *S. mansoni* expresses P2X7 receptors-like molecules and also enzymes responsible for the clearance of extracellular ATP (ectonucleotidases), suggesting that purinergic signaling is conserved in these parasites and is important for the host-parasite interplay. However, the impact of the chronic inflammation trigged by schistosomiasis on macrophage P2X7 receptor function remains unknown.

Here, we evaluated P2X7 receptor function and expression in macrophage from *S. mansoni*-infected mice. Our data show that peritoneal macrophage P2X7 receptor function is attenuated during schistosomiasis and that this is associated with high peritoneal levels of TGF-β1, the important inflammatory mediator present in the chronic phase of the disease. We also show that TGF-β1 downregulates P2X function *in vitro*, mimicking the effect of *S. mansoni* infection.

2. Materials and Methods

2.1. Reagents and Antibodies. The following primary antibodies were used in this work: rabbit polyclonal anti-P2X7 receptors (APR-004 and APR-008; Alomone Labs, Israel); mouse monoclonal anti-β-actin (Santa Cruz Biotechnology, USA); rat anti-F4/80 (Biolegend, USA); rat anti-F4/80 FITC (AbD Serotec, USA). ATP, 3′-O-(4-benzoyl)-ATP (BzATP), PMSF, sodium orthovanadate, aprotinin, leupeptin, BSA, ionomycin, and EGTA were from Sigma Chemical Co., (USA). Fura-2-AM was from Molecular Probes (USA) and A740003 was from Tocris (USA). RPMI, foetal bovine serum (FBS), and penicillin/streptomycin solutions were from GIBCO BRL (USA). TGF-β1 was from R&D Systems (Minneapolis, MN, USA). Stock solutions were prepared in DMSO (2.5 mM Fura-2-AM and 10 mM A740003), water (10 mM BzATP), RPMI (5 ng/mL TGF-β1), or a buffered physiological saline solution (10 mM ATP). The highest concentration of solvent used was 0.1% (v/v). All other reagents used were of analytical grade.

2.2. Mice. *Swiss*, C57BL/6 (wild type), and P2X7 receptor knockout (P2X7 KO) male mice were used in all procedures. *Swiss* and C57BL/6 mice were obtained from the animal facility of the Paulo de Goes Microbiology Institute (Federal University of Rio de Janeiro, Rio de Janeiro, Brazil). P2X7 KO mice (originally from The Jackson Laboratory, USA, stock number 005576) were maintained in the transgenic animal house of the Federal University of Rio de Janeiro. All mice were kept under a light/dark cycle of 12/12 h and with access to water and food ad libitum.

All experiments were conducted in compliance with the ethical standards of our institution (Ethics Committee of the Federal University of Rio de Janeiro; approved under the licenses DFBC-ICB-011 and IBCCF 154) and following

both the guidelines of the National Council on Experimental Animal Control (CONCEA, Brazil) and the Committee of Care and Use of Laboratory Animals (National Research Council, United States). All efforts were made to minimize both animals suffering and the number of animals used, on the basis of valid statistical evaluation.

2.3. Parasite and Infection. In this work, we used the BH strain of *S. mansoni*, obtained from infected *Biomphalaria glabrata* snails. *Swiss*, C57BL/6, and P2X7 KO mice (7-10 days old) were infected percutaneously with approximately 80 cercariae from both genders, for 8 min, as previously described [30]. Animals were used in experiments at least 45 days after infection (dpi) to allow for full establishment of the infection. Age-matched (60 to 80 days old) uninfected *Swiss*, C57BL/6, and P2X7 KO mice were used as controls. C57BL/6 and P2X7 KO mice survival was evaluated during infection.

2.4. Peritoneal Macrophages. To obtain mouse peritoneal macrophages, animals were anesthetized, sacrificed by cervical dislocation, and cleaned with 70% ethanol. The peritoneal cavity was washed with 5 mL of sterile phosphate buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 2.7 mM KCl, pH 7.4), and the peritoneal exudate was collected and centrifuged at 350 ×g, for 5 min, at 4°C. The pellet was resuspended in 1 mL of PBS and used to perform total leukocyte counts (cell viability, as estimated by Trypan blue exclusion, was always ≥95%). For macrophages culture, cells were resuspended in 1 mL of RPMI-1640 medium containing 2 g/L sodium bicarbonate, 1 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and plated in 35 mm Petri dishes or on 6-well plates. After 1 h of incubation at 37°C with 5% CO_2, nonadherent cells were removed by vigorous washing, and RPMI-1640 medium with 10% heat-inactivated FBS was added to the cultures, which were kept at 37°C with 5% CO_2 for 24 h until further use.

2.5. ATP-Induced Permeabilization Assay. To investigate macrophage responses to ATP, freshly harvested peritoneal macrophages (10^5 cells) from *Swiss* (uninfected or *S. mansoni*-infected) or C57BL/6 (wild type or P2X7 KO) mice were treated with 0.1, 0.5, or 1 mM ATP and 2.5 μM ethidium bromide (EB) in PBS (15 min; 37°C). EB was used as a tracer for P2X7 receptor activation (i.e., cell permeabilization) triggered by ATP. After incubation with ATP and EB, F4/80 positive cells were analyzed by flow cytometry (10,000 events/sample) using a FACScan system (BD Pharmingen, USA). Flow cytometry data analysis was performed using the WINMDI 2.9 software. In each histogram, a marker was added based on a gate of the F4/80 positive macrophages population, which limited the basal fluorescence. The specific ATP-induced permeabilization threshold was defined by comparison with the "baseline" profile of control cells not treated with ATP (i.e., incubated with EB only). The specific permeabilization is the percentage of EB positive cells after ATP stimulation in the F4/80 positive gated cells.

Alternatively, peritoneal macrophages were plated on 24-well plates (2×10^5 cells/well) and, after 24 h, treated with 5 ng/mL TGF-β1 or vehicle (RPMI), for 24 h, at 37°C and in 5% CO_2. Macrophages were washed in PBS (pH 7.4) and then incubated with 1 mM ATP and 5 μM EB in buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM CaCl, and 10 mM HEPES, pH 7.4) for 15 min at 37°C to evaluate the P2X7 receptor activation. The ratio between permeabilized (i.e., EB positive) and total cells was determined by direct counting of cells in five randomly chosen fields, using an Axiovert 100 microscope (Karl Zeiss, Oberkochen, Germany) equipped with an Olympus digital camera (Olympus American Inc., PA, USA). The specific ATP-induced permeabilization value (in %) was calculated by subtracting from the total % of permeabilized cells a "baseline" value corresponding to the % of permeabilized cells in control samples not treated with ATP (i.e., incubated with EB only).

2.6. Measurement of $[Ca^{2+}]_i$ in Macrophages. Measurements of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using Fura-2-AM were performed as previously described [31]. Briefly, macrophages were plated on glass bottom plates (MatTek, USA), at a density of 1×10^5 cells/plate, and maintained for 24 h at 37°C, with 5% CO_2. Then, the medium was removed and the cells were incubated for 40 min with 2.5 μM Fura-2-AM at room temperature. Cells were subjected to alternate cycles of illumination with 340 nm and 380 nm excitation wavelengths, and the emission was measured at 500 nm. Cells were washed twice with buffered physiological solution (in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 5, and HEPES 5, pH 7.4) and then stimulated with 100 μM 3′-O-(4-benzoyl)-ATP (BzATP) (at 37°C) in the absence or in the presence of 50 nM of the P2X7 receptor antagonist A740003 (preincubated for 45 min, at 37°C). After the maximum effect had been achieved, cells were treated with Ca^{2+} ionophore (10 μM ionomycin) and then with 6 mM EGTA to determine the molar concentration of Ca^{2+} according to the Grynkiewicz equation [32].

2.7. Determination of TGF-β1 Levels. The estimation of TGF-β1 levels in mouse peritoneal washes was performed using an ELISA immunoassay kit (Peprotech, Rocky Hill, NJ, USA), according to manufacturer's instructions. For peritoneal wash collection, mice were euthanized with CO_2 and the peritoneal cavity was washed with 1 mL of sterile PBS. Approximately 60% of the initial volume of PBS was recovered and centrifuged at 350 g for 5 min, at 4°C. Supernatants were collected and stored in liquid N_2 until further use (to determine TGF-β1 content).

2.8. Confocal Microscopy. Peritoneal macrophages from control and infected mice (10^5 cells/well) were treated with 5 ng/mL TGF-β for 24 h, fixed with 4% paraformaldehyde and 4% sucrose for 10 min in PBS, washed, and incubated for 30 min with 50 mM ammonium chloride (pH 8.0). After three washes in PBS, samples were blocked in PBS with 10% FBS and 0.1% BSA for 30 min, washed twice in PBS, and incubated overnight with a rabbit polyclonal antibody that

recognizes an extracellular epitope of P2X7 receptors (APR-008; Alomone Labs, Israel; Peptide KKGWMDPQSKGIQT-GRC, corresponding to amino acid residues 136–152 of mouse P2X7 receptor; extracellular loop) diluted to 1 : 400 in PBS and 0.1% BSA. Then, cells were washed and incubated with a rat anti-F4/80 antibody diluted to 1 : 50 in PBS and 0.1% BSA for 4 h, at 4°C. Samples were then labelled with secondary anti-rabbit-Alexa 488 and anti-rat-Alexa 546 antibodies diluted to 1 : 500 in PBS and 0.1% BSA for 1 h at 4°C. The secondary antibodies were used in the absence of primary antibodies as negative controls. After labelling, cells were mounted on slides using Vectashield with DAPI (Vector, Burlingame, CA, USA) and examined on a TCS-SP5 AOBS confocal microscope (Leica Microsystems, Germany). Images were analyzed using the ImageJ software. Each cell micrograph was analyzed using ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). The region of interest (ROI) was delimited in each cell and the respective mean fluorescence intensity was measured based on the pixel intensity. 10 cells per image were evaluated and the fluorescent values were used for statistical analysis. Data were also displayed in an orthogonal slice view that shows the raw pixel intensity values found mutually in each of three perpendicular planes.

2.9. Western Blotting. Macrophages adhered to 6-well plates were washed with sterile PBS and lysed with 200 μL RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Cell lysates were incubated at 4°C for 30 min and centrifuged at 8,100 ×g, for 10 min, at 4°C; the pellets were discarded and the supernatant had their protein content determined by the Lowry method [33]. Samples were run on 10% SDS–PAGE gels (15 μg protein/lane) and then transferred to nitrocellulose membranes using a semidry transfer system. Membranes were blocked for 1 h in Tris-buffered saline (TBS) with 2% nonfat milk and then incubated overnight with rabbit anti-P2X7 receptors (APR-004; Alomone Labs, Israel; 1 : 200; Peptide (C)KIRKEFPKTQGQYSGFKYPY, corresponding to amino acid residues 576–595 of P2X7 receptor intracellular, C-terminus) or mouse monoclonal anti-β-actin (1 : 20.000) primary antibodies. After 3 washes in TBS-Tween, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1 : 1.000) for 1 h and developed using enhanced chemiluminescence (ECL). Ponceau staining and labeling with a monoclonal anti-β-actin antibody were used as internal controls for protein loading. Densitometry of protein bands was performed using the ImageJ software, and the values for relative amounts of protein were normalized to the β-actin loading control.

2.10. Quantitative Real Time PCR (qRT-PCR). Peritoneal macrophages from uninfected *Swiss* mice (treated with 5 ng/mL TGF-β1 or with vehicle, for 24 h) or from *S. mansoni*-infected mice were used to isolate total RNA using the TRIzol reagent (Life Technologies), according to the manufacturer's instructions. Total RNA was quantified using an ND-1000 spectrophotometer (NanoDrop), and cDNA was synthesized from 500 ng of total RNA using the high capacity cDNA reverse transcription kit with RNase inhibitor (Invitrogen). The SYBR Select Master Mix (Applied Biosystems) was used for qRT-PCR, to detect double-stranded DNA synthesis. Reactions were carried out in a final volume of 10 μL, using 2 μL of diluted cDNA (1 : 10) and 300 nM of each of the reverse and forward primers. The following primers were used for qRT-PCR: for *P2rx7*, 5′ AATCG-GTGTGTTTCCTTTGG 3′ (forward) and 5′ CCGGGT-GACTTTGTTTGTCT 3′ (reverse); for *Actb*, 5′ TATGC-CAACACAGTGCTGTCTGG 3′ (forward) and 5′ TACTC-CTGCTTGCTGATCCACAT 3′ (reverse); and for *Gapdh*, 5′ GGTCATCCCAGAGCTGAACG 3′ (forward) and 5′ TTGCTGTTGAAGTCGCAGGA 3′ (reverse).

Reactions were performed in a 7500 Fast Real-Time System (Applied Biosystems), using the following PCR conditions: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 35 s at 60°C, and 15 s at 72°C. At the end of cycling protocol, a melting-curve analysis (with fluorescence measurements from 60 to 99°C) was performed. Relative expression levels were determined using the Sequence Detection Software v.2.0.5 (Applied Biosystems), and the efficiency per sample was calculated using the LinRegPCR 11.0 software (manufacturer). *β-Actin* and *Gapdh* were used as internal controls to calculate relative *P2rx7* mRNA levels, by the comparative threshold cycle ($\Delta\Delta C_T$) method with efficiency correction, using the mean qRT-PCR efficiency for each amplicon, as previously described [34, 35].

2.11. Statistical Analysis. Data were expressed as mean and SEM. Differences between two or more groups were analyzed by Student's t-test or one-way analysis of variance (ANOVA) followed by post hoc Newman-Keuls test, respectively, considering $P < 0.05$.

3. Results

3.1. P2X7 Receptor Function Is Reduced in Peritoneal Macrophages during Schistosomiasis. We used several approaches to examine the impact of the chronic inflammation triggered by *S. mansoni* infection on macrophage P2X7 receptor function. Activation of P2X7 receptors by ATP opens plasma membrane pores that allow molecules greater than 900 kDa (such as the fluorescent dye EB) to enter the cells [17, 36]. Thus, we used ATP-induced cell permeabilization (as evidenced by staining with EB) as a tool to compare the P2X7 receptor activation in peritoneal macrophage from infected and uninfected *Swiss* mice. Incubations with 0.1–1 mM ATP induced cell permeabilization in macrophages from uninfected mice; however, this effect was reduced in macrophages from infected mice (Figure 1(a)). ATP also induced permeabilization in macrophages from C57BL/6 wild type mice but, as expected, not in macrophages from P2X7 KO mice (Figure 1(b)).

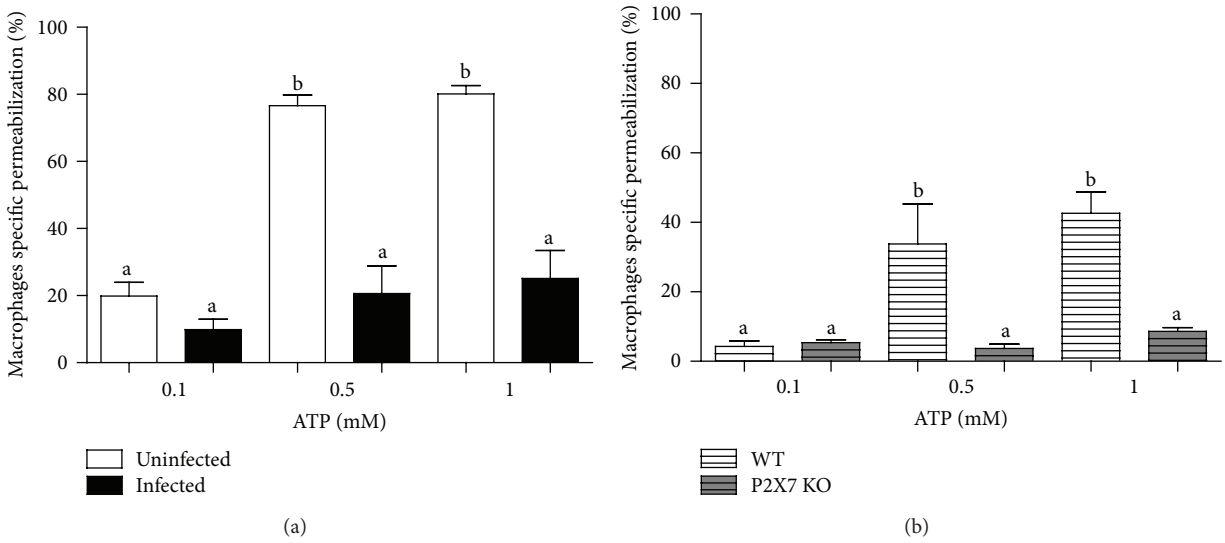

Figure 1: Evaluation of ATP-induced permeabilization in peritoneal macrophages, based on ethidium bromide (EB) uptake. (a) Peritoneal macrophages from *S. mansoni*-infected *Swiss* mice (black bars) were less sensitive to ATP-induced permeabilization than those from uninfected mice (white bars). (b) Peritoneal macrophages from C57BL/6 wild type mice (hatched white bars) respond to ATP-induced permeabilization unlike those from P2X7R KO mice (hatched gray bars). ATP-induced permeabilization values represent the % of EB uptake above basal levels by F4/80 positive cells and were expressed as mean and SEM. $N = 12$ (*Swiss*) and $n = 6 - 9$ (C57BL/6) replicates using different animals. (a) and (b) differ from each other with $P < 0.001$ (one-way ANOVA followed by post hoc Newman-Keuls test).

We also used the potent P2X7 agonist BzATP to verify if P2X7 receptor activation increased the cytosolic Ca^{2+} concentration in macrophages from infected and uninfected *Swiss* mice, since ATP-induced Ca^{2+} influx is a hallmark of P2X7 activation [36]. The basal levels of cytosolic Ca^{2+} (before BzATP addition) were similar in both groups (Figure 2(a)). After treatment with 100 μM BzATP, macrophages from uninfected mice showed a typical P2X7 receptors activation profile at 37°C, characterized by a pronounced biphasic increase in intracellular Ca^{2+} levels (Figure 2(a)) [36]. However, the P2X7 receptor-mediated Ca^{2+} influx was considerably less pronounced in macrophages from infected mice when compared to that observed in macrophages from uninfected mice (Figures 2(a) and 2(b)). We also assessed changes in intracellular Ca^{2+} in the presence of the selective P2X7 receptor antagonist A740003. Treatment with 50 nM A740003 did not alter basal intracellular Ca^{2+} levels, but it had a strong negative effect on the BzATP-induced Ca^{2+} influx, and this effect was similar in macrophages from both groups. These results confirmed that the increase in intracellular Ca^{2+} levels after addition of BzATP was due to P2X7 receptor activation. These data are in agreement with the reduction in ATP-induced permeabilization observed in macrophages from infected mice (Figure 1(a)) and suggest that P2X7 receptor signaling is downregulated during schistosomiasis.

3.2. S. Mansoni-Infected Mice Have Higher Levels of Peritoneal TGF-β1, Associated with Decreased P2X7-Dependent Macrophage Permeabilization. The anti-inflammatory cytokine TGF-β1 prevents P2X7 receptor upregulation in monocytes [37]. Moreover, in the chronic phase of schistosomiasis, there is a gradual increase of serum Th2 cytokines [21]. Indeed, we observed a significant increase in TGF-β1 levels in the peritoneal cavity of mice infected with *S. mansoni* (Figure 3).

To investigate if TGF-β1 could modulate P2X7 receptor function in peritoneal macrophages similar to that described for monocytes [37], we harvested peritoneal macrophages from uninfected animals and treated these cells with 5 ng/mL TGF-β1 for 24 h before assessing their sensitivity to ATP-induced permeabilization (using EB as a tracer). In the absence of ATP, TGF-β1 treatment did not induce cell permeabilization; however, TGF-β1 reduced the permeabilizing effect of 1 mM ATP by approximately 50%, when compared to controls treated with ATP only (Figure 4). We also performed the permeabilization after 48 h of TGF-β treatment and we observed that, even after 48 h, the P2X7-induced permeabilization was also reduced (data not shown). The analysis of the concentration-response curve using different concentrations of TGF-β showed that only the concentrations 5 ng/mL and 10 ng/mL were able to reduce the permeabilization intensity in macrophages (data not shown).

3.3. P2X7 Receptor Protein and mRNA Levels Do Not Change after Infection with S. Mansoni or Treatment with TGF-β1. To evaluate whether the reduction in P2X7 receptors function observed in schistosomiasis and in TGF-β1-treated cells was due, at least in part, to changes in P2X7 receptor expression, we quantified both P2X7 protein expression and

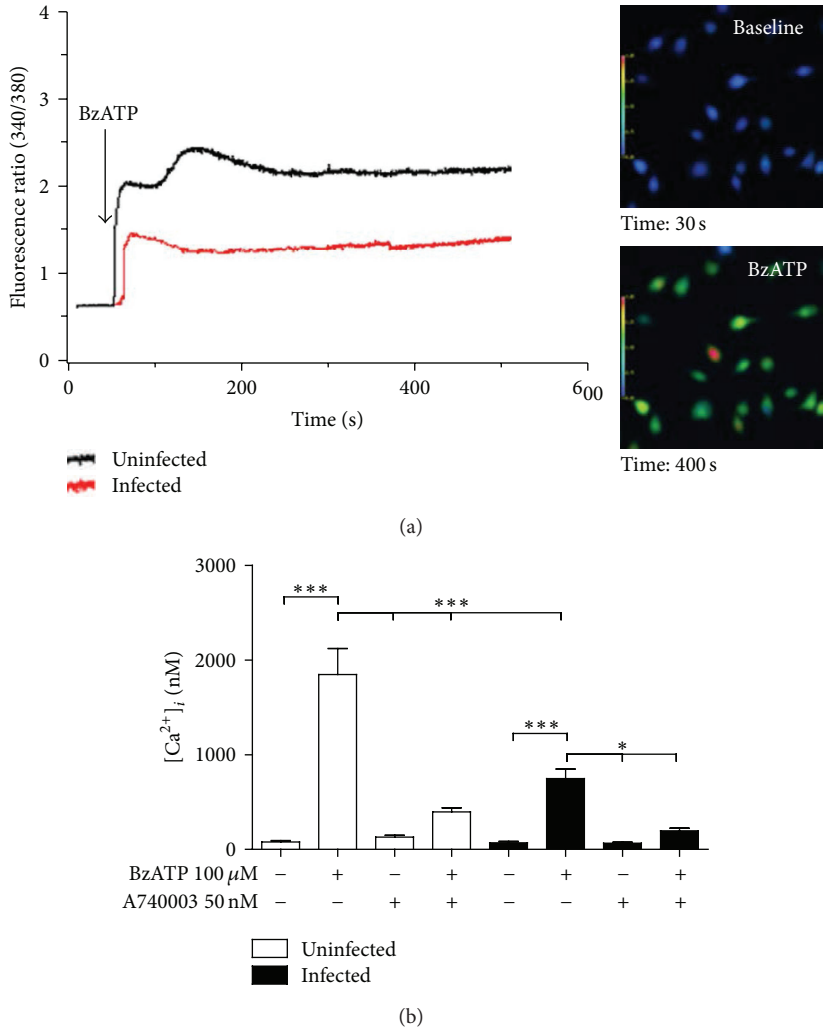

FIGURE 2: Intracellular Ca^{2+} measurement (using Fura-2-AM) in macrophages subjected to treatment with the P2X7 agonist BzATP and/or with the antagonist A740003. (a) Changes in intracellular Ca^{2+} levels in macrophages from control (black line) and *S. mansoni*-infected (red line) mice. Inset: representative images from macrophages (control) before (baseline) and after treatment with 100 μM BzATP. (b) Changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in macrophages from control (white bars) or *S. mansoni*-infected (black bars) mice. Three plates were used for each condition/animal, 10 cells/plate were chosen randomly for imaging, and cells were obtained from three animals. Data were expressed as mean and SEM. $^{*}P < 0.05$; $^{***}P < 0.001$ (one-way ANOVA followed by post hoc Newman-Keuls test).

the transcription from the *P2rx7* locus by Western blotting and qRT-PCR, respectively. We detected similar levels of P2X7 protein and mRNA in macrophages from uninfected and infected groups (Figures 5(a) and 5(c)). Moreover, the levels of P2X7 protein and mRNA in macrophages from uninfected mice were not altered by treatment with 5 ng/mL TGF-β1 for 24 h (Figures 5(b) and 5(c)).

3.4. Schistosomiasis and Treatment with TGF-β1 Reduce Cell Surface P2X7 Levels in Peritoneal F4/80 Positive Macrophages. P2X7 receptors are targets of posttranslational modifications that regulate their insertion into plasma membrane [38], and the proinflammatory effects of P2X7 receptors (including IL-1β release and NO and ROS production) depend on receptor localization at the cell surface. Moreover, prolonged exposure of macrophages to ATP may induce P2X7 receptors removal from the cell surface by internalization [39, 40].

Since the reduction in P2X7 function during *S. mansoni* infection was not due to decreases in protein or mRNA levels, we hypothesized that changes in receptor structure or localization occurred during infection and downregulated receptor function, similar to that described for other P2X receptors [41]. To test this hypothesis, we analyzed P2X7 receptor localization in nonpermeabilized F4/80 (a common surface marker of mature macrophages) positive macrophages from uninfected and *S. mansoni*-infected mice using an antibody that recognizes an extracellular P2X7 epitope. Confocal microscopy analysis of anti-P2X7 labeled cells suggested that the levels of cell surface P2X7 receptors were reduced in macrophages from infected mice (Figures 6(a) and 6(b)). Interestingly, there was also a reduction in cell

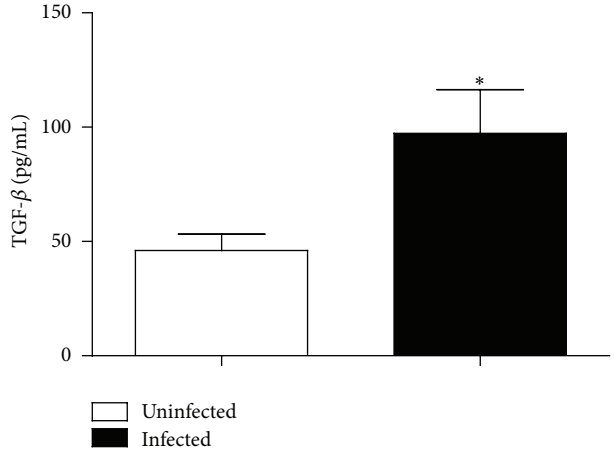

Figure 3: Peritoneal TGF-β1 levels in uninfected (white) and *S. mansoni*-infected mice (black). Data were expressed as mean and SEM. $n = 6$ (uninfected) or 4 (infected) mice. $^{*}P < 0.05$ (Student's t-test).

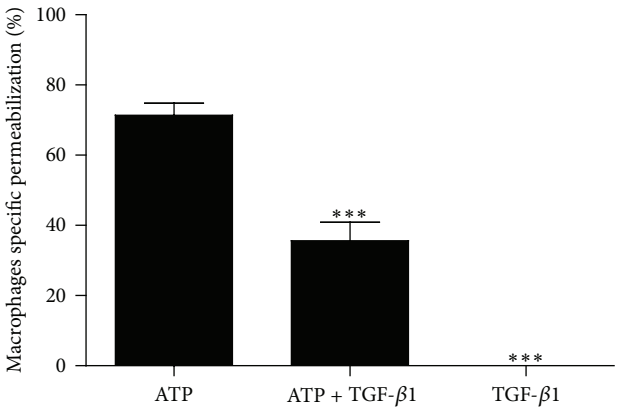

Figure 4: ATP-induced permeabilization assays (based on ethidium bromide uptake) by peritoneal F4/80 positive macrophages of uninfected mice treated with TGF-β1. Cells treated with 5 ng/mL TGF-β1 or vehicle for 24 h were stimulated with 1 mM ATP. ATP-induced permeabilization values represent the % of EB uptake above basal levels and were expressed as mean and SEM. $n = 8$–21 replicates using 6 animals ($^{***}P < 0.001$ versus ATP, one-way ANOVA followed by post hoc Newman-Keuls test).

surface P2X7 in macrophages from uninfected mice treated with TGF-β1 (Figures 6(a) and 6(b)). Relative quantification of the immunofluorescence data showed that there was a 1.6-fold decrease (approximately) in the intensity of cell surface P2X7 fluorescence in macrophages from infected mice, when compared to that observed in uninfected control mice (Figure 6(b)).

3.5. Reduced Survival of P2X7 KO Mice Infected with S. Mansoni. Next, to evaluate the participation of P2X7 receptors during the disease, we infected C57BL/6 wild type and P2X7 KO mice and followed the progression of infection. The survival curves showed that mortality in P2X7 KO infected mice started at 28 dpi and reached 100% at 60 dpi. In contrast, no death was observed in C57BL/6 wild type mice (Figure 7).

4. Discussion

During schistosomiasis, macrophages may display classical or activated phenotypes. The latter phenotype is present mainly in macrophages surrounding eggs within liver granulomas and was recently shown to be important in the control of tissue fibrosis [42]. Considering that schistosomiasis is a chronic inflammatory disease and that macrophages are involved in schistosomiasis pathogenesis [24], we sought to evaluate the function of P2X7 receptors in macrophages from mice infected with *S. mansoni*, since these receptors play an important role in inflammatory processes.

Here, we used a combination of ATP-induced permeabilization and intracellular Ca^{2+} measurement assays to show that, during the chronic phase of schistosomiasis, there is a reduction in the ATP-dependent P2X7 receptor function in macrophages (Figures 1 and 2), similar to that observed in mesenteric endothelial cells using the same experimental model [43].

While the function and expression of macrophage P2X7 receptors are positively regulated by proinflammatory cytokines such as IFN-γ [44, 45], Gadeock and coworkers [37] showed that the upregulation of P2X7 receptors in THP-1 monocytes is negatively regulated by TGF-β. On the other hand, peripheral blood mononuclear cells (PBMCs) from *S. mansoni*-infected mice produce high levels of TGF-β [27], and this cytokine is important to limit liver inflammation and favor host survival [27, 28]. Overall, these data are in agreement with our results also showing that the levels of peritoneal TGF-β1 are increased in mice infected with *S. mansoni* (Figure 3) and that TGF-β1, in a concentration close to the ones observed in the serum of chronic patients [46], reduces by approximately 50% the P2X7-dependent macrophage permeabilization triggered by ATP (Figure 4), mimicking the attenuated P2X7 response observed during *S. mansoni* infection (Figure 1(a)). Taken together, these results support our hypothesis that the downregulation of P2X7 function observed in schistosomiasis is a result of the increase in TGF-β1 levels during infection. Regarding TGF-β levels, despite the difference between the *in vitro* treatment concentration (5 ng/mL) and the *in vivo* peritoneal measurement (100 pg/mL), we must consider that we injected 1 mL of PBS in the peritoneal cavity of infected animals before removing the body fluid for cytokine determination. Therefore the samples were diluted, and, as a consequence, peritoneal TGF-β levels *in vivo* may be higher than the estimation *in vitro*. Moreover, previous data showed that individuals chronically infected with schistosomiasis have TGF-β serum levels around 20 ng/mL [46] and the serum level estimated in *S. mansoni*-infected mice (19.98 ± 2.37 ng/mL) is compatible with the value observed in patients [47]. Therefore we conclude that the TGF-β concentration used in present work is compatible with the disease, and the peritoneal TGF-β concentration in the infected animals is probably higher than estimated. Finally, TGF-β in the ng/mL range of concentration is able to modulate P2X7R function.

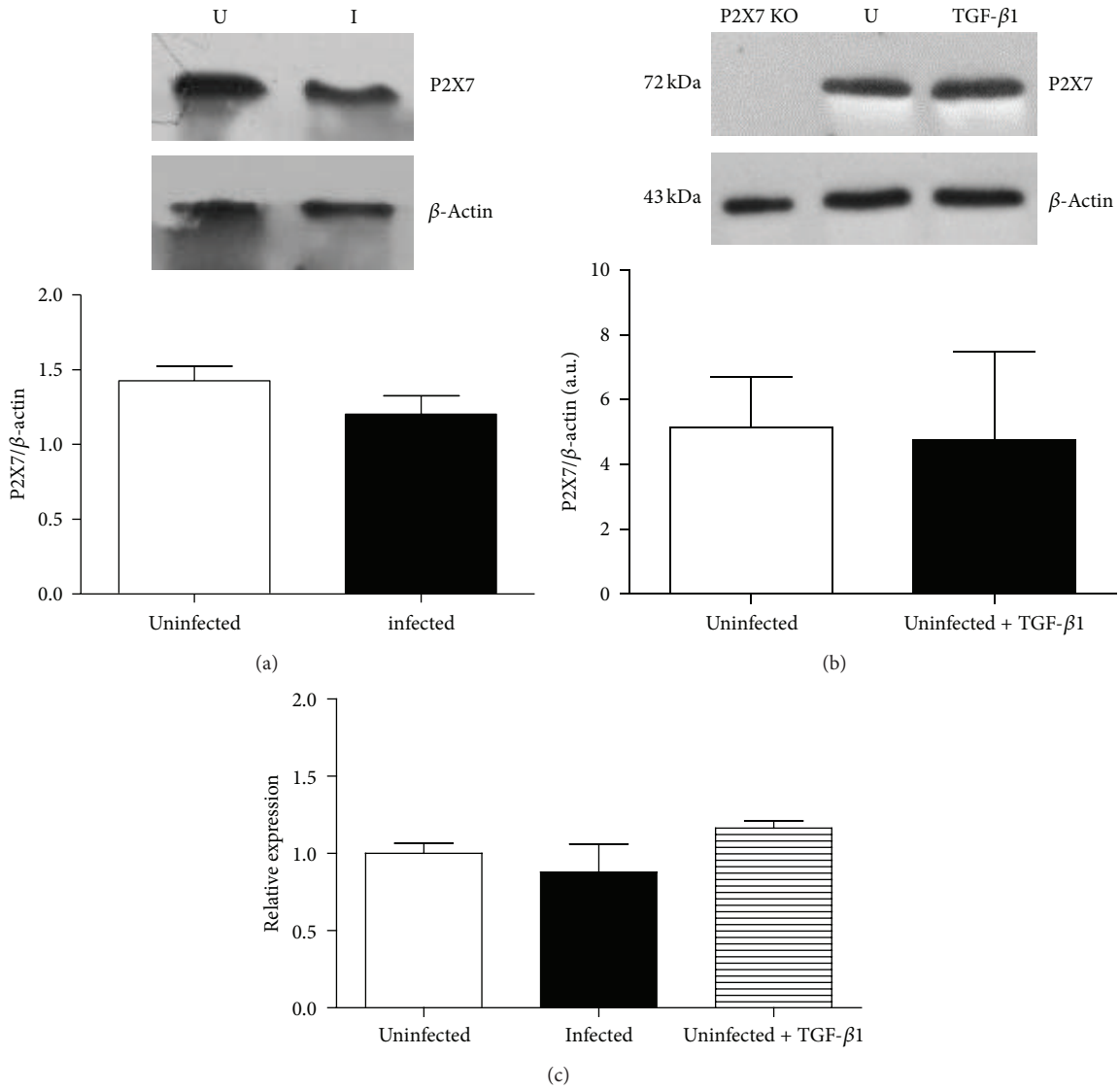

Figure 5: P2X7 receptors expression in peritoneal macrophages. (a) Macrophages from uninfected (white bar) and *S. mansoni*-infected (black bar) mice express similar levels of P2X7 protein. $n = 9$ replicates from three different animals, for each group. The images above are representative Western blots (U = uninfected and I = infected). (b) P2X7 receptors expression in peritoneal macrophages from control mice (white bar, untreated) or TGF-β1-treated cells (5 ng/mL, 24 h; black bar) are also similar. The images above are representative Western blots (U = uninfected; TGF-β1= TGF-β1-treated macrophages). Lysates from P2X7 KO macrophages were used as negative controls for P2X7 protein expression. Cells lysates were obtained from three animals for each group. (c) Quantitative RT-PCR (qRT-PCR) analysis of P2X7 receptor mRNA levels in peritoneal macrophages. Similar levels of P2X7 mRNA were found in uninfected (white bar), *S. mansoni*-infected mice (black bar), and TGF-β1-treated (5 ng/mL, 24 h; hatched bar) mouse macrophages. $N = 3$–5 samples per group (using different animals).

Western blotting and qRT-PCR data (using whole cell lysates) excluded the possibility that the reduction in P2X7 receptor function during *S. mansoni* infection or after treatment with TGF-β1 resulted from decreased levels of P2X7 protein or mRNA. In fact, immunofluorescence microscopy analysis of nonpermeabilized macrophages expressing F4/80 on their cell surface revealed that there was a lower density of surface P2X7 receptors in cells from infected mice compared to those from uninfected, control animals (Figure 6). Since total P2X7 protein levels were similar in both groups, we propose that there is reduction of P2X7 receptor in plasma membrane of macrophages from infected mice. The disappearance of immunoreactivity from plasma membrane could be related to some conformational changes of the receptor, an interaction with other proteins masking the extracellular epitope recognized by the antibody or the P2X7 receptor internalization. However, TGF-β treatment *in vitro* mimicked both the reduced cell surface receptor expression and function observed in macrophages obtained from infected animals. Consequently, a plausible explanation for the reduction in P2X7 function (without corresponding decreases in total protein or mRNA levels) during chronic

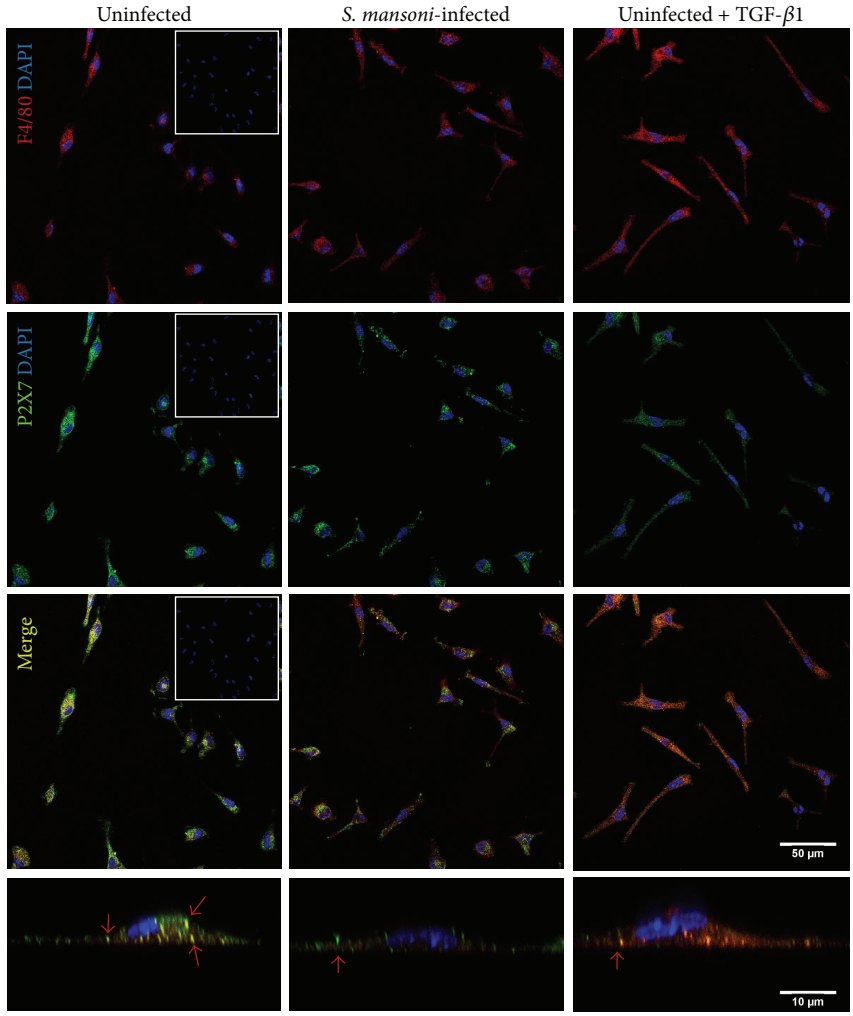

FIGURE 6: Confocal microscopy analysis of P2X7 receptor expression on the cell surface of macrophages. Cells were labelled with antibodies recognizing P2X7 receptors (green) and F4/80+ (red) as well as with DAPI (blue). (a) Representative images showing macrophages from uninfected (left column), *S. mansoni*-infected (middle column), and TGF-β1-treated (right column; 5 ng/mL, for 24 h) mice. The bottom row shows orthogonal slices of the representative labelled cells, with red arrows marking P2X7 expression on the plasma membrane. (b) Quantitative analysis of confocal microscopy data. The mean fluorescence intensity, showing cell surface P2X7 receptor expression in peritoneal macrophages from uninfected mice (white bar), *S. mansoni*-infected (black bar), and TGF-β1-treated (hatched bar; 5 ng/mL, for 24 h) mice, was obtained from pixels intensity using ImageJ software (see methods). Inset: negative controls (macrophages incubated with secondary antibodies only). $n = 6$ replicates from 3 animals for each condition ($^{***}P < 0.001$ versus control, $P < 0.01$ TGF-β1 versus infected group; one-way ANOVA followed by post hoc Newman-Keuls test).

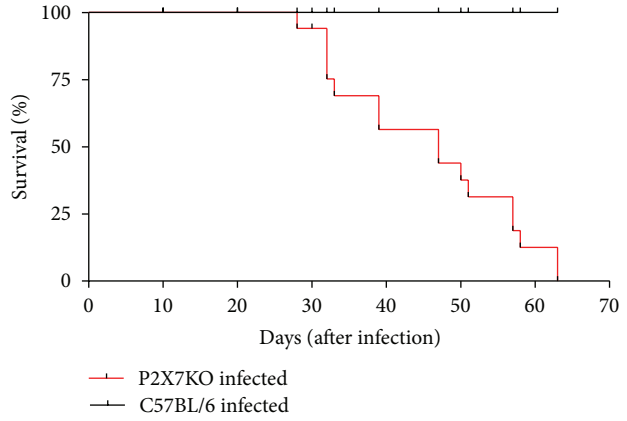

Figure 7: Survival curve of C57BL/6 wild type or P2X7 KO infected with approximately 80 cercariae of *S. mansoni*. (P2X7 KO: $n = 9$ females and 7 males; C57BL/6 wild type: 4 females and 10 males). $^{***}P < 0.0001$ using the Mantel-Cox log-rank test.

schistosomiasis could be related to a reduced cell surface expression of P2X7 receptor.

Despite the work of Gadeock et al. [37], data investigating the link between TGF-β1 and P2X7 receptors function and/or expression are still missing. Since control macrophage treatment with TGF-β1 mimicked the profile of cell surface P2X7 receptors expression and function observed in live macrophages obtained from infected mice, it is supposed that TGF-β1 could be involved in the P2X7 receptors reduced function observed in the disease. To the best of our knowledge, this is the first report that shows a direct effect of TGF-β1 on macrophage P2X7 receptor function. However, we do not exclude that other cytokines could contribute to the P2X7 reduced function in macrophages from *S. mansoni*-infected mice, since in the later phase of the disease beyond the increase of TGF-β levels there is also the increase of IL-4, IL-5, IL-10, and IL-13.. Moreover, it was shown that IL-4 and IL-10 also inhibit EB uptake in rat alveolar macrophage [48]. Furthermore, previous data showed that TGF-β, IL-10, and IL-4 inhibit macrophage cytotoxicity which could be an important strategy used by *S. mansoni* to evade macrophage-mediated immune destruction [49].

Patients with schistosomiasis may be more susceptible to secondary infections, and prior infection with *Schistosoma* also increases the severity of secondary infections with *Leishmania, Toxoplasma gondii,* and *Salmonella* [50]. In addition, a previous report showed that macrophages from chronically infected mice have reduced phagocytic activity [51]; however, little is known about the mechanisms underlying this phenomenon. Recently, Wiley and Gu [52] showed that P2X7 receptors on the surface of monocytes/macrophages may act as scavenger receptors for bacteria in the absence of ATP. Therefore, the reduction of P2X7 receptor function in *S. mansoni*-infected animals could limit bacterial (and possibly protozoal) phagocytosis during schistosomiasis. This phenomenon might explain the increased susceptibility of patients infected with *S. mansoni* to secondary infections with other parasites and also the higher severity of secondary infections in these patients.

Kusner and Adams [53] showed that ATP kills *Mycobacterium tuberculosis* in human macrophages and that this effect depends on P2X7 receptor-mediated PLD activation. This mechanism is also important for the ATP-mediated killing of *Chlamydia trachomatis* in murine peritoneal macrophages [13]. Moreover, our group showed that the acute infection of macrophages with *Chlamydia psittaci* reduces P2X7 receptor-mediated cell permeabilization and Ca^{2+} influx, thus inhibiting macrophage apoptosis [54]. Considering that *C. psittaci* are obligatory intracellular parasites, this phenomenon could be an attempt to limit immunological responses by reducing ATP-mediated apoptosis and, consequently, favoring parasite survival. An important new aspect of P2X7 receptors signaling highlighted in the present work is that the function of macrophage P2X7 receptors may also be modulated by the extracellular parasite *S. mansoni*.

Altogether, our data suggest that, during schistosomiasis, the function of macrophage P2X7 receptors is reduced and that TGF-β1 plays a key putative role in the downregulation of receptor signaling. We hypothesize that macrophage purinergic receptors are differentially modulated during disease progression and that this phenomenon has a key role in the immune response against *S. mansoni*. In this context, our data show that P2X7 receptor knockout animals (P2X7 KO) are much more susceptible to death during *S. mansoni* infection than WT mice highlighting this P2 purinergic receptor and reinforcing the necessity of future studies exploring in deep its importance in *S. mansoni*-induced disease. Given that purinergic signaling is also found in *S. mansoni*, understanding the interplay between host and parasite purinergic signaling pathways is now important to clarify if future therapeutic approaches targeting these signaling pathways would be useful against schistosomiasis.

5. Conclusions

Altogether, our data show that *S. mansoni* infection reduced P2X7 function in peritoneal macrophages during the chronic phase of the disease. Furthermore, the peritoneal cavity of infected mice had increased levels of TGF-β1, and this cytokine reduced P2X7 receptor function in macrophages from uninfected mice. Thus, immunomodulation by TGF-β1 could limit P2X7-dependent inflammatory effects in macrophages from *S. mansoni* patients and may provide an explanation for the increased susceptibility of these patients to infections by others pathogens.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[2] S. J. Galli, N. Borregaard, and T. A. Wynn, "Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils," *Nature Immunology*, vol. 12, no. 11, pp. 1035–1044, 2011.

[3] M. E. Bianchi, "DAMPs, PAMPs and alarmins: all we need to know about danger," *Journal of Leukocyte Biology*, vol. 81, no. 1, pp. 1–5, 2007.

[4] N. Saïd-Sadier and D. Ojcius, "Alarmins, inflammasomes and immunity," *Biomedical Journal*, vol. 35, no. 6, pp. 437–449, 2012.

[5] H. Kono and K. L. Rock, "How dying cells alert the immune system to danger," *Nature Reviews Immunology*, vol. 8, no. 4, pp. 279–289, 2008.

[6] A. La Sala, D. Ferrari, F. Di Virgilio, M. Idzko, J. Norgauer, and G. Girolomoni, "Alerting and tuning the immune response by extracellular nucleotides," *Journal of Leukocyte Biology*, vol. 73, no. 3, pp. 339–343, 2003.

[7] M. R. Elliott, F. B. Chekeni, P. C. Trampont et al., "Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance," *Nature*, vol. 461, no. 7261, pp. 282–286, 2009.

[8] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[9] A. C. Morandini, L. E. Savio, and R. Coutinho-Silva, "The role of p2x7 receptor in infectious inflammatory diseases and the influence of ectonucleotidases," *Biomedical Journal*, vol. 37, no. 4, pp. 169–177, 2014.

[10] G. Burnstock, "Purine and pyrimidine receptors," *Cellular and Molecular Life Sciences*, vol. 64, no. 12, pp. 1471–1483, 2007.

[11] F. Di Virgilio, "Purinergic signalling in the immune system. A brief update," *Purinergic Signalling*, vol. 3, no. 1-2, pp. 1–3, 2007.

[12] G. Burnstock and G. E. Knight, "Cellular distribution and functions of P2 receptor subtypes in different systems," *International Review of Cytology*, vol. 240, pp. 31–304, 2004.

[13] R. Coutinho-Silva, L. Stahl, M. Raymond et al., "Inhibition of chlamydial infectious activity due to P2X 7R-dependent phospholipase D activation," *Immunity*, vol. 19, no. 3, pp. 403–412, 2003.

[14] R. Coutinho-Silva and D. M. Ojcius, "Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites," *Microbes and Infection*, vol. 14, no. 14, pp. 1271–1277, 2012.

[15] B. D. Humphreys and G. R. Dubyak, "Modulation of P2X7 nucleotide receptor expression by pro- and anti- inflammatory stimuli in THP-1 monocytes," *Journal of Leukocyte Biology*, vol. 64, no. 2, pp. 265–273, 1998.

[16] R. Coutinho-Silva, L. A. Alves, W. Savino, and P. M. Persechini, "A cation non-selective channel induced by extracellular ATP in macrophages and phagocytic cells of the thymic reticulum," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1278, no. 1, pp. 125–130, 1996.

[17] R. Coutinho-Silva and P. M. Persechini, "P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J774 cells," *The American Journal of Physiology—Cell Physiology*, vol. 273, no. 6, pp. C1793–C1800, 1997.

[18] D. Ferrari, C. Pizzirani, E. Adinolfi et al., "The P2X7 receptor: a key player in IL-1 processing and release," *The Journal of Immunology*, vol. 176, no. 12, pp. 3877–3883, 2006.

[19] Y. Qu, L. Franchi, G. Nunez, and G. R. Dubyak, "Nonclassical IL-1β secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages," *The Journal of Immunology*, vol. 179, no. 3, pp. 1913–1925, 2007.

[20] L. Gudipaty, J. Munetz, P. A. Verhoef, and G. R. Dubyak, "Essential role for Ca^{2+} in regulation of IL-1β secretion by P2X7 nucleotide receptor in monocytes, macrophages, and HEK-293 cells," *American Journal of Physiology-Cell Physiology*, vol. 285, no. 2, pp. C286–C299, 2003.

[21] E. J. Pearce and A. S. MacDonald, "The immunobiology of schistosomiasis," *Nature Reviews Immunology*, vol. 2, no. 7, pp. 499–511, 2002.

[22] M. S. Wilson, M. M. Mentink-Kane, J. T. Pesce, T. R. Ramalingam, R. Thompson, and T. A. Wynn, "Immunopathology of schistosomiasis," *Immunology and Cell Biology*, vol. 85, no. 2, pp. 148–154, 2007.

[23] G. Schramm and H. Haas, "Th2 immune response against *Schistosoma mansoni* infection," *Microbes and Infection*, vol. 12, no. 12-13, pp. 881–888, 2010.

[24] L. Barron and T. A. Wynn, "Macrophage activation governs schistosomiasis-induced inflammation and fibrosis," *European Journal of Immunology*, vol. 41, no. 9, pp. 2509–2514, 2011.

[25] M. C. Melro and M. Mariano, "Extra-tissular Schistosoma mansoni egg granulomata in the peritoneal cavity of mice," *Memorias do Instituto Oswaldo Cruz*, vol. 82, supplement 4, pp. 245–252, 1987.

[26] C. Chuah, M. K. Jones, M. L. Burke, D. P. McManus, and G. N. Gobert, "Cellular and chemokine-mediated regulation in schistosome-induced hepatic pathology," *Trends in Parasitology*, vol. 30, pp. 141–150, 2014.

[27] I. O. Farah, P. W. Mola, T. M. Kariuki, M. Nyindo, R. E. Blanton, and C. L. King, "Repeated exposure induces periportal fibrosis in Schistosoma mansoni- infected baboons: role of TGF-β and IL-4," *Journal of Immunology*, vol. 164, no. 10, pp. 5337–5343, 2000.

[28] D. R. Herbert, T. Orekov, C. Perkins, and F. D. Finkelman, "IL-10 and TGF-β redundantly protect against severe liver injury and mortality during acute schistosomiasis," *The Journal of Immunology*, vol. 181, no. 10, pp. 7214–7220, 2008.

[29] R. Bhardwaj and P. J. Skelly, "Purinergic signaling and immune modulation at the schistosome surface?" *Trends in Parasitology*, vol. 25, no. 6, pp. 256–260, 2009.

[30] H. L. Lenzi, W. D. S. Romanha, R. M. Santos et al., "Four whole-istic aspects of schistosome granuloma biology: fractal

arrangement, internal regulation, autopoietic component and closure," *Memorias do Instituto Oswaldo Cruz*, vol. 101, no. 1, pp. 219–231, 2006.

[31] S. Salomone, C. L. M. Silva, N. Morel, and T. Godfraind, "Facilitation of the vasorelaxant action of calcium antagonists by basal nitric oxide in depolarized artery," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 354, no. 4, pp. 505–512, 1996.

[32] G. Grynkiewicz, M. Poenie, and R. Y. Tsien, "A new generation of Ca^{2+} indicators with greatly improved fluorescence properties," *Journal of Biological Chemistry*, vol. 260, no. 6, pp. 3440–3450, 1985.

[33] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent.," *The Journal of biological chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[34] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, article e45, 2001.

[35] J. M. Ruijter, C. Ramakers, W. M. H. Hoogaars et al., "Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data," *Nucleic Acids Research*, vol. 37, no. 6, article e45, 2009.

[36] P. M. Persechini, R. C. Bisaggio, J. L. Alves-Neto, and R. Coutinho-Silva, "Extracellular ATP in the lymphohematopoietic system: P2Z purinoceptors and membrane permeabilization," *Brazilian Journal of Medical and Biological Research*, vol. 31, no. 1, pp. 25–34, 1998.

[37] S. Gadeock, J. N. S. N. Tran, J. G. Georgiou et al., "TGF-β1 prevents up-regulation of the P2X7 receptor by IFN-γ and LPS in leukemic THP-1 monocytes," *Biochimica et Biophysica Acta: Biomembranes*, vol. 1798, no. 11, pp. 2058–2066, 2010.

[38] P. Gonnord, C. Delarasse, R. Auger et al., "Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts," *The FASEB Journal*, vol. 23, no. 3, pp. 795–805, 2009.

[39] J. F. Hiken and T. H. Steinberg, "ATP downregulates P2X7 and inhibits osteoclast formation in RAW cells," *American Journal of Physiology—Cell Physiology*, vol. 287, no. 2, pp. C403–C412, 2004.

[40] Y. H. Feng, L. Wang, Q. Wang, X. Li, R. Zeng, and G. I. Gorodeski, "ATP stimulates GRK-3 phosphorylation and β-arrestin-2-dependent internalization of P2X7 receptor," *The American Journal of Physiology—Cell Physiology*, vol. 288, no. 6, pp. C1342–C1356, 2005.

[41] L. E. Robinson and R. D. Murrell-Lagnado, "The trafficking and targeting of P2X receptors," *Frontiers in Cellular Neuroscience*, vol. 7, article 233, 2013.

[42] J. T. Pesce, T. R. Ramalingam, M. M. Mentink-Kane et al., "Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis," *PLoS Pathogens*, vol. 5, no. 4, Article ID e1000371, 2009.

[43] S. D. D. S. Oliveira, R. Coutinho-Silva, and C. L. M. Silva, "Endothelial P2X7 receptors'expression is reduced by schistosomiasis," *Purinergic Signalling*, vol. 9, no. 1, pp. 81–89, 2013.

[44] D. K. Blanchard, S. McMillen, and J. Y. Djeu, "IFN-γ enhances sensitivity of human macrophages to extracellular ATP-mediated lysis," *Journal of Immunology*, vol. 147, no. 8, pp. 2579–2585, 1991.

[45] L. Welter-Stahl, C. M. da Silva, J. Schachter et al., "Expression of purinergic receptors and modulation of P2X7 function by the inflammatory cytokine IFNγ in human epithelial cells," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1788, no. 5, pp. 1176–1187, 2009.

[46] R. C. Ferreira, S. M. Montenegro, A. L. Domingues et al., "Almeida MO: TGF beta and IL13 in schistosomiasis mansoni associated pulmonary arterial hypertension ; a descriptive study with comparative groups," *BMC Infectious Diseases*, vol. 14, article 282, 2014.

[47] N. M. El-Lakkany, O. A. Hammam, W. H. El-Maadawy, A. A. Badawy, A. A. Ain-Shoka, and F. A. Ebeid, "Anti-inflammatory/anti-fibrotic effects of the hepatoprotective silymarin and the schistosomicide praziquantel against *Schistosoma mansoni*-induced liver fibrosis," *Parasites & Vectors*, vol. 5, no. 1, article 9, 2012.

[48] I. Lemaire and N. Leduc, "Purinergic $P2X_7$ receptor function in lung alveolar macrophages: pharmacologic characterization and bidirectional regulation by Th1 and Th2 cytokines," *Drug Development Research*, vol. 59, no. 1, pp. 118–127, 2003.

[49] I. P. Oswald, R. T. Gazzinelli, A. Sher, and S. L. James, "IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity," *Journal of Immunology*, vol. 148, no. 11, pp. 3578–3582, 1992.

[50] A. Abruzzi and B. Fried, "Coinfection of Schistosoma (Trematoda) with Bacteria, Protozoa and Helminths," *Advances in Parasitology*, vol. 77, pp. 1–85, 2011.

[51] M. I. Muniz-Junqueira, A. Prata, and C. E. Tosta, "Phagocytic and bactericidal function of mouse macrophages to Salmonella typhimurium in schistosomiasis mansoni," *The American Journal of Tropical Medicine and Hygiene*, vol. 46, no. 2, pp. 132–136, 1992.

[52] J. S. Wiley and B. J. Gu, "A new role for the P2X7 receptor: a scavenger receptor for bacteria and apoptotic cells in the absence of serum and extracellular ATP," *Purinergic Signalling*, vol. 8, no. 3, pp. 579–586, 2012.

[53] D. J. Kusner and J. Adams, "ATP-induced killing of virulent Mycobacterium tuberculosis within human macrophages requires phospholipase D," *Journal of Immunology*, vol. 164, no. 1, pp. 379–388, 2000.

[54] R. Coutinho-Silva, J. Perfettini, P. M. Persechini, A. Dautry-Varsat, and D. M. Ojcius, "Modulation of P2Z/$P2X_7$ receptor activity in macrophages infected with *Chlamydia psittaci*," *American Journal of Physiology—Cell Physiology*, vol. 280, no. 1, pp. C81–C89, 2001.

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Research Article

Feed-Forward Inhibition of CD73 and Upregulation of Adenosine Deaminase Contribute to the Loss of Adenosine Neuromodulation in Postinflammatory Ileitis

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Purinergic signalling is remarkably plastic during gastrointestinal inflammation. Thus, selective drugs targeting the "purinome" may be helpful for inflammatory gastrointestinal diseases. The myenteric neuromuscular transmission of healthy individuals is fine-tuned and controlled by adenosine acting on A_{2A} excitatory receptors. Here, we investigated the neuromodulatory role of adenosine in TNBS-inflamed longitudinal muscle-myenteric plexus of the rat ileum. Seven-day postinflammation ileitis lacks adenosine neuromodulation, which may contribute to acceleration of gastrointestinal transit. The loss of adenosine neuromodulation results from deficient accumulation of the nucleoside at the myenteric synapse despite the fact that the increases in ATP release were observed. Disparity between ATP outflow and adenosine deficit in postinflammatory ileitis is ascribed to feed-forward inhibition of ecto-5′-nucleotidase/CD73 by high extracellular ATP and/or ADP. Redistribution of NTPDase2, but not of NTPDase3, from ganglion cell bodies to myenteric nerve terminals leads to preferential ADP accumulation from released ATP, thus contributing to the prolonged inhibition of muscle-bound ecto-5′-nucleotidase/CD73 and to the delay of adenosine formation at the inflamed neuromuscular synapse. On the other hand, depression of endogenous adenosine accumulation may also occur due to enhancement of adenosine deaminase activity. Both membrane-bound and soluble forms of ecto-5′-nucleotidase/CD73 and adenosine deaminase were detected in the inflamed myenteric plexus. These findings provide novel therapeutic targets for inflammatory gut motility disorders.

1. Introduction

The enteric nervous system (ENS) undergoes a series of adaptive responses to different pathological conditions (e.g., inflammatory and/or ischemic insults) [1, 2]. For instance, enteric neurons rapidly change their structure, function, or chemical phenotype in order to maintain gut homeostasis. Even if the inflammatory insult is brief and the damage circumscribed, its repercussion on enteric neurons may be long-lasting leading to significant changes in intestinal function, which can be observed in remote regions from the inflammation site. The postinflammatory status is frequently accompanied by substantial increases in enteric motility [3].

Inflammation of the gastrointestinal (GI) tract causes marked changes in the release of purines leading to subsequent adaptive modifications of purinoceptors expression and/or function (reviewed in [4]). The underlying mechanisms of disturbed purinergic modulation are not completely understood, in part because the study of purinoceptors may be hampered by the presence of distinct nucleotide release sites and cell surface enzymes that rapidly break down extracellular nucleotides into nucleosides [5]. In healthy individuals, ATP is released predominantly from stimulated enteric neurons [6], but its release from nonneuronal cells (e.g., smooth muscle fibres, interstitial cells of Cajal) might also occur [7]. Four members of

the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, namely, NTPDase1, NTPDase2, NTPDase3, and NTPDase8 and two members of the ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPP) family, NPP1 and NPP3, are located at the plasma membrane and hydrolyse extracellular nucleotides [5, 8, 9]. The relative contribution of distinct ecto-enzymes to the modulation of purinergic signalling depends not only on differential tissue and cell distribution, regulation of expression, and targeting to specific membrane domains but also on substrate preference and availability.

Regarding the substrate preference, NTPDase1 (CD39 or apyrase) dephosphorylates ATP directly into AMP, with minimal accumulation of ADP. NTPDase2 (ATPase) is a preferential nucleoside triphosphatase that hydrolises ADP 10 to 15 times less efficiently than ATP, leading to minimal AMP accumulation [8]. NTPDase3 and NTPDase8 are functional intermediates between NTPDase1 and NTPDase2 [8]. Because of their involvement in physiological processes, namely, blood clotting, vascular inflammation, immune reactions, and certain types of cancer, NTPDases are now considered as potential drug targets [10]. As for NPP1 and NPP3, they release nucleoside 5′-monophosphate from a variety of nucleotides, but intriguingly, their phosphorylated product (e.g., AMP) binds to NPPs with a higher affinity than substrates do and thus inhibits catalysis [9]. Finally, AMP is hydrolysed to adenosine and inorganic phosphate by ecto-5′-nucleotidase (CD73), which is concentrated in the myenteric smooth muscle cell layer [7]. Interestingly, ecto-5′-nucleotidase (CD73) may be cleaved from cell membranes through hydrolysis of the GPI anchor by phosphatidylinositol-specific phospholipases or by proteolysis while retaining its catalytic activity in the soluble form [11].

At the myenteric neuromuscular synapse, ATP is primarily metabolized into AMP, which is then dephosphorylated into adenosine; alternative conversion of ATP into ADP is more relevant at high ATP concentrations [12]. Thus, gradients of ATP and its breakdown products (ADP and adenosine) may provide fine tuning control of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischemia, and inflammation, when extracellular ATP reaches high levels (see [13, 14]). ATP transiently facilitates acetylcholine (ACh) release from non-stimulated nerve terminals via prejunctional P2X (probably P2X2) receptors. Hydrolysis of ATP directly into AMP and subsequent formation of adenosine activates inhibitory A_1 receptors in smooth muscle fibers [15] and in ganglionic cell bodies of myenteric neurons [16], where it downmodulates the release of excitatory neurotransmitters, such as substance P [17] and ACh [18]. Besides the well characterized inhibitory A_1 receptors, myenteric nerve terminals are endowed with prejunctional A_{2A} receptors mediating facilitation of ACh release [16, 18, 19].

Previous findings from our group demonstrated that the ectonucleotidase pathway contributes only partially to the total interstitial adenosine concentration in the myenteric plexus [19]. Adenosine released as such from either neuronal or nonneuronal cells seems to be the main source of endogenous adenosine in the enteric nervous system [18]. This release is sought to be mediated by facilitated diffusion via nucleoside transporters, which can be regulated by endogenous signaling molecules, such as ACh via muscarinic M_3 receptors [16, 19]. On the other hand, we demonstrated that the extracellular deamination of adenosine by adenosine deaminase (ADA) represents the most effective mechanism regulating synaptic adenosine levels in the myenteric plexus-longitudinal muscle (LM-MP) of the ileum from healthy animals [18, 19]. ADA is a ubiquitous purine metabolic enzyme localized on the cell surface (ecto-ADA) [20]. Upon proteolytic cleavage from cell membranes, soluble forms of ADA (exo-ADA) retain their catalytic activity and may be found in interstitial fluids [21]. We detected a relatively high ADA activity (~0.6 U/mL) in superfusates collected after stimulating LM-MP preparations, which is in keeping with the hypothesis that "ADA secretion" may restrict endogenous adenosine actions to the synaptic region near the release/production sites [19]. ADA secretion increases in various diseases and may be originated from monocyte/macrophage lineages, thus reflecting the involvement of the cellular immune system. de Man and colleagues reported that chronic intestinal inflammation enhanced the enteric contractile activity in part due to an impairment of purinergic modulation of cholinergic nerve activity [14].

Because, adenine nucleotides and nucleosides (and ADA itself) may be released from activated inflammatory cells [22], as well as from neighbouring neuronal and nonneuronal enteric cells [23, 24], there is an increasing interest in the neuromodulatory effects exerted by adenosine during inflammatory insults. The therapeutic potential of adenosine-related compounds for controlling intestinal motility and inflammation [14, 25] prompted us to investigate the kinetics of adenosine formation/inactivation cycle in order to understand the overall homeostatic role of the nucleoside on enteric excitability in postinflammatory ileitis caused by intraluminal instillation of TNBS in the rat.

2. Material and Methods

2.1. Animals. Rats of either sex (Wistar, ~200 g) (Charles River, Barcelona, Spain) were kept at a constant temperature (21°) and a regular light- (06.30–19.30 h) dark (19.30–06.30 h) cycle, with food and water) and a regular light- (06.30–19.30 h) dark (19.30–06.30 h) cycle, with food and water *ad libitum*. All studies involving animal experiments are reported in agreement with ARRIVE guidelines. Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC).

2.2. TNBS-Induced Intestinal Inflammation Model. Intestinal inflammation was produced by the instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the lumen of the rat ileum. After a fasting period of 4–8 hours with free access to drinking water, rats undergo median laparotomy under

anaesthesia with an association of medetomidine (10 mg/Kg) and ketamine (75 mg/Kg) given subcutaneously. At the end of the procedure animals were retrieved with atipamezole (10 mg/Kg). The terminal ileal loop was gently exteriorized, and TNBS (40 mM) was injected through the enteric wall into the lumen of the ileum, 10 centimeters proximal to the ileocolonic junction. Controls received 1 mL of 0.9% saline. Sixty minutes after surgery, the rats were allowed to eat and drink *ad libitum*. After surgery, pain was controlled with tramadol hydrochloride (10 mg/Kg). To have a control of time course of body weight loss and recovery after injection of TNBS, rats were weighed prior to TNBS administration and daily following surgery. Animals with intestinal inflammation (TNBS) transiently lose weight for three to four days after surgery and regain weight thereafter. Seven days after surgery, animals were sacrificed following an overnight fasting period.

2.3. Histological Findings and Gastrointestinal Motility. The postinflammatory phase of TNBS-induced ileitis was characterized in haematoxylin-eosin stained sections using Pontell and Jergens criteria, which is based in the loss of normal tissue architecture, epithelial damage, and infiltration of inflammatory cells [3, 26–28]. Evaluation of gastrointestinal (GI) motility was performed *in vivo* by oral administration of a gavage containing the methylene blue dye. Dye progression in the GI tract was evaluated after 30 minutes, both in control and TNBS-injected animals: the total length of the small intestine was used to normalize data.

2.4. [^{3}H]-Acetylcholine Release Experiments. Sections of the rat ileum not including the terminal five centimetres were used. The longitudinal muscle strip with the myenteric plexus attached was separated from the underlying circular muscle according to the method of Paton and Vizi [29]. This preparation is highly enriched in cholinergic neurons, mainly excitatory neurons projecting to the longitudinal muscle (25%) that receive inputs from intrinsic primary afferents (26%) and from ascending and descending pathways (17%) [30].

The procedures used for labelling the preparations and measuring evoked [^{3}H]-Acetylcholine ([^{3}H]-ACh) release were previously described [12, 16, 18, 19] and used with minor modifications. LM-MP strips were mounted in 3 mL capacity vertical perfusion chambers heated at 37°C. After a 30 min equilibration period, myenteric neurons were labelled for 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi nmol^{-1}) under electrical field stimulation (1 Hz-frequency, 1 ms pulse width). Following loading, the washout Tyrode's solution contained hemicholinium-3 (10 μM) to prevent choline uptake. After a 60 min period of washout, bath samples (3 mL) were automatically collected every 3 min using a fraction collector (Gilson, FC203B, France). Tritium content of the samples was measured by liquid scintillation spectrometry (% counting efficiency: 40 ± 2%).

Test drugs were added 15 min before S_2. The change in the ratio between the evoked [^{3}H]-ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. None of the drugs changed significantly ($P > 0.05$) basal tritium outflow.

2.5. Kinetic Experiments of the Extracellular Catabolism of Purines and HPLC Analysis. For the kinetic experiments of the extracellular catabolism of adenine nucleotides and adenosine, strips from the longitudinal muscle with the myenteric plexus attached (LM-MP) were mounted in a 2 mL organ bath. All experiments were performed at 37°C. Preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, and glucose 11.2. After a 30 min equilibration period, the preparations were incubated for 45 min with Tyrode's solution to eliminate endogenous interfering compounds. Following a washout period of 10 min, the preparations were then incubated with 30 μM of ATP, ADP, AMP, or adenosine (zero time). Samples of 75 μL were collected from the organ bath at different times up to 45 min for HPLC (with UV detection) analysis of the variation of substrate disappearance and product formation ([12, 18, 19]; see also [31]). The concentrations of the substrate and products were plotted as a function of time (progress curves). In some experiments, we measured unbound 5′-nucleotidase and adenosine deaminase activities in the fluid retained in the bath after removing the LM-MP preparation. We followed a similar experimental protocol to that used for studying the kinetics of the extracellular catabolism of adenine nucleotides and adenosine in the presence of the preparations.

2.6. Release of ATP and Adenine Nucleosides (Adenosine Plus Inosine). Experiments were performed using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection) and bioluminescence analysis. After the 30 min equilibration period, the preparations were incubated with 1.8 mL gassed Tyrode's solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. The preparations were electrically stimulated once, 15 min after starting sample collection (zero time), using 3000 square wave pulses of 1 ms duration delivered at a 5 Hz frequency. In these experiments, only the sample collected before stimulus application and the two samples collected after stimulation were retained for analysis. Bath aliquots (50–250 μL) were frozen in liquid nitrogen immediately after collection, stored at −20°C (the enzymes are stable for at least 4 weeks), and analysed within 1 week of collection by HPLC with diode array detection (Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 μM, 2.1 mm × 150 mm) equipped with a guard column (5 μm, 2.1 mm × 1 mm) using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 μL/min and the column temperature was

Table 1: Primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Species	Dilution	Supplier
Primary antibodies				
Adenosine receptor A_1	AB1587P	Rabbit	1 : 50	Chemicon
Adenosine receptor A_{2A}	A2aR21-A	Rabbit	1 : 150	Alpha Diagnostics
NTPDase1	rN1-$6_L I_4$	Rabbit	1 : 1000	http://ectonucleotidases-ab.com/
NTPDase2	rN2-6_L	Rabbit	1 : 400	http://ectonucleotidases-ab.com/
NTPDase3	rN3-$1_L I_5$	Rabbit	1 : 150	http://ectonucleotidases-ab.com/
NTPDase8	rN8-$8_C I_5$	Guinea-pig	1 : 600	http://ectonucleotidases-ab.com/
Ecto-5′-nucleotidase	rNu-$9_L I_5$	Rabbit	1 : 1000	http://ectonucleotidases-ab.com/
ADA	sc-25747	Rabbit	1 : 50	Santa Cruz
Secondary antibodies				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1 : 1000	Molecular probes
TRITC 568 anti-gp	706-025-148	Donkey	1 : 150	Molecular probes

maintained at 20°C. The autosampler was set at 4°C and 50 μL of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine. In parallel, the ATP content of the same samples was evaluated with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana) (see e.g., [31, 32]). Luminescence was determined using a multidetection microplate reader (Synergy HT, BioTek Instruments). Stimulation-evoked release of adenine nucleotides and nucleosides was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenine nucleotides and nucleosides determined after stimulus application.

2.7. Immunofluorescence Staining and Confocal Microscopy Observation. LM-MP fragments were isolated from the rat ileum as previously described. The LM-MP fragments were stretched to all directions and pinned onto Petri dishes coated with Sylgard. The tissues, then, were fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4°C. Following fixation, the preparations were washed three times for 10 min each using 0.1 M phosphate buffer. At the end of the washout period, tissues were cryoprotected during 16 h with a solution containing anhydrous glycerol 20% and phosphate buffer 0.1 M at 4°C and, then, stored at −20°C for further processing. Once defrosted, tissue fragments were washed with tamponated phosphate saline buffer (PBS) and incubated with a blocking buffer, consisting in foetal bovine serum 10%, bovine serum albumin 1%, triton X-100 0.3% in PBS, for 2 h; washout was facilitated by constant stirring of the samples. After blocking and permeabilization, samples were incubated with selected primary antibodies (see Table 1) diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4°C, for 48 h. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS supplemented with triton X 0.3% (3 cycles of 10 min), tissue samples were incubated with species-specific secondary antibodies in the dark for two hours, at room temperature. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield as mounting media (VectorLabs) and stored at 4°C. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

2.8. Antibody Production. All primary antibodies used in this study have previously been validated [33–37]: rabbit rN1-6L to rat NTPDase 1, rabbit rN2-6L to rat NTPDase2, rabbit rN3-1L to rat NTPDase3, guinea pig rN8-8C to rat NTPDase8, rabbit rNU-9L, and rabbit rNU-4L to rat ecto-5′-nucleotidase/CD73. Genetic immunization protocol was carried out with plasmids (pcDNA3.1) encoding each protein, in New Zealand rabbits for antibodies against rat NTPDase1, rat NTPDase2, rat NTPDase3, rat ecto-5′-nucleotidase, and Hartley guinea pigs for rat NTPDase8 antibodies. All procedures were approved by the Canadian Council on Animal Care and the Université Laval Animal Welfare Committee.

2.9. Materials and Solutions. Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine, inosine, hypoxanthine; 2,4,6-trinitrobenzenesulphonic acid (TNBS); choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol, fetal bovine serum (Sigma, St Louis, MO, USA); serum albumin, triton X-100, metanol, potassium dihydrogen phosphate (KH_2PO_4) (Merck, Darmstadt, Germany); dipyridamole (Boehringer Ingelheim, Germany); 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemicals, Natick, MA, USA); mibefradil dihydrochloride, tetrodotoxin (TTX), 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) (Tocris Cookson Inc., UK); [methyl-^{3}H]-choline chloride (ethanol solution, 80 Ci mmol^{-1}) (Amersham, UK); PicA reagent (Waters corporation, Milford, USA); ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana); medetomidine hydrochloride (Domitor, Pfizer Animal

Health); atipamezole hydrochloride (Antisedan, Orion, Espoo, Finland); ketamine hydrochloride (Imalgene, Merial, Lyon, France); Sodium chloride 0.9%, tramadol hydrochloride (Labesfal, Santiago de Besteiros, Portugal).

All drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed. The pH of the perfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

2.10. Presentation of Data and Statistical Analysis. The values are expressed as mean ± SEM, with n indicating the number of animals used for a particular set of experiments. Statistical analysis of data was carried out using paired or unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's modified t-test. $P < 0.05$ represents significant differences.

3. Results

3.1. Postinflammatory Ileitis Led to Increases in Gastrointestinal Transit in the Rat. Histology sections of the ileum of TNBS-injected rats were characterized using the criteria proposed by Pontell et al. [3] and Jergens [26] (see Section 2). One week after the TNBS treatment we observed a proliferative regeneration of the mucosal integrity. Postinflammatory infiltrates consisting mainly of eosinophils, lymphocytes, and macrophages were scarcely seen but when present extended to the submucosa, enteric plexuses, and muscular layers. At that time, the total ileal wall thickness of TNBS-treated rats was increased due to increase in thickness of both mucosal and muscular layers. The gastrointestinal motility was evaluated prior to sacrifice of the animals. This was done by measuring the progression of methylene blue dye gavage during 30 min. Figure 1 shows that methylene blue dye progression was significantly ($P < 0.05$) faster in TNBS-injected animals than in control animals, indicating that GI propulsion is increased in postinflammatory ileitis. Although TNBS-ileitis in the rat lacks a typical chronic phase, the postinflammatory phase is characterized by increases in motility (see e.g. [38]).

3.2. Adenosine Neuromodulation Is Impaired in Postinflammation Ileitis. Previous reports suggested that the purinergic control of cholinergic nerve activity may be significantly impaired during chronic intestinal inflammation, both in the colon of rabbits and guinea-pigs [39, 40], and in mice ileum [14]. Despite the differences in the mechanisms used to produce intestinal inflammation among these groups, all are unanimous on the indication for the need of more studies in order to investigate the underlying pathways responsible for disturbed purinergic neuromodulation. Hence, we focused our attention on the net tonic action of endogenous adenosine on electrically evoked [^{3}H]-ACh release from

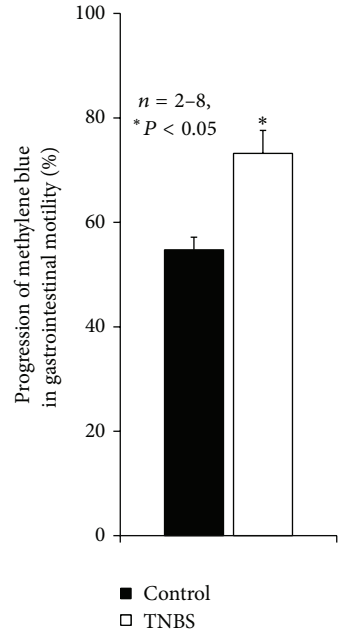


Figure 1: Progression of methylene blue dye gavage along the gastrointestinal tract during 30 minutes in control and TNBS-injected animals. The total length of the small intestine was used to normalize data. The vertical bars represent SEM. $^*P < 0.05$ (unpaired Student's t-test) represents significant differences as compared to the control situation.

myenteric motoneurons seven days after an inflammatory insult to the rat ileum. In preparations from healthy rats, the A_1 receptor antagonist, DPCPX (10 nM) increased the release of [^{3}H]-ACh by 27 ± 4% ($n = 4$) (Figure 2(a)). Conversely, selective blockade of adenosine A_{2A} receptors with ZM 241385 (50 nM) significantly decreased the evoked tritium outflow by 37 ± 10% ($n = 6$). The results indicate that endogenous adenosine exerts a dual role on evoked [^{3}H]-ACh release via the activation of inhibitory A_1 and excitatory A_{2A} receptors in the rat ileum (cf. [18, 19]). However, when similar experiments were conducted in the LM-MP seven days after ileal inflammation none of the adenosine receptor antagonists caused any measurable change on evoked transmitter release (Figure 2(a)). These results indicate that adenosine modulation of cholinergic nerve activity is severely impaired in postinflammation ileitis, as previously suggested using both myographic recordings and electrophysiology methods (see above).

Several authors hypothesized that purinoceptors down-regulation in the enteric nervous system can occur during prolonged contact with purines. This was predicted since purines, such as ATP and adenosine, can be released from activated inflammatory cells [22] in the vicinity of myenteric neurons [24]. To elucidate this contention, we performed immunolocalization studies by confocal microscopy to assess changes in the expression and localization of both A_1 and A_{2A} adenosine receptors that could contribute to explaining the lack of endogenous adenosine neuromodulatory tonus in the inflamed ileum. The immunoreactivity against A_1 receptors is located predominantly on cell bodies of myenteric neurons

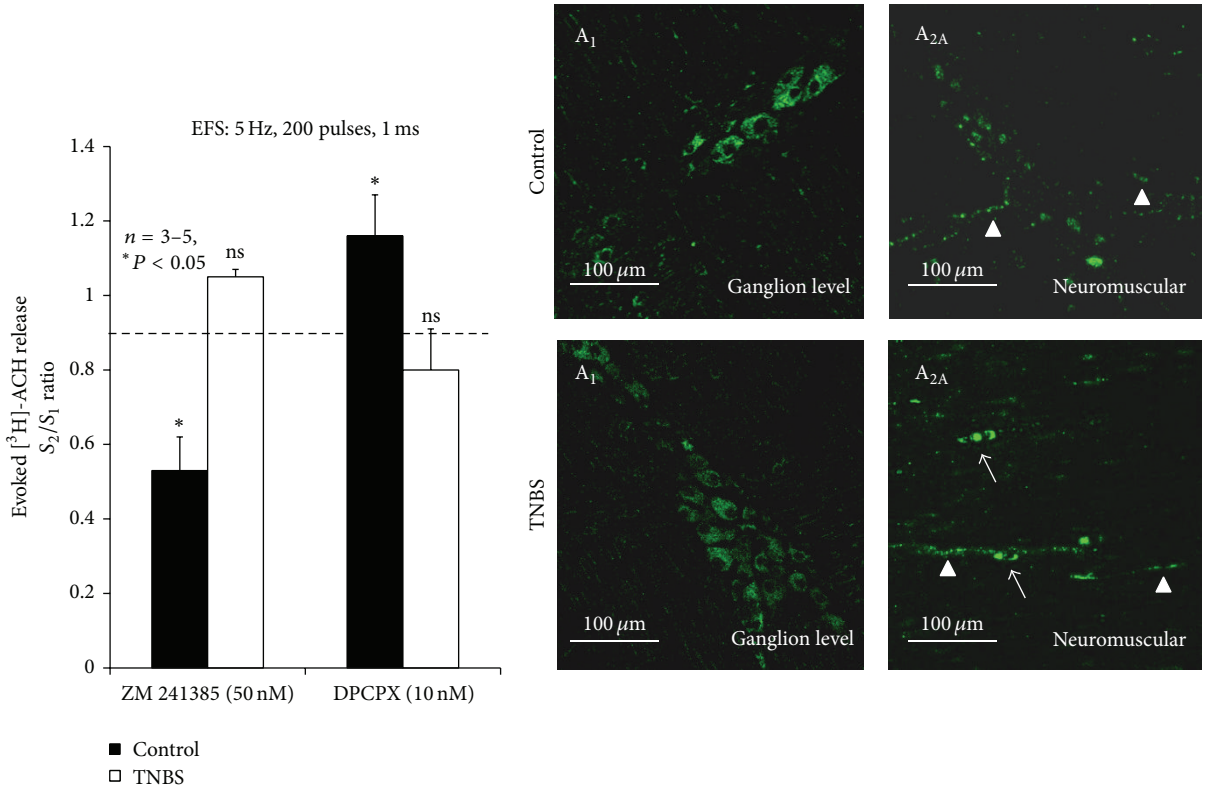


FIGURE 2: (a) Actions of selective adenosine A_1 and A_{2A} receptor antagonists on [^{3}H]-ACh release from myenteric motoneurons stimulated electrically (5 Hz, 200 pulses, 1 ms duration). DPCPX (10 nM, A_1 receptor antagonist) and ZM 241385 (50 nM, A_{2A} receptor antagonist) were added to the incubation media before the second period of stimulation (S_2) and were present throughout the assay. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained in control conditions, that is, with no drugs added. The data are means ± SEM of three to five individual experiments. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represent significant differences when compared to the situation where no drugs were added (dashed line). (b) Confocal images of whole-mount preparations of longitudinal muscle-myenteric plexus preparations of the ileum from control and TNBS-injected rats. Adenosine A_1 receptor immunoreactivity is predominantly present in ganglion neuronal cell bodies in control and TNBS-injected preparations. Adenosine A_{2A} receptor imunoreactivity is mainly present in nerve bundles and axon terminals of myenteric neurons (triangles). In inflamed preparations, adenosine A_{2A} receptor staining is also observed in few mononuclear cells infiltrating the neuromuscular layer (arrows).

of the ileum in control as well as in TNBS-injected rats (Figure 2(b)). The longitudinal muscle fibers also stain positively with the adenosine A_1 receptor antibody (data not shown). Yet, no significant differences were observed between control and inflamed preparations (Figure 2(b)). Likewise, the A_{2A} receptor immunoreactivity, which is more evident on myenteric nerve terminals, also did not significantly differ among control and inflamed tissues (Figure 2(b)). Interestingly, we observed staining against the A_{2A} receptor in few mononuclear cells (probably lymphocytes) infiltrating the neuromuscular level, which might be responsible for the immunosuppressant effect of A_{2A} receptors activation in experimental ileitis [41, 42]. Mononuclear infiltrates containing A_{2A}-positive immune cells may contribute to the increased mRNA expression of this receptor found in chronic inflamed intestinal tissues [43]. Although we cannot exclude at this stage differences in the intracellular signaling pathway triggered by activation of the two high affinity adenosine receptor subtypes, A_1 and A_{2A}, in the LM-MP of the rat ileum, our data suggest that receptors expression is fairly conserved in the ileum seven days following an inflammatory insult and, apparently, does not underscore the lack of adenosine neuromodulation tonus in this condition (see, e.g., [14, 43, 44]).

3.3. Postinflammatory Ileum Releases More ATP, Which Is Not Followed by Adenosine Formation. It has been hypothesized, but not yet proven, that disruption in ATP release and/or breakdown are most likely explanations for the suppression of purinergic neuromuscular transmission in inflamed regions of the distal colon [40]. It is possible that inflammatory mediators affect mitochondrial function, and therefore ATP synthesis, in the ulcerated region of the chronic inflamed intestine. Another possibility is that there may be increased expression of ectonucleotidases, which are responsible for the breakdown of ATP [12, 45], as has been demonstrated in the purinergic sympathetic regulation of submucosal blood vessels [45]. Here, we measured the extracellular

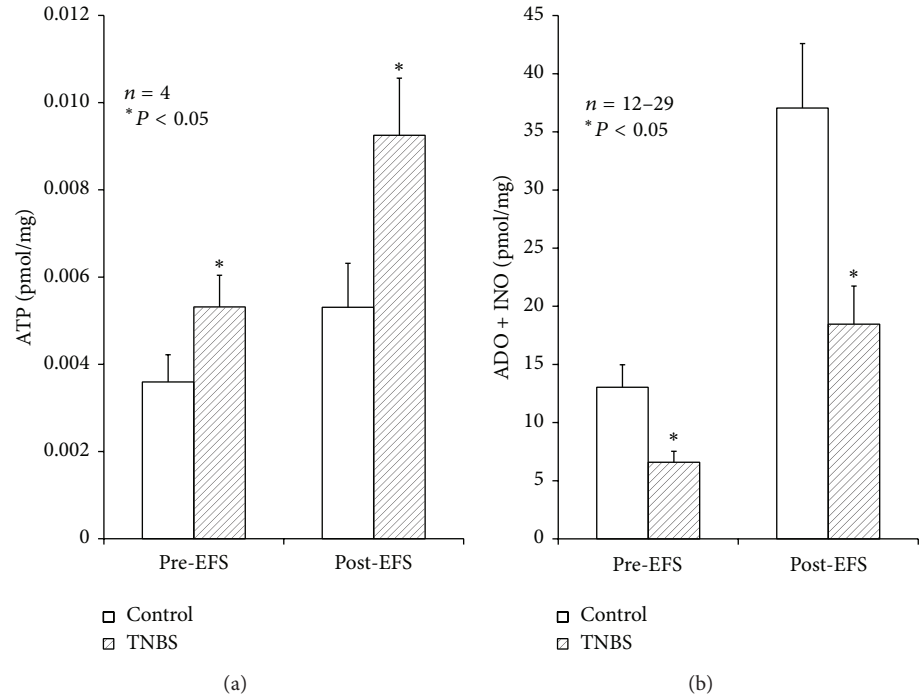


Figure 3: Effect of electrical field stimulation (EFS) on extracellular accumulation of purines in the LM-MP of control and inflamed rat ileum. The preparations were incubated with Tyrode's solution for 15 min and then subject to EFS (5 Hz frequency, 3000 pulses of 1 ms duration). Samples collected from the incubation media were analysed by HPLC to separate and quantify purine nucleosides; ATP outflow was assessed using the luciferin-luciferase bioluminescence assay. Illustrated are ATP (a) and purine nucleosides (b) content of bath samples collected immediately before (Pre-EFS) and after (Post-EFS) LM-MP stimulation expressed in pmol/mg of tissue. Data are means ± SEM of an n number of individual experiments. $^{*}P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represent significant differences as compared to control animals.

accumulation of ATP (by bioluminescence) in parallel with adenosine plus inosine content (by HPLC with diode array detection) in samples collected immediately before and after electrical stimulation of LM-MP preparations of the ileum of both control and TNBS-injected rats. Figure 3 shows that, in healthy animals, stimulation of the LM-MP at a frequency of 5 Hz (3000 pulses of 1 ms duration) yields increased amounts of ATP in the incubation fluid, which was followed by even higher extracellular accumulation of adenine nucleosides, consisting mostly of adenosine and inosine. This pattern was totally reversed seven days after inflammation of the rat ileum. Basal levels of ATP significantly ($P < 0.05$) increase (Figure 3(a)), whereas the baseline of endogenous adenosine plus inosine decreased (Figure 3(b)), in the fluid collected from TNBS-treated LM-MP of the rat ileum as compared to control tissues. Although the amount of ATP and adenine nucleosides increased from baseline following electric stimulation of LM-MP preparations in both animal groups, released ATP levels reached higher values in inflamed preparations than in controls (Figure 3(a)), but the opposite was observed regarding the content of adenosine plus inosine (Figure 3(b)). These results indicate that, in contrast to previous hypothesis, the amount of ATP released in basal conditions and following electric stimulation of TNBS-treated ileal preparations was significantly higher than in control tissues, although one could not discard at this stage that ATP accumulation could also result from a decrease in the extracellular breakdown of the nucleotide (see below). Despite this increase in ATP accumulation, the content of adenosine plus inosine in the superfusates from TNBS-treated animals was severely decreased, which might contribute to explaining the lack of endogenous adenosine neuromodulatory tonus in postinflammatory ileitis (Figure 2; see also [43]).

In healthy tissue, but not in the ileum following the inflammatory insult, stimulus-evoked adenosine release was partially dependent on neuronal activity. This was evidenced because pretreatment of the preparations with tetrodotoxin (TTX), applied in a concentration (1 μM) that essentially abolished evoked [^{3}H]-ACh release, reduced by about 50% adenine nucleosides outflow from control tissues with almost no effect in TNBS-treated preparations (Figure 4). These findings indicate that barely half of the extracellular adenosine released from stimulated LM-MP preparations from healthy rats comes from TTX-sensitive neuronal cells, but this source may be severely affected in the postinflammation phase of TNBS-ileitis due to neuronal dysfunction, as demonstrated by several authors. Under conditions leading to myenteric neuronal dysfunction such as chronic inflammation, ATP release may be shifted from a neuronal origin towards other cell sources (e.g., smooth muscle fibers, glial cells, and interstitial cells of Cajal, infiltrating immune cells)

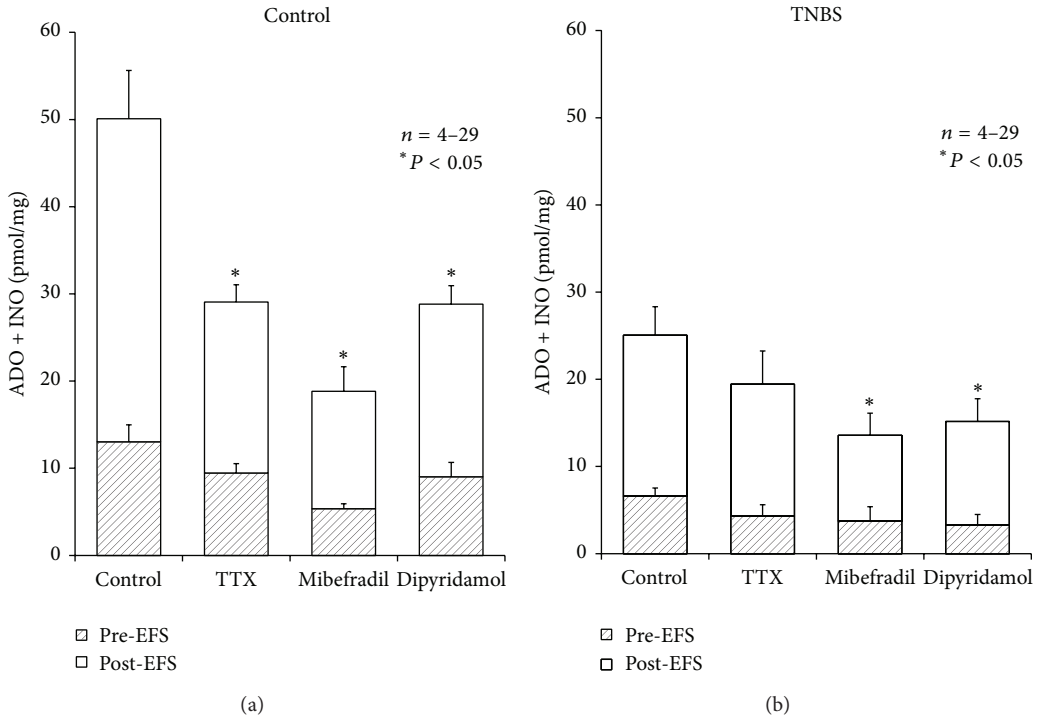


Figure 4: Influence of tetrodotoxin (TTX, 1 μM), mibefradil (3 μM), and dipyridamole (0.5 μM) on stimulation-evoked release of purine nucleosides (adenosine plus inosine) from the LM-MP of control and TNBS-injected rat ileum. Drugs were in contact with the preparations for at least 15 min before stimulus application (5 Hz frequency, 3000 pulses of 1 ms duration). Samples collected from the incubation media were analysed by HPLC with diode array detection. Illustrated is the purine nucleosides content of bath samples collected immediately before (Pre-EFS) and after (Post-EFS) stimulation of LM-MP expressed in pmol/mg of tissue. Data represent the means ± SEM of an n number of individual experiments. $^*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represents significant differences from the evoked amount of purine nucleosides detected in control conditions where no drugs were added to the preparations.

(see, e.g., [7]). As a matter of fact, evoked ATP release from TTX-resistant nonneuronal sources increased ($P < 0.05$) above baseline from 7.4 ± 0.6 fmol/mg ($n = 4$) in control animals to 13.2 ± 0.2 fmol/mg ($n = 4$) in TNBS-treated preparations.

3.4. Smaller Amounts of Adenosine Released from Inflamed Ileum Originate from Activated Pacemaker Interstitial Cells of Cajal via Equilibrative Nucleoside Transporters. Considering that adenosine may also be released as such from nonneuronal cells at the tripartite myenteric synapse, we tested whether stimulus-evoked smooth muscle contraction and activation of pacemaker interstitial cells of Cajal (ICCs) affected the outflow of adenine nucleosides from the ileum of control and TNBS-injected rats. Blockade of smooth muscle contractions with the Ca_v1 (L-type) voltage-sensitive calcium channel inhibitor, nifedipine (1–5 μM), did not affect the accumulation of adenine nucleosides in bath samples collected immediately before and after electric field stimulation to the LM-MP from healthy animals [19]. However, selective blockade of Ca_v3 (T-type) calcium channels located predominantly in ICCs with mibefradil (3 μM) significantly ($P < 0.05$) depressed the outflow of adenine nucleosides from both control and TNBS-treated preparations (Figure 4). The results indicate that ICCs are the main source of adenosine released from stimulated LM-MP of the ileum in the postinflammation phase. Data also indicate that ICCs cooperate with myenteric neurons to increase extracellular adenosine in preparations from healthy rats.

Given that adenosine is neither stored nor released as a classical neurotransmitter and previous findings from our laboratory demonstrated that the ectonucleotidase pathway contributes only partially to the total interstitial adenosine concentration in the myenteric plexus [19], we tested the involvement of equilibrative nucleoside transporters to extracellular adenosine released from tissues isolated from control and TNBS-injected rats. The nucleoside transport inhibitor, dipyridamole (0.5 μM), decreased proportionally, and by a similar magnitude to that obtained with mibefradil (3 μM) the outflow of adenine nucleosides from both control and inflamed tissues (Figure 4). Thus, it is likely that extracellular adenosine detected in ileum following an inflammatory insult originates predominantly from activated interstitial cells of Cajal via equilibrative nucleoside transporters.

Another potential source of endogenous adenosine in the rat ileum may be adenosine 3′,5′-cyclic monophosphate (cAMP) extruded from activated cells, which may be converted to AMP and then to adenosine by ecto-phosphodiesterase and ecto-5′-nucleotidase/CD73, respectively [46]. Despite the existence of a functional extracellular cAMP-adenosine pathway in the rat ileum, we were unable to detect any measurable amounts of cAMP by HPLC

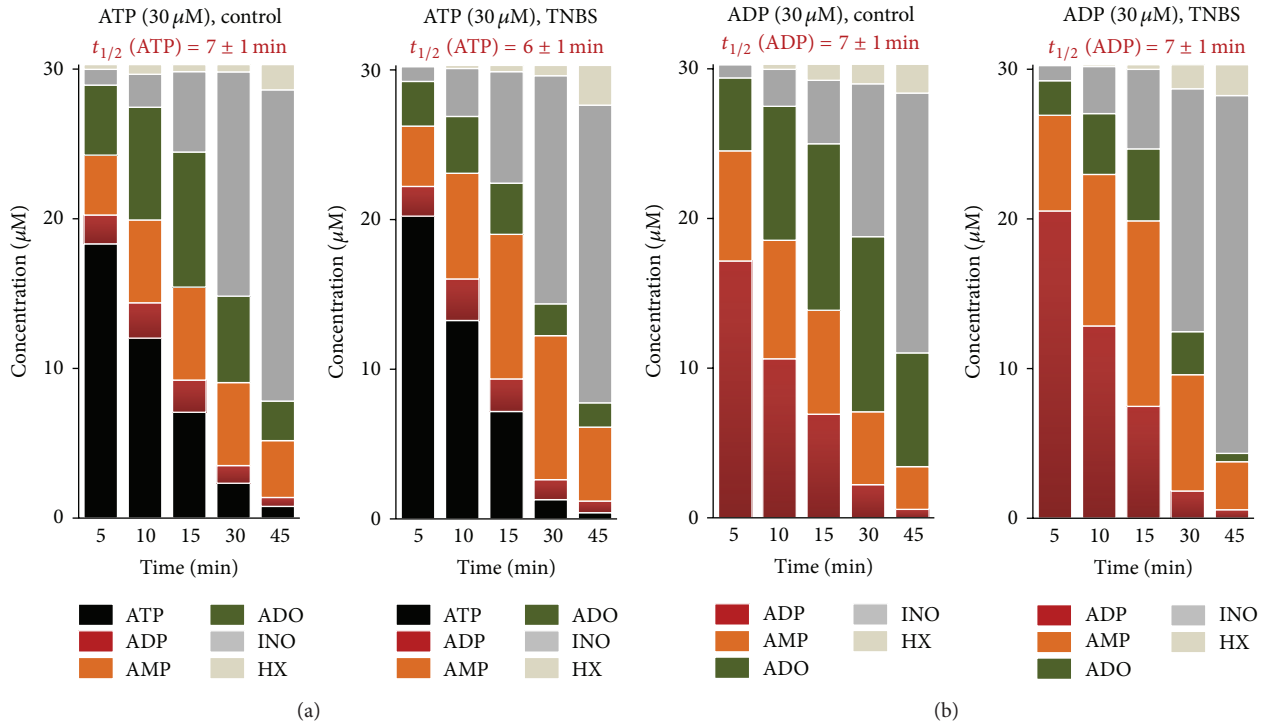


Figure 5: Kinetics of the extracellular catabolism of ATP (a) and ADP (b) in the LM-MP of the ileum of control and TNBS-injected rats. ATP and ADP (30 μM) were incubated at zero time (see Section 2). Collected samples were analysed by HPLC with UV detection to separate and quantify ATP (black), ADP (red), AMP (orange), ADO (green), INO (dark grey), and HX (light grey). Average results obtained in four experiments.

analysis (with diode array detection) in the samples used to quantify adenine nucleosides, even though we could identify a chromatographic peak corresponding to cAMP spectrum but with a higher retention time than adenosine using a 30-μM external standard (data not shown). These results suggest that, under the present experimental conditions, the cAMP-adenosine pathway does not account significantly for endogenous adenosine formation in the LM-MP of the rat ileum.

3.5. The Kinetics of the Extracellular Catabolism of Adenine Nucleotides (ATP and ADP) Was Not Different in Control and Postinflammation Preparations of the Rat Ileum. Despite the observation that the ileum in the postinflammation phase releases more ATP than control preparations, we observed a decrease in the extracellular adenosine content in samples collected both under basal and stimulated conditions. It is, therefore, possible that disruption of ATP catabolism into adenosine via ecto-NTPDases may occur in the inflamed LM-MP of the ileum. Figure 5 illustrates the time course of the extracellular catabolism of ATP and ADP in the LM-MP of the rat ileum from control and TNBS-treated rats. No significant differences ($P > 0.05$) were observed among the half-degradation times of ATP (30 μM) and ADP (30 μM) in control and TNBS-treated preparations, that is, the half degradation time of the two adenine nucleotides ranged from 6 to 7 min independently of the experimental condition. Extracellular ATP (30 μM) was metabolized into ADP, AMP, adenosine, inosine, and hypoxanthine, whose concentrations increased with time. Higher amounts of AMP as compared to ADP were detected in the bath at all time points following ATP (30 μM) application (Figure 5(a)). ADP (30 μM) catabolism led to AMP, adenosine, inosine, and hypoxanthine (Figure 5(b)). Interestingly, we detected differences in the accumulation of AMP and its degradation product, adenosine, between control and postinflamed preparations incubated either with ATP (30 μM) or ADP (30 μM). The extracellular AMP content increased (orange bars), whereas adenosine decreased (green bars), proportionally in the incubation fluid of TNBS-treated preparations as compared to those isolated from control rats following incubation with either ATP (30 μM) or ADP (30 μM) (Figure 5). Increases in AMP content of bath samples from postinflammation ileum reached the control levels 45 min after starting incubation with either ATP (30 μM) or ADP (30 μM), that is, when the concentration of adenine nucleotides reached minimum (Figure 5).

3.6. Feed-Forward Inhibition of Ecto-5′-Nucleotidase/CD73 by Adenine Nucleotides Controls Adenosine Formation in Postinflammation Ileitis. Data from Figure 5 clearly indicate that differences in adenosine formation from the extracellular catabolism of adenine nucleotides in postinflammation ileitis implicate AMP dephosphorylation by ecto-5′-nucleotidase, which is the rate limiting enzyme for adenosine formation in the rat myenteric plexus [12]. Surprisingly, we did not

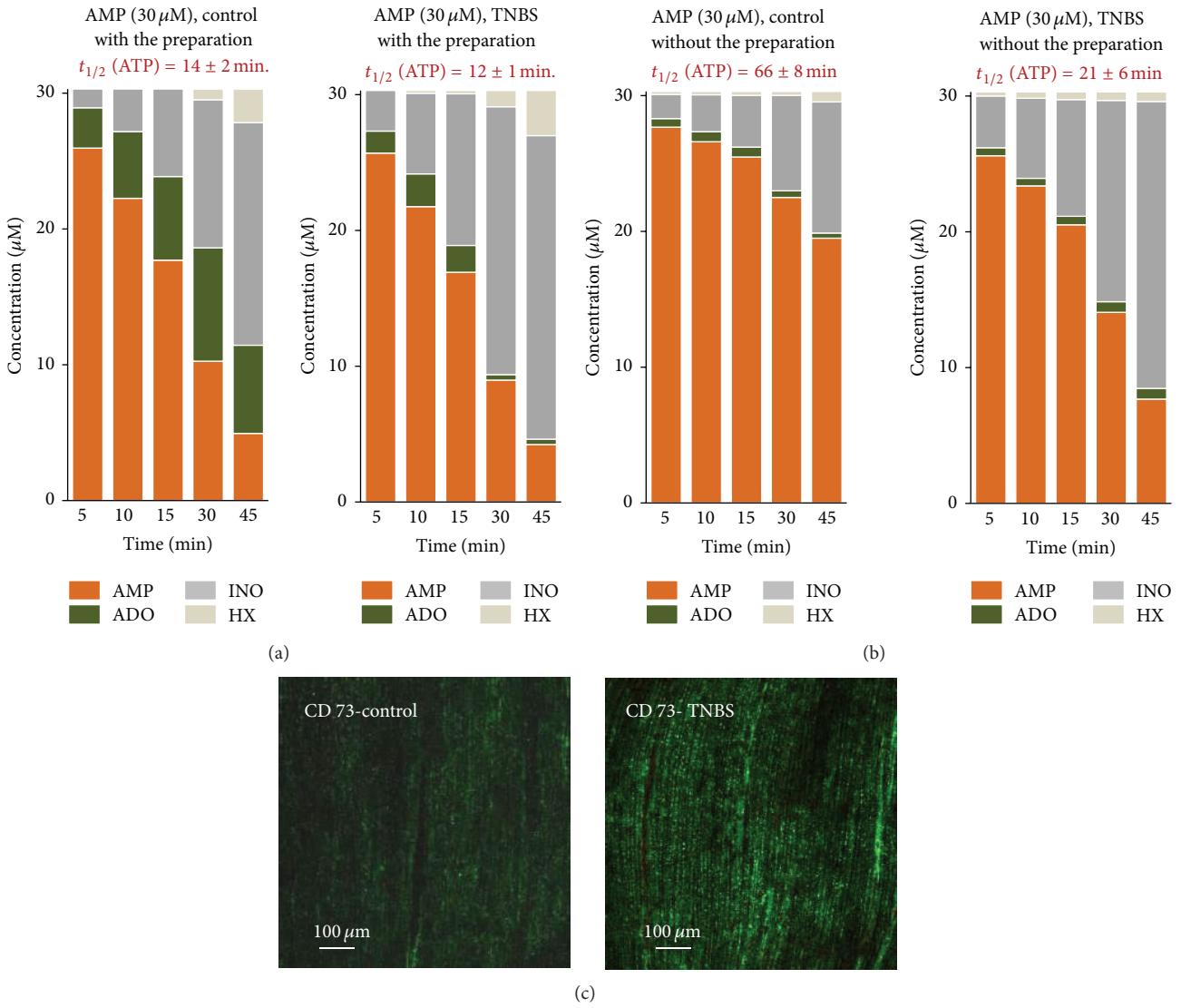


Figure 6: Kinetics of the extracellular catabolism AMP (30 μM) in the LM-MP of the ileum of control and TNBS-injected rats (a). The residual ecto-5′-nucleotidase/CD73 activity (soluble form) in the incubation fluid after removing the preparations from the organ bath, is also shown (b). AMP (30 μM) was incubated at zero time (see Section 2). Collected samples were analysed by HPLC with UV detection to separate and quantify AMP (orange), ADO (green), INO (dark grey) and HX (light grey). Average results obtained in four experiments. Panel (c) shows that immunoreactivity against ecto-5′-nucleotidase/CD73 in longitudinal smooth muscle fibers of the ileum from TNBS-treated rats is much more evident than in control animals.

observe significant changes ($P > 0.05$) in the half degradation time of AMP (30 μM) between control and TNBS-treated preparations (Figure 6(a)). If any difference has to be mentioned, is the fact that the inflamed ileum accumulated proportionally less adenosine in the extracellular milieu as compared to control tissues when AMP (30 μM) was used as substrate (Figure 6(a)). Overall, the results indicate that adenosine is being rapidly converted into inosine by ADA in the extracellular space of both control and TNBS-treated preparations (see below), given that stoichiometry of AMP conversion into adenosine, inosine and hypoxanthine is kept unaltered ruling out a significant contribution of the nucleoside uptake system (compared to [18]).

Since ecto-5′-nucleotidase/CD73 may be cleaved from its membrane GPI anchor by proteolysis retaining its catalytic activity in the incubation fluid, we decided to test whether binding of this enzyme to the plasma membrane was somehow affected during chronic inflammation in order to explain the differences detected in adenosine formation from the hydrolysis of adenine nucleotides. To this end, we evaluated the 5′-nucleotidase activity in the fluid remaining in the bath after removing the preparation following a 45-min incubation period (Figure 6(b)). Under these experimental conditions, the half-life of AMP (30 μM) was significantly ($P < 0.05$) decreased in TNBS-treated preparations ($t_{1/2} = 21 \pm 6$ min, two-times slower than with the tissue present) as compared to controls ($t_{1/2} = 66 \pm 8$ min, almost five-times slower than with the tissue present). Data suggest that postinflammation ileitis causes an increase in unbound soluble forms of 5′-nucleotidase, which contribute to dephosphorylation

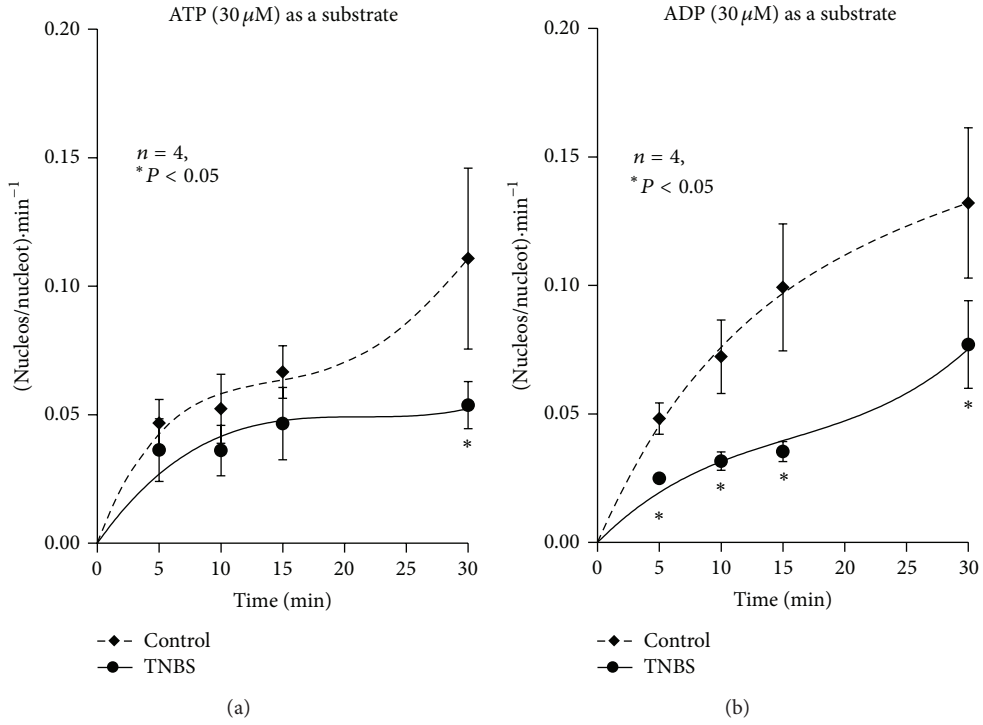


Figure 7: Activity of ecto-5′-nucleotidase/CD73 when ATP (a) and ADP (b) were used as substrates of the ectonucleotidase cascade in the LM-MP of the ileum of control and TNBS-injected rats. ATP and ADP (30 μM) were incubated at zero time. Ecto-5′-nucleotidase/CD73 activity was evaluated by quantifying the ratio [Nucleosides] : [Nucleotides]/min. Average results obtained in four experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. $^{*}P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represents significant differences as compared to control animals.

of extracellular AMP into adenosine without changing the global enzyme activity in the tissue. Notwithstanding, functional repercussions may be expected from this fact taking into consideration that ecto-5′-nucleotidase/CD73 is concentrated in the myenteric smooth muscle cell layer of healthy ileum [7], whereas unbound soluble forms of the enzyme may generate adenosine from extracellular AMP way from the most common site at the myenteric neuromuscular synapse in the inflamed ileum.

Given that we did not observe any significant differences in the AMP catabolism between control and TNBS-treated preparations (see Figure 6(a)) and we, still, detected increases in ecto-5′-nucleotidase/CD73 immunoreactivity in the longitudinal neuromuscular layer of the inflamed rat ileum (Figure 6(c); see also [43]), we hypothesized that ecto-5′-nucleotidase/CD73 could be feed-forwardly inhibited by high extracellular levels of ATP and/or ADP which bind to the catalytic site of the enzyme blunting adenosine formation from AMP in inflamed preparations (Figure 3; see also [47]). The ecto-5′-nucleotidase activity was evaluated by quantifying the ratio [Nucleosides] : [Total nucleotides] per min, which is a direct measure of the activity of ecto-5′-nucleotidase, using either ATP (30 μM) or ADP (30 μM) as substrates. Figure 7(a) shows that this ratio increased progressively with time upon consumption of ATP or ADP in the incubation media. The activity of ecto-5′nucleotidase/CD73 was significantly ($P < 0.05$) impaired in the LM-MP of the ileum following an inflammatory insult, particularly when ADP (30 μM) was used as substrate. These findings agree with data from Figure 5, showing that adenosine formation is delayed with a compensatory increase in AMP accumulation in the inflamed ileum as compared to control preparations which is compatible with our hypothesis that ATP and/or ADP feed-forwardly inhibit ecto-5′-nucleotidase/CD73 in postinflammation ileitis.

3.7. Tissue Distribution Changes of NTPDase2, NTPDase3, and NTPDase8 in Postinflammation Ileitis. The relative amounts of ATP and/or ADP near the ecto-5′-nucleotidase/CD73, which is concentrated in the myenteric smooth muscle layer ([7]; see also Figure 6(c)), are paramount to predict the magnitude of feed-forward inhibition of the enzyme by released adenine nucleotides. This may be determined by the expression and colocalization of NTPDases responsible for the kinetics of the catabolism of adenine nucleotides in control as well as in inflamed ileum. Figure 8 shows that in control LM-MP preparations the immunoreactivity against NTPDase2 is restricted to ganglion cell bodies and large ramifications (primary meshwork) of the myenteric plexus (compared to [48]). This contrasts with the localization of NTPDase3 immunoreactivity, which is also evident on myenteric nerve trunks and terminals corresponding to secondary and tertiary neuronal meshworks, respectively [49]. At this time, we cannot exclude the presence of this

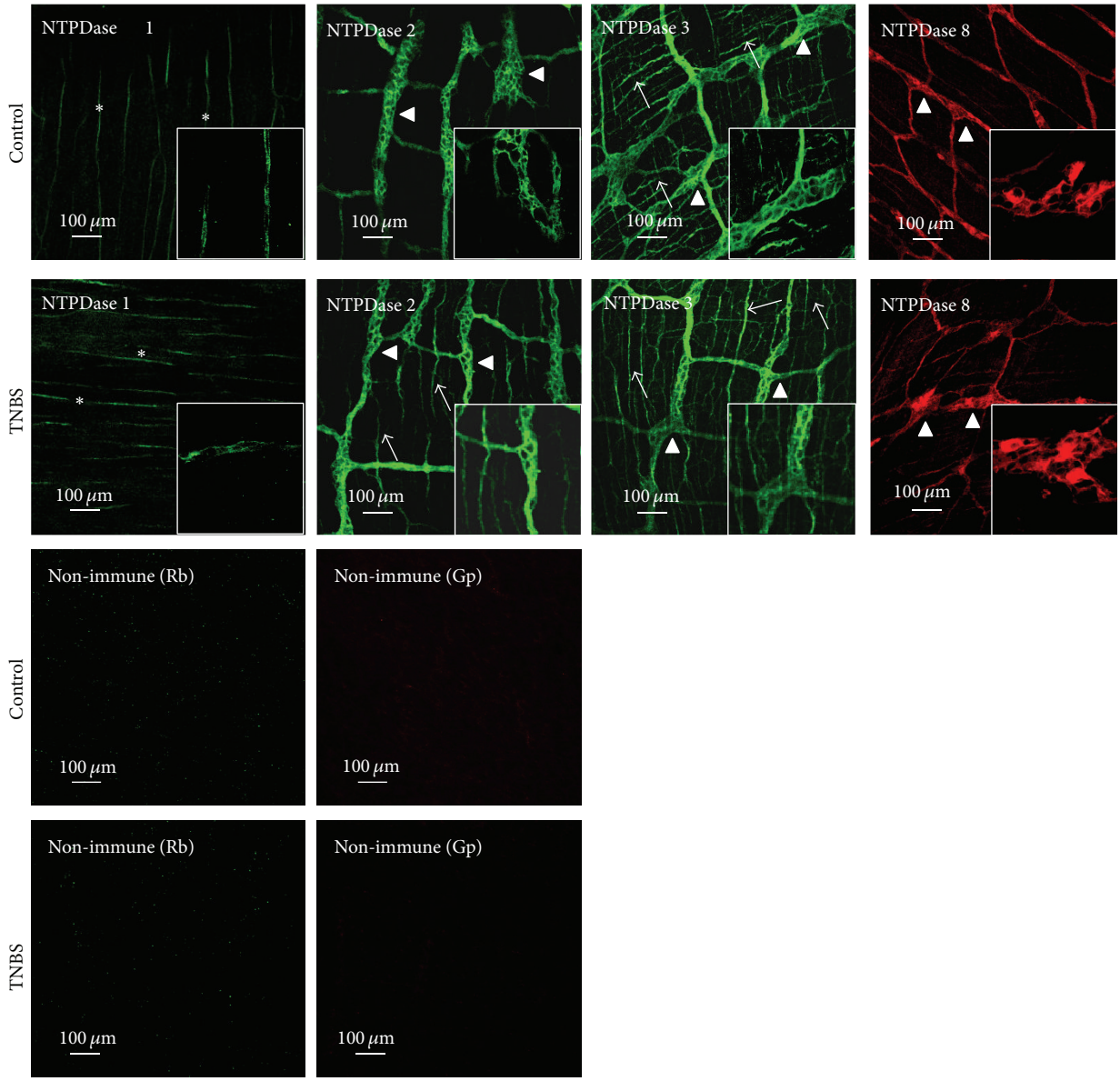


Figure 8: Localization of NTPDase1, NTPDase2, NTPDase3, and NTPDase8 immunoreactivity in single confocal images of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum of control and TNBS-injected rats. In healthy animals, NTPDase 1 immunoreactivity is present only in blood vessels (asterisks); NTPDase2 immunoreactivity is present predominantly in ganglion neuronal cell bodies (triangles) and large ramifications (primary meshwork) of the myenteric plexus, whereas NTPDase3 is also evident on myenteric axon terminals (arrows). In TNBS-treated preparations the NTPDase2 staining acquires a pattern that is very similar to that of NTPDase3, with NTPDase2 immunoreactivity also appearing in nerve bundles and axon terminals of myenteric neurons. NTPDase8 stains few ganglion neuronal cell bodies and large ramifications (primary meshwork) of the myenteric plexus; this pattern did not significantly change among control and TNBS-treated animals. No staining was obtained when nonimmune sera from host species (rabbit, Rb, and guinea-pig, Gp) were used instead of interest primary antibodies.

enzyme on intramuscular ICCs. This pattern changes in the postinflammation ileum. In TNBS-treated preparations, the NTPDase2 immunoreactivity is also observed in the secondary and tertiary neuronal meshwork, meaning that ADP formation from released ATP by stimulated enteric neurons [6] and smooth muscle fibres [7] may contribute to prolong feed-forward inhibition of muscle-bound ecto-5′-nucleotidase/CD73 at the myenteric neuromuscular synapse following an inflammatory insult. Apparently no significant differences were found in the expression and localization of NTPDase3 in the inflamed LM-MP of the rat ileum. Regarding NTPDase8, we were able to demonstrate its presence in few neuronal cell bodies of myenteric ganglia, with much lesser expression at the neuromuscular layer (Figure 8). Phenotypic characterization of NTPDase8 positive neuronal cell bodies deserves future investigations. Like NTPDase3, we found no gross changes in NTPDase8 immunoreactivity between control and TNBS-treated animals. We focused our

attention on these three ectoenzymes, because we detected no immunoreactivity against NTPDase1 in the LM-MP of the rat ileum besides few blood vessels (Figure 8).

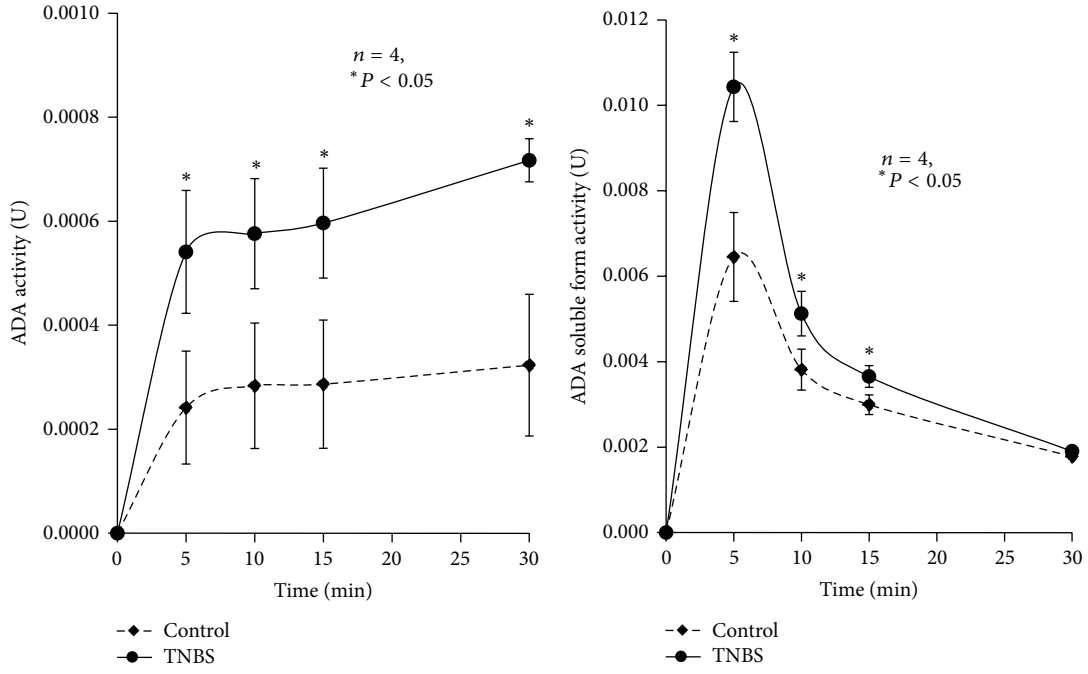
3.8. Postinflammation Ileitis Is Accompanied by Higher Adenosine Deaminase (ADA) Activity: Contribution of Membrane-Bound (ecto-ADA) and Soluble (exo-ADA) Forms of the Enzyme. The bath concentrations of adenosine (30 μM) decrease progressively with time yielding to the formation of inosine and hypoxanthine in the LM-MP of the rat ileum (see [19]). The extracellular catabolism of ADO (30 μM) was faster ($t_{1/2}$ = 24 ± 3 min, n = 4) in TNBS-treated than in control preparations ($t_{1/2}$ = 52 ± 10 min, n = 4). The rate of extracellular adenosine inactivation can be best appreciated by calculating adenosine deaminase (ADA) activity in the present experimental conditions, which is represented in Figure 9(a). Data indicate that the LM-MP of the ileum following an inflammatory insult has increasing amounts of ADA that we confirmed by immunofluorescence confocal microscopy (Figure 9(b)). ADA immunoreactivity was detected both at the ganglion level, as well as at the neuromuscular layer probably attached to smooth muscle fibers. In TNBS-treated preparations, ADA staining was also observed in mononuclear cells, most probably T lymphocytes and macrophages, infiltrating the myenteric plexus at the ganglion level [50–52]. Likewise, Antonioli and colleagues [53] demonstrated by Western blot analysis increases in the expression of ADA at the level of inflamed colonic tissues.

Like the ecto-5′-nucleotidase/CD73, extracellular ADA can be found attached to the plasma membrane (ecto-ADA) [20], as well as in soluble forms after proteolytic cleavage from cell membranes (exo-ADA) [21]. Both forms retain catalytic activity. Figure 9(a), also shows that the activity of the soluble form of ADA retained in the incubation fluid is highest during the first 5 min after removing the LM-MP preparation from the bath. Soluble ADA activity was not accompanied by any modification of lactate dehydrogenase activity, thus indicating that its activity is not due to damaged cells (see e.g., [19]). The soluble ADA activity was further increased in the incubation fluid which had been in contact for 45 min with preparations treated with TNBS. This implies that ADA is secreted in high amounts from the inflamed ileum, thus contributing to explaining the lack of adenosine neuromodulatory tonus. Even though inosine may accumulate in postinflammation ileitis as a result of increased extracellular adenosine deamination (see Figures 5 and 6), we failed to detect any variation on evoked [^{3}H]-ACh release when inosine was applied to the incubation fluid of control and TNBS-treated preparations in concentrations as high as 1 mM (data not shown). Catabolic degradation of adenosine by soluble ADA may also result in the impairment of immune modulation by endogenous adenosine (via A_{2A} receptors on mononuclear cells, see Figure 2(b)) and the consequent worsening of inflammation and tissue injury [53].

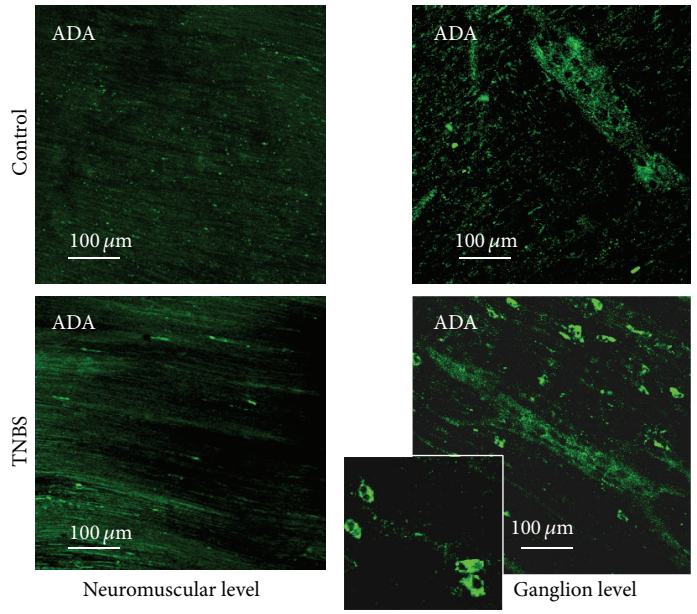
4. Discussion and Conclusions

Enteric plasticity comprises a wide range of structural and/or functional changes in enteric neurons, glial cells, and pacemaker interstitial cells of Cajal, which are located between the muscle layers of gastrointestinal tract (GI). Different types of pathophysiological conditions drive to distinct adaptive responses. Enteric neurons are known to control all gastrointestinal functions. They are able to rapidly change their structure, function, or chemical coding to maintain the gut homeostasis (e.g., during inflammatory disorders) [52]. Despite heterogeneity of enteric adaptive responses, involvement of ubiquitous purine nucleotides, and nucleosides provides fine tuning control of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischemia, and inflammation (reviewed in [20]). Numerous studies have described the potential role of purinergic function regulators in inflammation and immunity [44, 54–56]. There is recent evidence that blockade of adenosine kinase, which results in increased endogenous levels of adenosine, downregulates the inflammatory response in experimental colitis [57]. In addition, Mabley et al. [58] showed that inosine resulting from the breakdown of adenosine effectively reduced the inflammatory response in a murine model of colitis. Despite this, we showed in this study that inosine accumulation might not be sufficient to explain the purinergic neuromodulatory changes observed in postinflammation ileitis. These, and many other findings, suggest that adenosine can mediate both proinflammatory (via A_1 or A_{2B} receptors) and/or anti-inflammatory/immunosuppressant activities (mainly through A_{2A} and A_3 receptors) in inflammatory gut diseases, and they have potential for the treatment of these diseases (reviewed in [44]). However, much is unknown to what extent the immunemodulatory role of purines during inflammation interferes with neuromodulation of the enteric nervous system [14].

Control of cellular activity by purines depends on the activation of two families of membrane-bound purinoceptors, P1 and P2, which are sensitive to adenosine and to adenine and/or uracil nucleotides, respectively. Interestingly, P1 and P2 purinoceptors are also present on immune cells and there is evidence that adenosine and ATP are generated at sites of inflammation (see, for a review [59]). Purinoceptors activation is fine-tuned regulated by the extracellular conversion of released adenine and uracil nucleotides leading to the formation of other biologically active products, namely, ADP/UDP and adenosine, via a cascade of membrane-bound ectonucleotidases [8, 60]. Thus, cellular expression and topology of ectonucleotidase enzymes, as well as factors that extrinsically affects their catalytic activity (e.g., ionic concentration, nucleotide binding) create gradients of adenine nucleotides and their breakdown products, which may discriminate the nearby purinoceptor that is more likely to be activated. For instance, under basal conditions ATP transiently facilitates acetylcholine from nonstimulated myenteric neurons via prejuctional P2X (probably P2X2) receptors; however, increases in the amount of ATP released from stimulated neurons create conditions favourable to ADP accumulation at the myenteric synapse, leading to down-regulation of transmitter release via the $P2Y_1$ receptor [12]. Strategic manipulation of the activity of ectonucleotidases has



(a)



(b)

Figure 9: (a) Activity of ADA during the extracellular catabolism of adenosine in the LM-MP of the ileum of control and TNBS-injected rats. Adenosine (30 μM) was incubated at zero time. Average results obtained in four experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. $^{*}P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represent significant differences as compared to control animals. (b) Localization of ADA immunoreactivity in single confocal images of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum of control and TNBS-injected rats, taken both at ganglia and neuromuscular layers. Please note that ADA immunoreactivity is much more exuberant in preparations from TNBS-treated rats than in control animals. The figure insert details ADA staining in mononuclear inflammatory cells infiltrating myenteric ganglia.

been proposed as a novel therapeutic approach to modify pathogenic purinergic signalling cascades (reviewed in [61]).

At the myenteric plexus, the rate limiting enzyme responsible for extracellular adenosine formation from released adenine nucleotides is ecto-5′-nucleotidase/CD73 [12, 18]. This enzyme acts in a concerted manner with adenosine inactivation pathways, both cellular uptake and adenosine deaminase (ADA), to control extracellular levels of the nucleoside and, thereby, the activation of co-localized P1 receptors [19]. Interestingly, although much less has been explored in functional terms, the enzymes most relevant to control adenosine levels in the extracellular milieu, both ecto-5′-nucleotidase/CD73 and ADA, may be cleaved from their anchor to the plasma membrane while retaining their catalytic activity in the soluble form [20]. Coincidently or not, significant increases in ecto-5′-nucleotidase/CD73 and ADA mRNA expression were observed in inflamed colonic tissues [62]. Fragile docking of these enzymes to the plasma membrane is more likely to occur within the context of inflammatory reactions, given that recruitment of inflammatory cells release a huge number of cytokines, chemokines and enzymes, some of these with proteolytic activity. One of these enzymes is glycosylphosphatidylinositol-specific phospholipase, which plays a role in inflammation since it can hydrolyse the GPI anchor of several membrane proteins and its hydrolytic products up-regulate cytokine expression in macrophages [63]. The widespread distribution of soluble forms of ecto-5′-nucleotidase/CD73 and ADA create conditions to unbalanced bulk production and/or inactivation of adenosine way from its original location, thus affecting mass organ function. Likewise, inflammatory infiltrates including T lymphocytes, which are endowed with NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 enzymes, may additionally contribute to adenosine accumulation disturbance and to unpredictable P1-receptor-mediated responses in inflamed tissues. In light of these compelling hypotheses, this work was designed to investigate the kinetics of the catabolism of adenine nucleotides and adenosine accumulation in the myenteric plexus of the ileum following an inflammatory insult.

Data from this study evidenced a disparity between higher amounts of ATP detected in the extracellular fluid released from TNBS-treated ileal preparations and deficits in the adenosine levels measured in the same samples, both under baseline conditions and after electrical stimulation. This puzzling contradiction has not been detected before. On the contrary, indirect evidences made by other authors led to the hypothesis that the disruption of ATP release could be due to mitochondrial dysfunction induced by inflammatory mediators [40, 45], which was not confirmed in the present study. In postinflammation ileitis, higher ATP amounts can be originated from infiltrating immune and non-neuronal cells, such as glial cells, interstitial cells of Cajal and smooth muscle contractions (see e.g., [7]; reviewed in [64]), considering the proposed inflammatory neuronal dysfunction. Hemichannels containing pannexins may act as conduits for ATP release from non-neuronal cells in response to physiological and pathological stimuli. These channels are able to form signalling complexes with the purinergic P2X7 receptor, which activation leads to large pore formation and consequently to further release of ATP [65, 66]. These authors proposed a model where high extracellular levels of ATP chronically activate neuronal P2X7, pannexins, and caspases. This relationship between P2X7 and pannexins has been related to the loss of enteric neurons during inflammatory conditions [52, 66, 67]. Moreover, our data add some information to explain the impairment of purinergic signalling during intestinal inflammation detected in other reports [14, 39, 40] which has been expanded in this study by showing a lack of adenosine neuromodulatory control of acetylcholine release from postinflammation myenteric plexus. We provided compelling evidences that most of the adenosine originated in the inflamed myenteric plexus of the rat ileum was derived from activated ICC's by the release of the nucleoside as such via dipyridamole-sensitive equilibrative transporters. This also agrees with previous findings from our group showing that adenosine formation from released ATP, via the ecto-nucleotidase cascade, contributes only partially to the total interstitial adenosine concentration in the myenteric plexus of healthy rats [19].

The apparent disturbance of adenosine generated from the extracellular catabolism of released ATP may be due to feed-forward inhibition of ecto-5′-nucleotidase/CD73 as a consequence of high extracellular levels of ATP, and particularly ADP, accumulated in the inflamed myenteric synapse (cf. [47]). ATP and ADP are competitive inhibitors of ecto-5′-nucleotidase/CD73 with inhibition constants in the low micromolar range. This indicates that they can bind to the active site of this enzyme, like AMP, but they cannot be hydrolysed [11, 68]; recovery of ecto-5′-nucleotidase/CD73 activity occurs once extracellular ATP and ADP levels decrease below the micromolar range. Redistribution of NTPDase2 from ganglion cell bodies, which is the preferential localization of this enzyme in healthy animals, towards myenteric nerve terminals affords the most probable explanation for the unpredicted ADP accumulation in postinflammation neuromuscular synapse. This is possible because NTPDase2 is a preferential nucleoside triphosphatase hydrolysing ADP 10 to 15 times less efficiently than ATP [8], and we detected no changes in the distribution of NTPDase3 and NTPDase8 present in the myenteric plexus. Notably, no evidence of NTPDase1 expression was detected in the rat myenteric plexus, besides a few blood vessels. Thus, during postinflammation ileitis the hydrolysis of ATP into adenosine at the myenteric synapse may be transiently interrupted by feed-forward inhibition of ecto-5′-nucleotidase/CD73, which makes a shift from P1 receptors activation (by adenosine) to a preferential P2 receptors activation (by ATP or ADP). These data are in agreement with the hydrolysis of ATP by NTPDase2 and ecto-5′-nucleotidase/CD73 in the rat liver [69]. Indeed, depending with which NTPDase subtype ecto-5′-nucleotidase/CD73 co-localizes different amounts of adenosine will be generated [69]. The functional interpretation of these findings in order to justify the increase in gastrointestinal motility may be complicated by disturbances in compartmentalization originated by the release of significant amounts of soluble forms of the enzyme [11, 20]. Nevertheless, our results fully agree with

other authors suggesting that ecto-5′-nucleotidase/CD73 might play a significant role in the modulation of purinergic signalling during enteric inflammation [44, 55].

Previous findings from our group demonstrated that extracellular deamination by ADA is the most efficient mechanism regulating synaptic adenosine levels and, consequently, tonic activation of facilitatory A_{2A} receptors in myenteric nerve terminals of healthy rats [18]. Besides the very high level of activity of ecto-ADA, a less-efficient nucleoside transport system sensitive to dipyridamole may also contribute to inactivation of extracellular adenosine. Owing to the effectiveness of both inactivation mechanisms, endogenous adenosine actions may be restricted to the release/production region at the myenteric cholinergic synapse, while exogenously added adenosine seems to activate preferentially extrajunctional inhibitory A_1 receptors [16, 18]. A question remains unanswered regarding the tonic effect of endogenous adenosine on low affinity excitatory A_3 receptors, although selective agonists to this receptor have been shown to beneficially influence inflammation in experimental models [53, 70]. Tandem localization of adenosine A_3 (on cell bodies) and A_{2A} (on nerve varicosities) receptors along myenteric neurons explains why the A_3 receptor activation may be prevented by ZM 241385 (50 nM), a selective A_{2A} receptor antagonist with low affinity for A_3 receptors ($K_i > 10\,\mu$M). This implies that endogenous adenosine acts preferentially on prejunctional A_{2A} under normal conditions making the activation of low affinity A_3 receptors by adenosine high unlikely during the postinflammatory phase of TNBS-ileitis due to suppression/inactivation of the nucleoside (this study). The putative activation of A_3 receptors by endogenously formed inosine via ADA also cannot explain the loss of the neuromodulatory influence of nucleosides in postinflammation ileitis. While we are uncertain regarding the extracellular levels of adenosine at both particular locations, both *in vivo* and *in vitro* models suggest that the balance between inhibitory A_1 and excitatory A_{2A} may be important in regulating intestinal motility. For instance, de Man et al. reported that chronic intestinal inflammation enhanced enteric contractile activity in part due to a loss of the cholinergic neuromodulation mediated by A_1 receptors [14]. It is worth noting that human postganglionic myenteric neurons co-express adenosine A_1 and A_{2A} receptors, which exhibit a heterogenous distribution [71]. Therefore, there is a considerable interest in the neuroprotective effects exerted by adenosine during inflammatory (and ischemic) insults, and it is conceivable that adenosine accumulation disturbances may contribute to enteric excitability during pathological conditions.

Besides the proposed interruption of the ectonucleotidase cascade at the level of ecto-5′-nucleotidase/CD73 leading to AMP accumulation and low adenosine formation in the inflamed myenteric plexus, our data also show that ADA activity is significantly enhanced in postinflammation ileitis thus contributing to decrease the extracellular adenosine levels and, thereby, tonic activation of P1 receptors. ADA is the enzyme responsible for the deamination of adenosine into inosine. This enzyme has a wide phylogenetic distribution and its amino acid sequence is highly conserved, suggesting that ADA is a key enzyme in purine metabolism. Besides the classical intracellular form, ADA is reported to bind to the cell surface of T lymphocytes, via the activation cell marker CD26 [72]. The presence of ADA in nerve terminals has also been demonstrated in many brain regions and it was hypothesize that ADA-containing terminals may release adenosine or the enzyme itself, which could serve to regulate purinergic neurotransmission [73, 74]. In the rat myenteric plexus, we first described that ADA activity increased in bath samples collected after tissue stimulation in parallel with the interstitial accumulation of adenosine and its metabolites [19]. This implies that the amount of adenosine detected following stimulation of the preparation was largely underestimated as compared to the levels of the nucleoside in the biophase. Secretion of ADA may occur in several diseases, namely those affecting hematopoietic and immune [75]. To our knowledge, this is the first study reporting an increase in the activity of soluble ADA in the chronic inflamed myenteric plexus of the rat ileum. Our findings unravel the mechanism by which inhibition of ADA might attenuate inflammation in experimental colitis [76].

In conclusion, data indicate that postinflammation ileitis suppresses adenosine neuromodulation, which may contribute to increase gastrointestinal motility. Impairment of adenosine neuromodulation is most probably due to deficient accumulation of the nucleoside at the myenteric synapse despite the paradoxical increase in ATP release. Discrepancy between ATP outflow and adenosine deficiency in the chronic inflamed ileum can be ascribed to feed-forward inhibition of ecto-5′-nucleotidase/CD73 by high extracellular levels of ATP and/or ADP. Interestingly, redistribution of NTPDase2, but not of NTPDase3, from ganglion cell bodies to myenteric nerve terminals leads to preferential ADP accumulation from released ATP, thus contributing to prolong inhibition of muscle-bound ecto-5′-nucleotidase/CD73 and to delay adenosine formation at the inflamed neuromuscular synapse. On the other hand, depression of endogenous adenosine accumulation may also occur due to enhancement of ADA activity. We observed a remarkably increase in the activity of soluble forms of ecto-5′-nucleotidase/CD73 and ADA in the postinflammatory phase of TNBS-ileitis, which may contribute to unbalanced bulk production and/or inactivation of adenosine way from its location in the native tissue thus affecting organ function. Thus, our findings suggest that pharmacological inhibition of ADA, and/or the use of AMP-derived prodrugs able to selectively activate P1 receptor co-localized with ecto-5′-nucleotidase/CD73, represent promising therapeutic strategies to ameliorate motility disturbances and immune reactivity inherent to inflammatory enteric disorders.

Abbreviations

ACh: Acetylcholine
ADA: Adenosine deaminase
ADO: Adenosine
ADP: Adenosine diphosphate
AMP: Adenosine monophosphate
ATP: Adenosine triphosphate
CNS: Central nervous system

DSS:	Dextran sodium sulphate
E-NTPDase:	Ectonucleoside triphosphate diphosphohydrolase
E-NPP:	Ecto-nucleotide pyrophosphatases/phosphodiesterases
EFS:	Electrical field stimulation
ENS:	Enteric nervous system
GI:	Gastrointestinal
GPI:	Glycosylphosphatidylinositol
HPLC:	High performance liquid chromatography
HX:	Hypoxanthine
IBD:	Inflammatory bowel disease
ICC's:	Interstitial cells of Cajal
INO:	Inosine
LM-MP:	Longitudinal muscle-myenteric plexus
Min:	Minutes
PBS:	Phosphate saline buffer
PLP:	Periodate-lysine-paraformaldehyde
TTX:	Tetrodotoxin
TNBS:	2,4,6-trinitrobenzenesulfonic acid.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] K. A. Sharkey and B. A. Kroese, "Consequences of intestinal inflammation on the enteric nervous system: neuronal activation induced by inflammatory mediators," *Anatomical Record*, vol. 262, pp. 79–90, 2001.

[2] V. Vasina, G. Barbara, L. Talamonti et al., "Enteric neuroplasticity evoked by inflammation," *Autonomic Neuroscience*, vol. 126-127, pp. 264–272, 2006.

[3] L. Pontell, P. Castelucci, M. Bagyánszki et al., "Structural changes in the epithelium of the small intestine and immune cell infiltration of enteric ganglia following acute mucosal damage and local inflammation," *Virchows Archiv*, vol. 455, no. 1, pp. 55–65, 2009.

[4] J. A. Roberts, M. K. Lukewich, K. A. Sharkey, J. B. Furness, G. M. Mawe, and A. E. Lomax, "The roles of purinergic signaling during gastrointestinal inflammation," *Current Opinion in Pharmacology*, vol. 12, no. 6, pp. 659–666, 2012.

[5] F. Kukulski, SA. Lévesque, and J. Sévigny, "Impact of ectoenzymes on P2 and P1 receptor signaling," *Advances in Pharmacology*, vol. 61, pp. 263–299, 2011.

[6] T. D. White and R. A. Leslie, "Depolarization-induced release of adenosine 5'-triphosphate from isolated varicosities derived from the myenteric plexus of the guinea pig intestine," *Journal of Neuroscience*, vol. 2, no. 2, pp. 206–215, 1982.

[7] K. Nitahara, A. Kittel, S. D. Liang, and E. S. Vizi, "A_1-Receptor-mediated effect of adenosine on the release of acetylcholine from the myenteric plexus: Role and localization of ecto-ATPase and 5′-nucleotidase," *Neuroscience*, vol. 67, no. 1, pp. 159–168, 1995.

[8] F. Kukulski, S. A. Lévesque, É. G. Lavoie et al., "Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8," *Purinergic Signalling*, vol. 1, no. 2, pp. 193–204, 2005.

[9] C. Stefan, S. Jansen, and M. Bollen, "NPP-type ectophosphodiesterases: unity in diversity," *Trends in Biochemical Sciences*, vol. 30, no. 10, pp. 542–550, 2005.

[10] F. P. Gendron, O. Benrezzak, B. W. Krugh, Q. Kong, G. A. Weisman, and A. R. Beaudoin, "Purine signaling and potential new therapeutic approach: possible outcomes of NTPDase inhibition," *Current Drug Targets*, vol. 3, no. 3, pp. 229–245, 2002.

[11] N. Sträter, "Ecto-5′-nucleotidase: structure function relationships," *Purinergic Signalling*, vol. 2, no. 2, pp. 343–350, 2006.

[12] M. Duarte-Araújo, C. Nascimento, M. A. Timóteo, M. T. Magalhães-Cardoso, and P. Correia-de-Sá, "Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on acetylcholine release from myenteric motoneurons," *British Journal of Pharmacology*, vol. 156, no. 3, pp. 519–533, 2009.

[13] E. Milusheva, B. Sperlagh, B. Kiss et al., "Inhibitory effect of hypoxic condition on acetylcholine release is partly due to the effect of adenosine released from the tissue," *Brain Research Bulletin*, vol. 24, no. 3, pp. 369–373, 1990.

[14] J. G. de Man, T. C. Seerden, B. Y. de Winter, E. A. van Marck, A. G. Herman, and P. A. Pelckmans, "Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation," *British Journal of Pharmacology*, vol. 139, no. 1, pp. 172–184, 2003.

[15] J. Nicholls, V. R. Brownhill, and S. M. O. Hourani, "Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae," *British Journal of Pharmacology*, vol. 117, no. 1, pp. 170–174, 1996.

[16] C. Vieira, F. Ferreirinha, I. Silva, M. Duarte-Araújo, and P. Correia-de-Sá, "Localization and function of adenosine receptor subtypes at the longitudinal muscle-myenteric plexus of the rat ileum," *Neurochemistry International*, vol. 59, no. 7, pp. 1043–1055, 2011.

[17] N. A. Moneta, T. J. McDonald, and M. A. Cook, "Endogenous adenosine inhibits evoked substance P release from perifused networks of myenteric ganglia," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 272, no. 1, pp. G38–G45, 1997.

[18] M. Duarte-Araújo, C. Nascimento, M. A. Timóteo, T. Magalhães-Cardoso, and P. Correia-de-Sá, "Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A_{2A} and extrajunctional inhibitory A_1 receptors," *British Journal of Pharmacology*, vol. 141, no. 6, pp. 925–934, 2004.

[19] P. Correia-de-Sá, S. Adães, M. A. Timóteo et al., "Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: on the role of secreted adenosine deaminase," *Autonomic Neuroscience: Basic and Clinical*, vol. 126-127, pp. 211–224, 2006.

[20] M. Pettengill, S. Robson, M. Tresenriter et al., "Soluble ecto-5′-nucleotidase (5′-NT), alkaline phosphatase, and adenosine deaminase (ADA1) activities in neonatal blood favor elevated extracellular adenosine," *The Journal of Biological Chemistry*, vol. 288, no. 38, pp. 27315–27326, 2013.

[21] R. Franco, V. Casadó, F. Ciruela et al., "Cell surface adenosine deaminase: much more than an ectoenzyme," *Progress in Neurobiology*, vol. 52, no. 4, pp. 283–294, 1997.

[22] D. L. Marquardt, H. E. Gruber, and S. I. Wasserman, "Adenosine release from stimulated mast cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 19I, pp. 6192–6196, 1984.

[23] R. H. Stead, M. F. Dixon, N. H. Bramwell, R. H. Riddell, and J. Bienenstock, "Mast cells are closely apposed to nerves in the human gastrointestinal mucosa," *Gastroenterology*, vol. 97, no. 3, pp. 575–585, 1989.

[24] J. Bogers, T. Moreels, J. de Man et al., "Schistosoma mansoni causing diffuse enteric inflammation and damage of the enteric nervous system in the mouse small intestine," *Neurogastroenterology & Motility*, vol. 12, pp. 431–440, 2000.

[25] L. Antonioli, M. Fornai, R. Colucci et al., "Regulation of enteric functions by adenosine: pathophysiological and pharmacological implications," *Pharmacology and Therapeutics*, vol. 120, no. 3, pp. 233–253, 2008.

[26] A. E. Jergens, "Inflammatory bowel disease. Current perspectives," *Veterinary Clinics of North America-Small Animal Practice*, vol. 29, no. 2, pp. 501–521, 1999.

[27] M. A. Engel, C. A. Kellermann, T. Rau, G. Burnat, E. G. Hahn, and P. C. Konturek, "Ulcerative colitis in AKR mice is attenuated by intraperitoneally administered anandamide," *Journal of Physiology and Pharmacology*, vol. 59, no. 4, pp. 673–689, 2008.

[28] S. Wirtz and M. F. Neurath, "Mouse models of inflammatory bowel disease," *Advanced Drug Delivery Reviews*, vol. 59, no. 11, pp. 1073–1083, 2007.

[29] W. D. Paton and E. S. Vizi, "The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip," *British Journal of Pharmacology*, vol. 35, no. 1, pp. 10–28, 1969.

[30] M. Costa, S. J. H. Brookes, P. A. Steele, I. Gibbins, E. Burcher, and C. J. Kandiah, "Neurochemical classification of myenteric neurons in the guinea-pig ileum," *Neuroscience*, vol. 75, no. 3, pp. 949–967, 1996.

[31] A. R. Pinheiro, D. Paramos-de-Carvalho, M. Certal et al., "Histamine induces ATP release from human subcutaneous fibroblasts, via pannexin-1 hemichannels, leading to Ca^{2+} mobilization and cell proliferation," *The Journal of Biological Chemistry*, vol. 288, pp. 27571–27583, 2013.

[32] M. A. Timóteo, I. Carneiro, I. Silva et al., "ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial $P2Y_6$ receptors," *Biochemical Pharmacology*, vol. 87, pp. 371–379, 2014.

[33] K. Enjyoji, J. Sévigny, Y. Lin et al., "Targeted disruption of *cd39*/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation," *Nature Medicine*, vol. 5, no. 9, pp. 1010–1017, 1999.

[34] P. Heine, N. Braun, J. Sévigny, S. C. Robson, J. Servos, and H. Zimmermann, "The C-terminal cysteine-rich region dictates specific catalytic properties in chimeras of the ectonucleotidases NTPDase1 and NTPDase2," *European Journal of Biochemistry*, vol. 268, no. 2, pp. 364–373, 2001.

[35] M. MartínSatué, E. G. Lavoie, J. Pelletier et al., "Localization of plasma membrane bound NTPDases in the murine reproductive tract," *Histochemistry and Cell Biology*, vol. 131, pp. 615–628, 2009.

[36] D. L. Bartel, S. L. Sullivan, É. G. Lavoie, J. Sévigny, and T. E. Finger, "Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds," *Journal of Comparative Neurology*, vol. 497, no. 1, pp. 1–12, 2006.

[37] M. Fausther, J. Lecka, F. Kukulski et al., "Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 292, no. 3, pp. G785–G795, 2007.

[38] T. G. Moreels, J. G. De Man, J. M. C. Dick et al., "Effect of TNBS-induced morphological changes on pharmacological contractility of the rat ileum," *European Journal of Pharmacology*, vol. 423, no. 2-3, pp. 211–222, 2001.

[39] I. Depoortere, T. Thijs, and T. L. Peeters, "Generalized loss of inhibitory innervation reverses serotonergic inhibition into excitation in a rabbit model of TNBS-colitis," *British Journal of Pharmacology*, vol. 135, no. 8, pp. 2011–2019, 2002.

[40] D. S. Strong, C. F. Cornbrooks, J. A. Roberts, J. M. Hoffman, K. A. Sharkey, and G. M. Mawe, "Purinergic neuromuscular transmission is selectively attenuated in ulcerated regions of inflamed guinea pig distal colon," *Journal of Physiology*, vol. 588, no. 5, pp. 847–859, 2010.

[41] M. Odashima, G. Bamias, J. Rivera-Nieves et al., "Activation of A_{2A} adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease," *Gastroenterology*, vol. 129, no. 1, pp. 26–33, 2005.

[42] I. C. Cavalcante, M. V. Castro, A. R. F. Barreto et al., "Effect of novel A_{2A} adenosine receptor agonist ATL 313 on Clostridium difficile toxin A-induced murine ileal enteritis," *Infection and Immunity*, vol. 74, no. 5, pp. 2606–2612, 2006.

[43] L. Antonioli, M. Fornai, R. Colucci et al., "Differential recruitment of high affinity A_1 and A_{2A} adenosine receptors in the control of colonic neuromuscular function in experimental colitis," *European Journal of Pharmacology*, vol. 650, no. 2-3, pp. 639–649, 2011.

[44] L. Antonioli, P. Pacher, E. S. Vizi, and G. Haskó, "CD39 and CD73 in immunity and inflammation," *Trends in Molecular Medicine*, vol. 19, no. 6, pp. 355–367, 2013.

[45] S. Neshat, M. Devries, A. R. Barajas-Espinosa, L. Skeith, S. P. Chisholm, and A. E. Lomax, "Loss of purinergic vascular regulation in the colon during colitis is associated with upregulation of CD39," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 296, no. 2, pp. G399–G405, 2009.

[46] M. C. Giron, A. Bin, P. Brun et al., "Cyclic AMP in rat ileum: evidence for the presence of an extracellular cyclic AMP-adenosine pathway," *Gastroenterology*, vol. 134, no. 4, pp. 1116–1126, 2008.

[47] M. T. Magalhães-Cardoso, M. F. Pereira, L. Oliveira, J. A. Ribeiro, R. A. Cunha, and P. Correia-de-Sá, "Ecto-AMP deaminase blunts the ATP-derived adenosine A_{2A} receptor facilitation

of acetylcholine release at rat motor nerve endings," *Journal of Physiology*, vol. 549, no. 2, pp. 399–408, 2003.

[48] N. Braun, J. Sévigny, S. C. Robson, K. Hammer, M. Hanani, and H. Zimmermann, "Association of the ecto -ATPase NTPDase2 with glial cells of the peripheral nervous system," *GLIA*, vol. 45, no. 2, pp. 124–132, 2004.

[49] E. G. Lavoie, B. D. Gulbransen, M. Martín-Satué, E. Aliagas, K. A. Sharkey, and J. Sévigny, "Ectonucleotidases in the digestive system: focus on NTPDase3 localization," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 300, no. 4, pp. G608–G620, 2011.

[50] M. Martin, J. Huguet, J. J. Centelles, and R. Franco, "Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex: possible role of adenosine deaminase as costimulatory molecule," *The Journal of Immunology*, vol. 155, no. 10, pp. 4630–4643, 1995.

[51] B. A. Conlon and W. R. Law, "Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses," *Clinical and Experimental Immunology*, vol. 138, no. 1, pp. 14–20, 2004.

[52] S. E. Lakhan and A. Kirchgessner, "Neuroinflammation in inflammatory bowel disease," *Journal of Neuroinflammation*, vol. 7, article 37, 2010.

[53] L. Antonioli, M. Fornai, R. Colucci et al., "The blockade of adenosine deaminase ameliorates chronic experimental colitis through the recruitment of adenosine A_{2A} and A_3 receptors," *Journal of Pharmacology and Experimental Therapeutics*, vol. 335, no. 2, pp. 434–442, 2010.

[54] S. C. Robson, J. Sévigny, and H. Zimmermann, "The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance," *Purinergic Signalling*, vol. 2, no. 2, pp. 409–430, 2006.

[55] N. A. Louis, A. M. Robinson, C. F. MacManus, J. Karhausen, M. Scully, and S. P. Colgan, "Control of IFN-αA by CD73: implications for mucosal inflammation," *The Journal of Immunology*, vol. 180, no. 6, pp. 4246–4255, 2008.

[56] I. Sotnikov and N. A. Louis, "CD73-dependent regulation of interferon AlphaA and interleukin-10 in the inflamed mucosa," *The Scientific World Journal*, vol. 10, pp. 2167–2180, 2010.

[57] B. Siegmund, F. Rieder, S. Albrich et al., "Adenosine kinase inhibitor GP515 improves experimental colitis in mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 296, pp. 99–105, 2001.

[58] J. G. Mabley, P. Pacher, L. Liaudet et al., "Inosine reduces inflammation and improves survival in a murine model of colitis," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 284, no. 1, pp. G138–G144, 2003.

[59] B. N. Cronstein, "Adenosine, an endogenous anti-inflammatory agent," *Journal of Applied Physiology*, vol. 76, no. 1, pp. 5–13, 1994.

[60] G. G. Yegutkin, "Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade," *Biochimica et Biophysica Acta*, vol. 1783, no. 5, pp. 673–694, 2008.

[61] G. Burnstock and M. Williams, "P2 purinergic receptors: modulation of cell function and therapeutic potential," *Journal of Pharmacology and Experimental Therapeutics*, vol. 295, no. 3, pp. 862–869, 2000.

[62] L. Antonioli, M. Fornai, O. Awwad et al., "Role of the A_{2B} receptor-adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rats," *British Journal of Pharmacology*, vol. 171, pp. 1314–1329, 2014.

[63] K. D. O'Brien, C. Pineda, W. S. Chiu, R. Bowen, and M. A. Deeg, "Glycosylphosphatidylinositol-specific phospholipase D is expressed by macrophages in human atherosclerosis and colocalizes with oxidation epitopes," *Journal of the American Heart Association*, vol. 99, no. 22, pp. 2876–2882, 1999.

[64] G. Burnstock and S. Lavin, "Interstitial cells of Cajal and purinergic signalling," *Autonomic Neuroscience*, vol. 97, no. 1, pp. 68–72, 2002.

[65] E. F. Diezmos, S. L. Sandow, I. Markus et al., "Expression and localization of pannexin-1 hemichannels in human colon in health and disease," *Neurogastroenterology and Motility*, vol. 25, no. 6, pp. e395–e405, 2013.

[66] B. D. Gulbransen, M. Bashashati, S. A. Hirota et al., "Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis," *Nature Medicine*, vol. 18, no. 4, pp. 600–604, 2012.

[67] G. J. Sanger, "New target profiling for gastrointestinal disorders: where are we going?" *Current Opinion in Pharmacology*, vol. 12, no. 6, pp. 629–631, 2012.

[68] M. Fausther, J. Lecka, E. Soliman et al., "Coexpression of ecto-5'-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 302, no. 4, pp. 447–459, 2012.

[69] R. A. Cunha, "Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals," *Neurochemical Research*, vol. 26, no. 8-9, pp. 979–991, 2001.

[70] J. Guzman, J. G. Yu, Z. Suntres et al., "ADOA3R as a therapeutic target in experimental colitis: proof by validated high-density oligonucleotide microarray analysis," *Inflammatory Bowel Diseases*, vol. 12, no. 8, pp. 766–789, 2006.

[71] F. L. Christofi, "Unlocking mysteries of gut sensory transmission: is adenosine the key?" *News in Physiological Sciences*, vol. 16, no. 5, pp. 201–207, 2001.

[72] G. Cristalli, S. Costanzi, C. Lambertucci et al., "Adenosine deaminase: functional implications and different classes of inhibitors," *Medicinal Research Reviews*, vol. 2, pp. 105–128, 2001.

[73] J. I. Nagy, J. D. Geiger, and W. A. Staines, "Adenosine deaminase and purinergic neuroregulation," *Neurochemistry International*, vol. 16, no. 3, pp. 211–221, 1990.

[74] J. I. Nagy, T. Yamamoto, H. Uemura, and W. P. Schrader, "Adenosine deaminase in rodent median eminence: detection by antibody to the mouse enzyme and co-localization with adenosine deaminase-complexing protein (CD26)," *Neuroscience*, vol. 73, no. 2, pp. 459–471, 1996.

[75] J. P. J. Ungerer, H. M. Oosthuizen, S. H. Bissbort, and W. J. H. Vermaak, "Serum adenosine deaminase: isoenzymes and diagnostic application," *Clinical Chemistry*, vol. 38, no. 7, pp. 1322–1326, 1992.

[76] L. Antonioli, M. Fornai, R. Colucci et al., "Inhibition of adenosine deaminase attenuates inflammation in experimental colitis," *Journal of Pharmacology and Experimental Therapeutics*, vol. 322, no. 2, pp. 435–442, 2007.

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Research Article

Sustained Release of Prostaglandin E_2 in Fibroblasts Expressing Ectopically Cyclooxygenase 2 Impairs P2Y-Dependent Ca^{2+}-Mobilization

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The nucleotide uridine trisphosphate (UTP) released to the extracellular milieu acts as a signaling molecule *via* activation of specific pyrimidine receptors (P2Y). P2Y receptors are G protein-coupled receptors expressed in many cell types. These receptors mediate several cell responses and they are involved in intracellular calcium mobilization. We investigated the role of the prostanoid PGE_2 in P2Y signaling in mouse embryonic fibroblasts (MEFs), since these cells are involved in different ontogenic and physiopathological processes, among them is tissue repair following proinflammatory activation. Interestingly, Ca^{2+}-mobilization induced by UTP-dependent P2Y activation was reduced by PGE_2 when this prostanoid was produced by MEFs transfected with COX-2 or when PGE_2 was added exogenously to the culture medium. This Ca^{2+}-mobilization was important for the activation of different metabolic pathways in fibroblasts. Moreover, inhibition of COX-2 with selective coxibs prevented UTP-dependent P2Y activation in these cells. The inhibition of P2Y responses by PGE_2 involves the activation of PKCs and PKD, a response that can be suppressed after pharmacological inhibition of these protein kinases. In addition to this, PGE_2 reduces the fibroblast migration induced by P2Y-agonists such as UTP. Taken together, these data demonstrate that PGE_2 is involved in the regulation of P2Y signaling in these cells.

1. Introduction

P2 receptors are purinergic receptors selective for adenosine 5′-trisphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5′-trisphosphate (UTP), and uridine 5′-diphosphate (UDP). These nucleotides act as extracellular signaling molecules and exert their activity by binding to and activating specific membrane receptors, designed P2 receptors [1, 2]. There are two families of P2 receptors structurally distinct: P2X ionotropic ion channel receptors and P2Y metabotropic G protein-coupled receptors [3–5]. Currently, seven P2X subtypes and eight P2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as to purines [6]. Receptors for purine and pyrimidine nucleotides are involved in many neuronal as well as nonneuronal mechanisms, including short-term purinergic signaling such as neurotransmission, neuromodulation, neurosecretion, immune responses, inflammation, platelet aggregation, and vasodilatation, and long-term purinergic signaling of cell proliferation, differentiation, motility, and death in development and regeneration [7].

At present, there are eight accepted P2Y receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 [8, 9]. The metabotropic receptors, coupled to phospholipase C

(PLC), can be activated by different nucleotides, depending on the P2Y receptors and the species studied [10]. In the aftermath of nucleotide release to the extracellular medium these receptors are stimulated leading to an intracellular increase in diacylglycerol (DAG) and inositol trisphosphate (IP3) followed by a release of calcium from intracellular stores [11, 12]. These receptors are widely distributed in several cell types; their existence in fibroblasts was first reported by Okada et al. [13]. Fibroblasts respond to inflammation and damage, being involved in the repair phase following tissue damage, or in other pathological circumstances, such as atherogenesis [14].

Prostaglandin E_2 (PGE_2) is an important chemical mediator generated from arachidonic acid *via* the cyclooxygenase pathway. The various biological effects of PGE_2 are mediated by four receptors called E-type prostanoid receptors (EP1 to EP4), which are G protein-coupled membrane receptors [15]. EP1 leads to mobilization of intracellular calcium. This transient change in intracellular Ca^{2+} alters the activity of many proteins, including several isoforms of PKC. Therefore, PGE_2 evokes Ca^{2+}- and PKC-mediated effects in cells expressing EP1 [16]. EP2 and EP4 signaling generates increased intracellular cyclic AMP (cAMP) levels, whereas EP3 leads to a reduction in intracellular cAMP levels [17, 18]. However, in addition to EP-mediated effects, PGs may exert other EP-independent actions, for example, through the purinergic signaling [19, 20].

Taken together, both signaling pathways generate DAG and IP3, promoting Ca^{2+} mobilization. This alteration may affect the activity of several proteins, such as PKC and, indeed, previous work have described that the signaling of G protein-coupled receptors is regulated by mechanisms involving protein kinases such as PKC [21]. Although it has been shown that PGE_2 is a potent inhibitor of the purinergic signaling mediated by some purinergic receptors [19, 20], less is known about the underlying cross-talk between PGs and P2 signaling as a mechanism integrating inflammation and the presence of extracellular nucleotides. In the present work we have investigated this interplay between PGs and P2 receptors in mouse embryonic fibroblasts (MEFs). Our data extend previous work in macrophages and suggest that this communication between the two pathways is functional in MEFs adding a new piece of knowledge to understand how fibroblast activity may be regulated by these dual signaling pathways.

2. Materials and Methods

2.1. Reagents. UTP, ionophores, and standard reagents were from Sigma-Aldrich (St Louis, MO, USA). DFU was from Merck (Rahway, NJ, USA). Prostaglandin E_2 was from Cayman Chemical (Ann Arbor, MI, USA). Gö6976, Gö6983, Gö6850, and inhibitors of standard signaling pathways were from Calbiochem (San Diego, CA, USA). Fura-2/AM was from Invitrogen (Carlsbad, CA, USA). Cytokines were from PeproTech (London, UK). Antibodies against P2Y2, P2Y4, and P2Y6 receptors were from Alomone Labs (Jerusalem, Israel) and other antibodies were from Santa Cruz Biotech (Santa Cruz, CA, USA), from Cell Signaling (Danvers, MA, USA), or from the sources previously described [22]. Reagents for electrophoresis were from Bio-Rad (Hercules, CA, USA) and Sigma-Aldrich. Tissue culture dishes were from Falcon (Lincoln Park, NJ, USA) and culture media were from Invitrogen.

2.2. Animals. COX-2 wild type (WT) and COX-2-deficient mice, with a mixed background 129SV and C57BL/6, were obtained from the Jackson Laboratory. Mice were housed under 12 h light/dark cycle and food and water were provided *ad libitum*. Animals were cared for according to a protocol approved by the Ethical Committee of our institution (following directive 2010/63/EU of the European Parliament).

2.3. Preparation of Mouse Embryonic Fibroblasts (MEFs), Cell Culture, and MEFs Immortalization. MEFs were prepared from E14.5 embryos from WT and COX-2-deficient mice. Briefly, female mice were euthanized by CO_2 at 14.5 after conception. Using scissors, the abdomen was opened and the embryos were isolated with their yolk sacs intact. The yolk sac was removed and retained for genotyping. The head and internal organs of each embryo were discarded. The dissected embryo was passed through an 18G needle to disperse the cells [23]. MEFs were cultured (2×10^6 cells per 60 mm dish or in 12-multiwell plates at a density of 2×10^5 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C, and 5% CO_2 [24, 25]. COX-$2^{+/+}$ and COX-$2^{-/-}$ primary MEFs were transfected with a SV40 large T-antigen expression vector using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions, to obtain immortalized MEF cell line (referred to as MEFs WT, KO, or KI-carrying the COX-2 transgene).

2.4. Transfection. To ectopically express COX-2, COX-2-deficient MEFs were transiently transfected with 4 μg of plasmid DNA (per well in a 6-well plate) using Lipofectamine 2000 reagent following the instructions of the supplier. Briefly, MEF cells at 70% confluence were exposed for 6–16 h to Lipofectamine reagent containing pPyCAGIP-COX-2 or control vector pPyCAGIP. At the end of this period, the transfection media were replaced with fresh medium containing 10% FBS. COX-2 expression was determined by Western blot.

2.5. Determination of PGE_2 by Enzyme Immunoassay. PGE_2 accumulation was measured in the culture medium. For the assay, WT and KI (COX-2-deficient MEFs overexpressing COX-2) MEFs were plated in 6-well plates at a density of 1.5×10^6 cells/well in 2 mL DMEM and treated in the absence or presence of LPS (200 ng/mL) plus cytokines (IFN-γ, TNF-α, and IL-1β, 20 ng/mL) for 18 h at 37°C. The culture supernatants were centrifuged at 12,000 ×g for 5 min and PGE_2 levels were determined by specific immunoassay (DetectX Prostaglandin E_2, Arbor Assays, Ann Arbor MI, USA), according to the manufacturer's instructions.

2.6. Calcium Dynamic Analysis. MEFs attached to coverslips were incubated in Locke's solution as previously described [20]. The effect of purinergic receptor agonists was assayed at near-maximal effective concentrations (100 μM UTP) [26]. In other studies, 5 μM PGE_2 was applied for 5 min before nucleotides perfusion in the presence of prostanoids. When pharmacological inhibitors were used, they were preincubated at the indicated concentrations and for the required times as specified in the text and figure legends and kept during prostanoid incubation and/or purinergic agonist stimulation. After dual excitation at 340 and 380 nm the fluorescence was recorded and analyzed. Background signals were subtracted from each wavelength and the F_{340}/F_{380} ratio was calculated [27]. Alternatively, in some cases (indicated in the corresponding figure legends), calcium mobilization was measured using the nonratiometric Fluo-4 direct probe (Invitrogen), following the instructions of the supplier. In this case, the changes in fluorescence were measured in a fluorescence microscope (Observer Z1, Plan Apochromat objective, Zeiss) equipped with a Cascade1K camera, analyzed using the Axiovision 4.8 imaging program and expressed as the percentage of responding cells. Video imaging of the calcium-dependent fluorescence fluxes was also acquired.

2.7. RNA Isolation and Quantitative PCR (qPCR) Analysis. 1 μg of total RNA, extracted with TRI Reagent (Ambion, Life Technologies), was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). Real-time PCR was conducted with SYBR Green (Roche) on a MyiQ Real-Time PCR System (Bio-Rad). The TaqMan probes for mouse EP1, EP2, EP3, EP4, P2Y2, and P2Y4 used in this study were purchased from Applied Biosystems and experiments for validation of amplification efficiency were performed for each TaqMan probe set [28, 29]. PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Each sample was run in duplicate and was normalized with the expression of 36B4. The fold induction (FI) was determined in a ΔΔCt based fold-change calculations (relative quantity, RQ, is $2^{-\Delta\Delta Ct}$).

2.8. Preparation of Total Protein Cell Extracts and Western Blot Analysis. Cells were homogenized in a buffer containing 10 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol, and a protease and phosphatase inhibitor cocktail (Sigma). The extracts were vortexed for 30 min at 4°C and after centrifuging for 15 min at 13,000 ×g, the supernatants were stored at −20°C. Protein levels were determined with Bradford reagent (Bio-Rad). For immunoblot analysis the protein extracts were analyzed as described using a Charged Coupling Device camera in a luminescent image analyzer (Molecular Imager, BioRad) to ensure the linearity of the band intensities. Values of densitometry were determined using Quantity One software (Bio-Rad).

2.9. MEFs Migration in Transwells. Migration assays were performed in 24 transwells (uncoated 8 μm porous transwells) according to the manufacturer's instructions (Corning Incorporated, NY). 5×10^4 MEFs were seeded in the upper chambers and cells were allowed to attach for 2 h. After thorough washing with PBS to remove nonadherent cells, MEFs were starved overnight. Cells were stimulated with combinations of the indicated stimuli (PGE_2 and UTP in the upper chamber and 10% FBS, UTP, or PGE_2 into 500 μL in the lower chamber, used as chemoattractants). The plates were incubated at 37°C overnight in the presence of 20 μg/mL of mitomycin C (Sigma-Aldrich) to inhibit cell proliferation. The membrane was fixed with paraformaldehyde (4%; pH 7.2) and stained with crystal violet solution (Sigma-Aldrich). The number of cells that migrated completely through the 8 μm pores was determined.

2.10. Statistical Analysis. The values in graphs correspond to the mean ± SD. The statistical significance was estimated with a Student's t-test for unpaired observation. Data were analyzed by the SPSS for Windows statistical package, version 21.

3. Results

3.1. Transgenic Expression of COX-2 Impairs P2Y-Dependent Ca^{2+}-Mobilization. MEFs expressing COX-2 release PGE_2 in the absence of additional stimuli. This accumulation was enhanced after proinflammatory stimulation with a combination of LPS, TNF-α, IL-1β, and IFN-γ (Figure 1(a)). In addition to this, the presence of PGE_2 inhibited UTP-dependent Ca^{2+}-mobilization in MEF cells, either when this PG is exogenously added or when produced by the COX-2 transgene (Figure 1(b)). A video imaging of the Ca^{2+}-transients in COX-2 WT and KI (expressing the COX-2 transgene) MEFs treated with different stimuli (PGs and UTP) is shown in Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/832103. Interestingly, when the medium of COX-2 KI MEFs is replaced by fresh medium containing the selective COX-2 inhibitor DFU (a coxib), the impaired P2Y signaling in response to UTP observed in the same cells without medium change was abolished (Figure S1). Analysis of the pathways involved in the impairment of P2Y-dependent Ca^{2+}-mobilization in MEFs from WT and COX-2 transfected cells (KI) showed a similar pattern of responses between both conditions for exogenous PGE_2 or when COX-2 was inhibited with DFU (Figure 2). The UTP mobilization of Ca^{2+} was similar between MEFs from WT or COX-2-deficient mice (data not shown), regardless of the treatment with DFU. In addition to this, a broad inhibitor of PKCs and some tyrosine kinases (staurosporine) partially rescued the response to UTP in KI cells. Interestingly, inhibition of novel PKCs (δ, ε, η, and θ) [30] and PKD (Gö6976 and CID755376, resp.), but not of the classic isoforms of PKC (α, β, and γ; inhibited with Gö6983), restored the response to UTP in the presence of PGE_2 due to the activity of COX-2. Opposite to this, activation of PKCs/PKD with the diacylglycerol analogue PDBu abolished the UTP-dependent Ca^{2+}-mobilization, whereas the inactive phorbol α-PDD was unable to influence the responses of both types of cells. PKA activation after treatment with a permeant

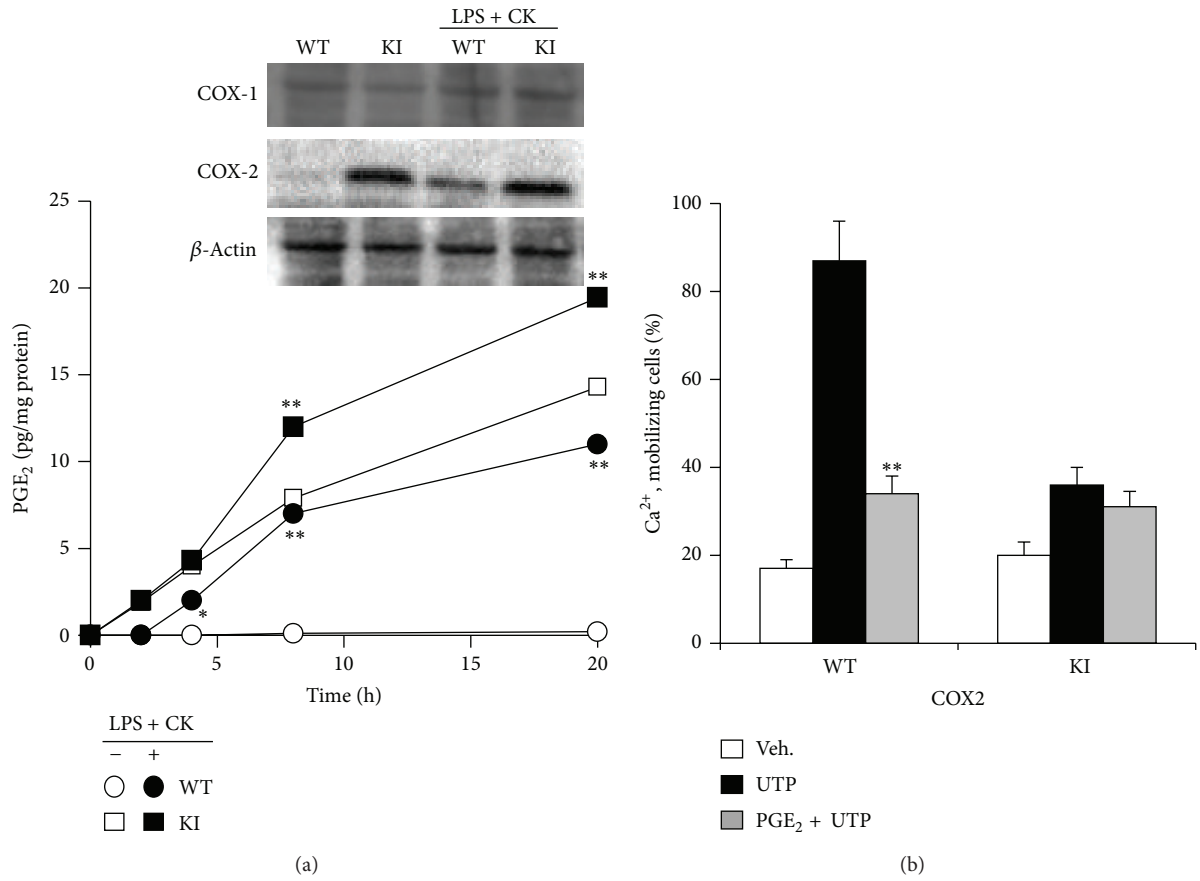

FIGURE 1: PGE_2 released in MEFs overexpressing COX-2 and effect on P2Y-dependent Ca^{2+}-mobilization. WT and KI (COX-2-deficient MEFs overexpressing COX-2) MEFs, treated in the absence or presence of LPS (200 ng/mL) plus cytokines (IFN-γ, TNF-α, and IL-1β, 20 ng/mL), were used. The protein levels of COX-1 and COX-2 and the PGE_2 released into the culture medium were determined by immunoblot and ELISA, respectively (a). The percentage of cells showing Ca^{2+}-mobilization in response to the P2Y agonist UTP (100 μM) was determined using the nonratiometric Fluo-4 assay (b). Results show a representative blot (a) and the mean + SD of three experiments for release of PGE_2 to the culture medium and Ca^{2+}-mobilization. $^{*}P < 0.05$, $^{**}P < 0.001$ versus the corresponding control.

cAMP analogue (dibutyril-cAMP) was also unable to affect UTP signaling (Figure 2). Together, these results suggest that novel PKCs and PKD are involved in the suppressive effect of PGE_2 on UTP-dependent Ca^{2+}-mobilization.

3.2. Transgenic Expression of COX-2 Accumulates P2Y4 Receptors in the Nucleus. To identify mechanisms involved in the impairment of P2Y signaling the distribution of these receptors in MEF cells constitutively synthesizing PGE_2 was analyzed. As Figure 3 shows, P2Y2, P2Y4, and P2Y6 were present in these cells; however, a significant proportion of P2Y4 receptors localized in the nucleus, a situation that was suppressed after inhibition of COX-2 with DFU. This was also observed in WT MEFs treated with PGE_2 (Supplementary Figure S2). Interestingly, the expression of EP1–4 PGE_2 receptors and P2Y2 and P2Y4 receptors was not influenced by the ectopic expression of COX-2 (Figures 4(a) and 4(b)). To further investigate the effect of PGE_2 on Ca^{2+}-mobilization, treatment of KI MEFs with the Ca^{2+} ionophore ionomycin resulted in identical calcium fluxes regardless of the incubation with DFU or PGE_2 (Figure 4(c)). Indeed, the shape of the Ca^{2+} fluxes exhibited similar profiles when the extracellular Ca^{2+} concentration was maintained high (0.5 mM) or low (0.1 mM). However, Ca^{2+}-mobilization in response to thapsigargin (i.e., after inhibition of the replenishment of the ER stores) was significantly inhibited in the presence of PGE_2 (Figure 4(d)). This latter condition was similar to the mobilization induced by thapsigargin in KI cells in the absence of medium replacement (i.e., with accumulation of PGE_2 in the culture medium, not shown).

3.3. Thapsigargin-Dependent Phosphorylation in MEFs. To evaluate the effect of PGE_2 on Ca^{2+}-mobilization, cells were treated with prostaglandin and immediately with thapsigargin. As Figure 5(a) shows, the phosphorylation of AKT was inhibited by PGE_2 and to lesser extents in a proinflammatory situation. Similar results were obtained for the phosphorylation of CaMKII and ACC, whereas AMPK phosphorylation was minimally affected by PGE_2. These data suggest a complex pattern of phosphorylation of these enzymes beyond P2Y activation, as previously described in macrophages [20].

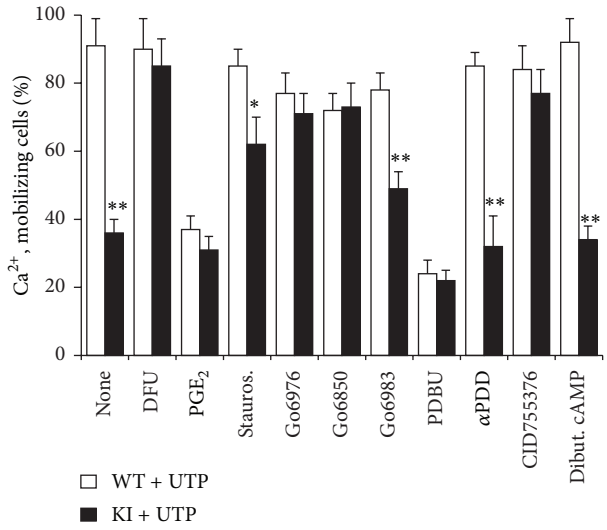

Figure 2: Characterization of targeting of PKC, PKD, and PKA on the effect of PGE_2 on the UTP-dependent Ca^{2+}-mobilization. WT or KI MEFs were washed with fresh medium and maintained in culture for 1 h to remove PGE_2 accumulated and then treated for 10 min with the indicated effectors, except for DFU that was added immediately after washing (1 μM DFU, an inhibitor of COX-2; 5 μM PGE_2; 100 nM staurosporine; 100 nM Gö6976; 5 μM Gö6850; 10 nM Gö6983, a selective inhibitor of classic PKCs; 200 nM PDBU; 200 nM αPDD; 200 nM CID755376, a selective inhibitor of PKD; 5 μM dibutyryl cAMP) and the percentage of cells showing Ca^{2+}-mobilization in response to UTP (100 μM) was determined using the nonratiometric Fluo-4 assay. Results show the mean + SD of three experiments for Ca^{2+}-mobilization. $^*P < 0.05$, $^{**}P < 0.001$ versus the same condition in the WT cells.

3.4. PGE_2 Inhibits P2Y-Dependent Cell Migration. MEFs migration is affected by extracellular nucleotides [31]. As Figure 5(b) shows, transwell migration analysis of MEFs carrying a COX-2 transgene and maintained in the presence of DFU showed a response that was increased in cells treated with UTP, a process that was attenuated after pretreatment with PGE_2. Interestingly, when PGE_2 was present in both the upper and lower compartments, cell migration was completely abolished stressing the effect of this prostaglandin in the regulation of MEFs motility.

4. Comment

Extracellular nucleotides, such as UTP, have been described as innate immune regulators acting via the P2 receptors [32, 33]. Indeed, P2 agonists are increasingly viewed as a new class of innate immune system mediators following their release at sites of inflammation as a result of infection or cell damage [34]. Indeed, interplay between PGs and P2Y response in the context of macrophage activation, polarization, and resolution of the inflammation has been described [20]. However, less is known regarding the role of P2Y receptors and PGE_2 in other cell types. For this reason, in this work, we provide new data on the fine regulation of P2Y signaling in MEFs using a specific agonist. Since fibroblasts play a role in the immune response [35], our data suggest that MEFs may play a central role in the regulation in the proresolution and tissue repair phase [36].

We have characterized the expression of P2Y2, P2Y4, and P2Y6 in MEFs, using functional and immunological approaches. Experiments were performed in MEFs from the WT and COX-2-deficient animals, carrying a COX-2 transgene. The release of PGE_2 impaired UTP-dependent Ca^{2+}-mobilization responses that could be attributed to the accumulation of PGE_2 in the culture medium. These data clearly establish a regulation of P2Y receptors by PGE_2 in MEF cells, in addition to other cells such as macrophages [20]. Interestingly enough, in the intact animal, this PGE_2 can be derived by several COX-2 expressing cells acting in a concerted way. Moreover, it has been described that P2X7 receptor activation is required for the release of PGE_2 in macrophages [37] which in turn could regulate P2Y responses. Taking this into account, it seems that there is a complex crosstalk between P2 receptors and PGE_2 release.

All of the cloned P2Y receptors activate phospholipase C resulting in IP3 generation and Ca^{2+} release from intracellular stores. However, the response of the P2Y receptors is regulated by mechanisms involving desensitization that comprises phosphorylation of the receptors by protein kinases such as protein kinase C (PKC), attenuating receptor signaling [38]. Moreover, previous studies have demonstrated that P2Y receptors desensitization has been attributed to PKC-dependent mechanisms [39, 40]. In the present work, we have provided evidence for the involvement of PGE_2, through PKC, in P2Y receptor desensitization, analyzing Ca^{2+} mobilization as read-out. We elucidated the main PKC isoenzyme responsible for the alterations of Ca^{2+} mobilization by choosing selective PKC inhibitors [30]. As controls, we used PGE_2 and DFU, a selective COX-2 inhibitor which restores the UTP response, as in MEF WT and in KI cells, suggesting the regulation of P2 receptors signaling by PGE_2. Also, we used Gö6976, for inhibiting the classic PKC isoforms, and Gö6850 that is structurally similar to the poorly selective PKC inhibitor staurosporine. Gö6850 shows high selectivity for PKCα, β1, β2, γ, δ, and ε isoenzymes [41]. Gö6983 is a pan-PKC inhibitor against PKCα, β, γ, and δ. Moreover, phorbol 12,13-dibutyrate (PDBu), a potent activator of PKC/PKD, and α-phorbol didecanoate (αPDD), which is an inactive derivate of PDBu, supported the role of these kinases in the regulation of P2Y activity by PGE_2. Furthermore, PKDs regulate diverse cellular processes such as P2 signaling [26]. Previous data described that activation of PKCδ acts as an upstream PDK1 activation step [42]. For this reason, we use a selective PKD1 inhibitor, CID755376. Taken together, these data indicate that activation of PKC/PKD reduced Ca^{2+}-mobilization by UTP. Using selective PKC and PKD inhibitors we hypothesized a key role for PKCs, although we cannot determine the specific isoforms involved in the alteration of Ca^{2+}-mobilization by PGE_2 after stimulation with UTP. The absence of effect after treatment with dibutyryl-cAMP indicates that the inhibition by PGE_2 is independent of PKA. These conclusions agree with previous evidence describing a regulation of P2Y signaling by PGE_2 [20]. Our data also indicate that the EP1–4 and P2Y receptors expression was not influenced by COX-2 activity.

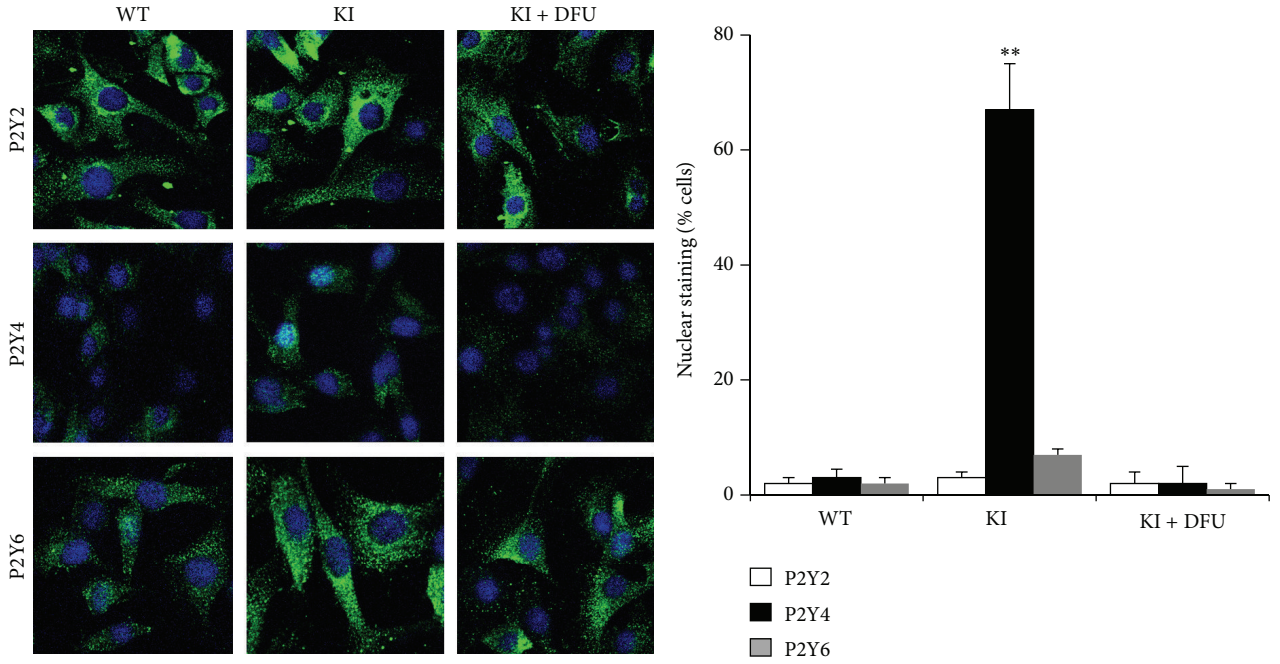

Figure 3: Subcellular distribution of P2Y2, P2Y4, and P2Y6 receptors in MEFs. WT or KI MEFs were cultured and, after changing the medium, were maintained in the absence or presence of 1 μM DFU for 2 h. Cells were fixed with paraformaldehyde (4%; pH 7.2) and permeabilized with cold methanol at RT. After incubation with anti-P2Y2, anti-P2Y4 and anti-P2Y6 antibodies (1 : 500) overnight at 4°C, cells were visualized by confocal microscopy using a FITC-conjugated secondary Ab (Alexa-Fluor 488, 1 : 1000). Nuclei were stained with Hoechst 33258. Coverslips were mounted in Prolong Gold antifade reagent (Molecular Probes) and the intensity of the fluorescence was measured using Image J software (NIH, Bethesda, MD, USA). Results show the mean + SD of three experiments. $^{**}P < 0.001$ versus the same condition in the WT cells.

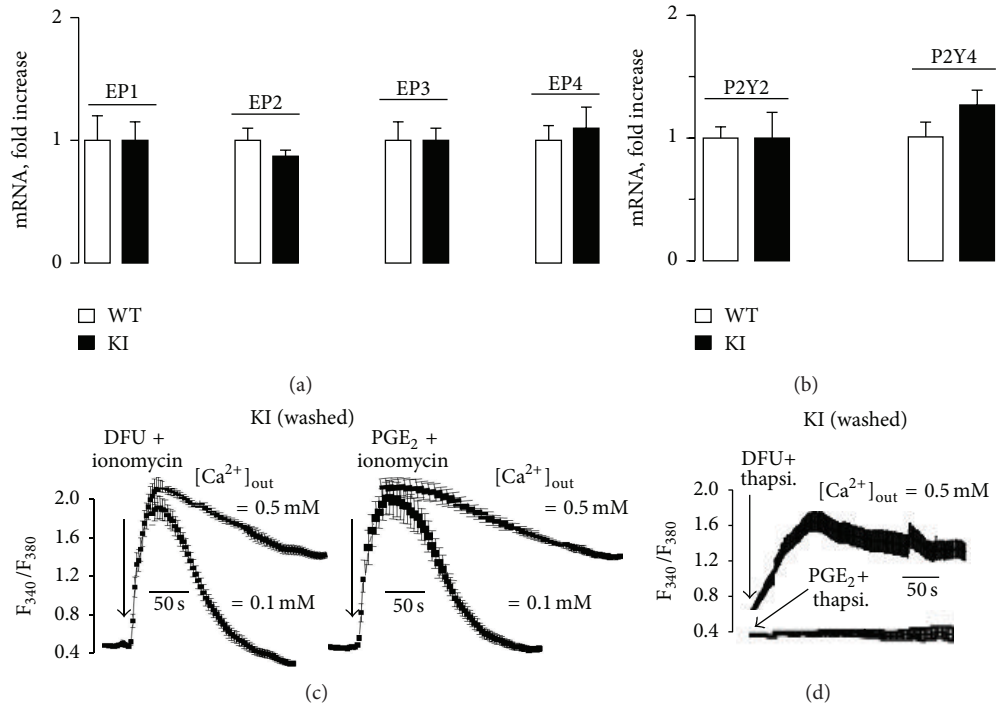

Figure 4: Characterization of EP1–4 and P2Y2–P2Y4 expression and effect of ionophores on Ca^{2+}-mobilization in MEF cells. The expression levels of the prostaglandin receptors EP1–4 and the levels of P2Y2 and P2Y4 were determined by qPCR (a-b). The response to 1 μM ionomycin (c) and 500 nM thapsigargin (d) on Ca^{2+}-mobilization was determined in MEFs overexpressing COX-2, using the dual excitation 340/380 nm protocol as described in Section 2. MEFs KI were washed with fresh medium to remove PGE_2 accumulated and maintained in the absence or presence of 1 μM DFU and 5 μM PGE_2. Different extracellular concentrations of calcium were used. Results show the mean + SD of three experiments (a-b) or a representative trace (c-d). $^{*}P < 0.05$ versus the same condition in WT cells.

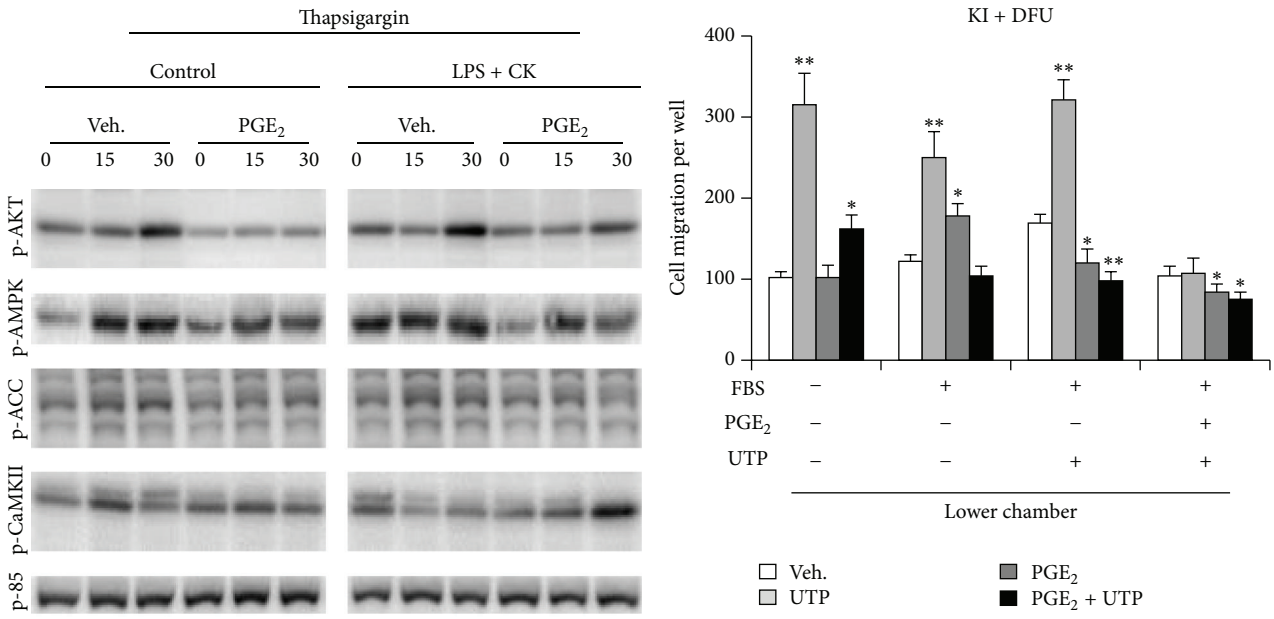

Figure 5: Effect of thapsigargin on Ca^{2+}-mobilization and migration of MEFs in response to UTP and chemotactic stimuli. WT or COX-2 KI cells were activated, or not, for 24 h with LPS (200 ng/mL) plus cytokines (IFN-γ, TNF-α, and IL-1β, 20 ng/mL) and then treated for 5 min with 500 nM thapsigargin and in the absence or presence of 5 μM PGE_2. The levels of the indicated phosphoproteins were determined by Western blot (a). The capacity of these cells to migrate in transwell was determined after incubation with 5 μM PGE_2 and/or 100 μM UTP. The migration was measured after 24 h of incubation in the absence or presence of different combinations of 10% FBS, PGE_2 (5 μM), or UTP (100 nM) in the lower wells (b). Results show a representative blot (a) out of three or the mean + SD of four experiments (b). $^{*}P < 0.05$; $^{**}P < 0.001$ versus the same condition in the absence of treatment in the upper chamber.

Interestingly, PGE_2 did not affect Ca^{2+} fluxes by ionomycin but suppressed the effect of thapsigargin, suggesting that PGE_2 alters Ca^{2+}-mobilization from intracellular stores.

PGE_2 promotes an internalization of P2Y4 in MEFs transfected with COX-2, an effect that is suppressed after inhibition of COX-2 with DFU. Based on these results, we hypothesize that the alteration in Ca^{2+}-mobilization in response to UTP in MEFs transfected with COX-2 might be due to a lower membrane localization of P2Y4 when PGE_2 production is enhanced. Moreover, the blockade in Ca^{2+}-mobilization by PGE_2 has an important reflect in terms of activation of different signaling pathways, including key regulators such as PKCs and energetic metabolism via AMPK activation and ACC inhibition.

Cell migration contributes to normal development and differentiation. Evidences in recent years have indicated that extracellular nucleotides can regulate the movement of "professional phagocytes" (macrophages, neutrophils, lymphocytes, and microglia) and other cell types (e.g., fibroblasts, endothelial cells, neurons, and keratinocytes) [43]. From a functional point of view, our data demonstrate that PGE_2 inhibits P2Y-dependent cell migration, regardless of chemoattractant. These observations are in agreement with Koizumi et al. and other authors who described that P2Y2,4,6 receptors participate in chemotactic actions [44]. In this way, recent studies have focused on stromal cells, such as macrophages and fibroblasts, playing a role in the inflammatory lesion. Here we describe a cross-regulation between PGE_2 and P2Y signaling that is independent of the PG receptors in MEFs. This mechanism is similar to that described by Través et al. [20], suggesting that macrophages and fibroblasts contribute to the regulation of inflammatory response and repair of tissue damage through aligned mechanisms involving P2Y signaling [35, 36]. Overall, the work suggests that targeting the stromal microenvironment is likely to be an important strategy for future anti-inflammatory therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

María Pimentel-Santillana and Paqui G. Través contributed equally to the work.

Acknowledgments

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References

[1] G. Burnstock, "A basis for distinguishing two types of purinergic receptor," in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*, R. W. Straub and L. Bolis, Eds., pp. 107–118, Raven Press, New York, NY, USA, 1978.

[2] R. A. North and E. A. Barnard, "Nucleotide receptors," *Current Opinion in Neurobiology*, vol. 7, no. 3, pp. 346–357, 1997.

[3] M. P. Abbracchio and G. Burnstock, "Purinoceptors: are there families of P2X and P2Y purinoceptors?" *Pharmacology and Therapeutics*, vol. 64, no. 3, pp. 445–475, 1994.

[4] G. Burnstock and C. Kennedy, "Is there a basis for distinguishing two types of P2-purinoceptor?" *General Pharmacology*, vol. 16, no. 5, pp. 433–440, 1985.

[5] M. E. Ferrero, "Purinoceptors in inflammation: fotential as anti-inflammatory therapeutic targets," *Frontiers in Bioscience*, vol. 16, no. 6, pp. 2172–2186, 2011.

[6] G. Burnstock, "Purinergic signalling: past, present and future," *Brazilian Journal of Medical and Biological Research*, vol. 42, no. 1, pp. 3–8, 2009.

[7] G. Burnstock and G. E. Knight, "Cellular distribution and functions of P2 receptor subtypes in different systems," *International Review of Cytology*, vol. 240, pp. 31–304, 2004.

[8] M. P. Abbracchio, G. Burnstock, J. Boeynaems et al., "International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy," *Pharmacological Reviews*, vol. 58, no. 3, pp. 281–341, 2006.

[9] A. Brunschweiger and C. E. Müller, "P2 receptors activated by uracil nucleotides: an update," *Current Medicinal Chemistry*, vol. 13, no. 3, pp. 289–312, 2006.

[10] G. Burnstock, "Purine and pyrimidine receptors," *Cellular and Molecular Life Sciences*, vol. 64, no. 12, pp. 1471–1483, 2007.

[11] G. Burnstock, G. E. Knight, and A. V. H. Greig, "Purinergic signaling in healthy and diseased skin," *Journal of Investigative Dermatology*, vol. 132, no. 3, part 1, pp. 526–546, 2012.

[12] S. Hashioka, Y. Wang, L. Little et al., "Purinergic responses of calcium-dependent signaling pathways in cultured adult human astrocytes," *BMC Neuroscience*, vol. 15, article 18, 2014.

[13] Y. Okada, T. Yada, T. Ohno-Shosaku, S. Oiki, S. Ueda, and K. Machida, "Exogenous ATP induces electrical membrane responses in fibroblasts," *Experimental Cell Research*, vol. 152, no. 2, pp. 552–557, 1984.

[14] S. Heeneman, J. P. Cleutjens, B. C. Faber et al., "The dynamic extracellular matrix: intervention strategies during heart failure and atherosclerosis," *The Journal of Pathology*, vol. 200, no. 4, pp. 516–525, 2003.

[15] V. Konya, G. Marsche, R. Schuligoi, and A. Heinemann, "E-type prostanoid receptor 4 (EP4) in disease and therapy," *Pharmacology and Therapeutics*, vol. 138, no. 3, pp. 485–502, 2013.

[16] T. Furuyashiki and S. Narumiya, "Roles of prostaglandin E receptors in stress responses," *Current Opinion in Pharmacology*, vol. 9, no. 1, pp. 31–38, 2009.

[17] M. A. Hull, S. C. W. Ko, and G. Hawcroft, "Prostaglandin EP receptors: targets for treatment and prevention of colorectal cancer?" *Molecular Cancer Therapeutics*, vol. 3, no. 8, pp. 1031–1039, 2004.

[18] A. Medeiros, C. Peres-Buzalaf, F. Fortino Verdan, and C. H. Serezani, "Prostaglandin E 2 and the suppression of phagocyte innate immune responses in different organs," *Mediators of Inflammation*, vol. 2012, Article ID 327568, 13 pages, 2012.

[19] M. Ito and I. Matsuoka, "Regulation of purinergic signaling by prostaglandin E2 in murine macrophages," *Journal of Pharmacological Sciences*, vol. 107, no. 4, pp. 443–450, 2008.

[20] P. G. Través, M. Pimentel-Santillana, L. M. G. Carrasquero et al., "Selective impairment of P2Y signaling by prostaglandin E2 in macrophages: implications for Ca^{2+}-dependent responses," *Journal of Immunology*, vol. 190, no. 8, pp. 4226–4235, 2013.

[21] R. Rodríguez-Rodríguez, P. Yarova, P. Winter, and K. Dora, "Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries," *British Journal of Pharmacology*, vol. 158, no. 6, pp. 1609–1620, 2009.

[22] J. C. Rodríguez-Prados, P. G. Través, J. Cuenca et al., "Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation," *Journal of Immunology*, vol. 185, no. 1, pp. 605–614, 2010.

[23] J. Xu, "Preparation, culture, and immortalization of mouse embryonic fibroblasts," in *Current Protocols in Molecular Biology*, chapter 28, unit 28.1, 2005.

[24] T. Inazumi, N. Shirata, K. Morimoto, H. Takano, E. Segi-Nishida, and Y. Sugimoto, "Prostaglandin E2-EP4 signaling suppresses adipocyte differentiation in mouse embryonic fibroblasts via an autocrine mechanism," *Journal of Lipid Research*, vol. 52, no. 8, pp. 1500–1508, 2011.

[25] C. Liu, S. La Rosa, and E. G. Hagos, "Oxidative DNA damage causes premature senescence in mouse embryonic fibroblasts deficient for Krüppel-like factor 4," *Molecular Carcinogenesis*, 2014.

[26] L. M. G. Carrasquero, E. G. Delicado, L. Sánchez-Ruiloba, T. Iglesias, and M. T. Miras-Portugal, "Mechanisms of protein kinase D activation in response to $P2Y_2$ and P2X7 receptors in primary astrocytes," *GLIA*, vol. 58, no. 8, pp. 984–995, 2010.

[27] C. Hervás, R. Pérez-Sen, and M. T. Miras-Portugal, "Coexpression of functional P2X and P2Y nucleotide receptors in single cerebellar granule cells," *Journal of Neuroscience Research*, vol. 73, no. 3, pp. 384–399, 2003.

[28] P. Marin-Garcia, J. Sánchez-Nogueiro, A. Diez et al., "Altered nucleotide receptor expression in a murine model of cerebral malaria," *The Journal of Infectious Diseases*, vol. 200, no. 8, pp. 1279–1288, 2009.

[29] J. Sánchez-Nogueiro, P. Marín-García, D. León et al., "Axodendritic fibres of mouse cerebellar granule neurons exhibit a diversity of functional P2X receptors," *Neurochemistry International*, vol. 55, no. 7, pp. 671–682, 2009.

[30] D. Breitkreutz, L. Braiman-Wiksman, N. Daum, M. F. Denning, and T. Tennenbaum, "Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium," *Journal of Cancer Research and Clinical Oncology*, vol. 133, no. 11, pp. 793–808, 2007.

[31] M. R. Elliott, F. B. Chekeni, P. C. Trampont et al., "Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance," *Nature*, vol. 461, no. 7261, pp. 282–286, 2009.

[32] J. E. Hazleton, J. W. Berman, and E. A. Eugenin, "Purinergic receptors are required for HIV-1 infection of primary human macrophages," *Journal of Immunology*, vol. 188, no. 9, pp. 4488–4495, 2012.

[33] L. Vitiello, S. Gorini, G. Rosano, and A. La Sala, "Immunoregulation through extracellular nucleotides," *Blood*, vol. 120, no. 3, pp. 511–518, 2012.

[34] A. Seregi, S. Doll, A. Schobert, and G. Hertting, "Functionally diverse purinergic P(2Y)-receptors mediate prostanoid synthesis in cultured rat astrocytes: the role of ATP-induced phosphatidyl-inositol breakdown," *Eicosanoids*, vol. 5, pp. S19–S22, 1992.

[35] D. Knight, "Epithelium-fibroblast interactions in response to airway inflammation," *Immunology and Cell Biology*, vol. 79, no. 2, pp. 160–164, 2001.

[36] M. Jordana, B. Sarnstrand, P. J. Sime, and I. Ramis, "Immune-inflammatory functions of fibroblasts," *European Respiratory Journal*, vol. 7, no. 12, pp. 2212–2222, 1994.

[37] M. Barbera-Cremades, A. Baroja-Mazo, A. I. Gomez, F. Machado, F. Di Virgilio, and P. Pelegrińn, "P2X7 receptor-stimulation causes fever via PGE2 and IL-1β release," *The FASEB Journal*, vol. 26, no. 7, pp. 2951–2962, 2012.

[38] A. B. Tobin, "G-protein-coupled receptor phosphorylation: where, when and by whom," *British Journal of Pharmacology*, vol. 153, no. 1, pp. S167–S176, 2008.

[39] A. R. Hardy, P. B. Conley, J. Luo, J. L. Benovic, A. W. Poole, and S. J. Mundell, "P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms," *Blood*, vol. 105, no. 9, pp. 3552–3560, 2005.

[40] S. J. Mundell, M. L. Jones, A. R. Hardy et al., "Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function," *Molecular Pharmacology*, vol. 70, no. 3, pp. 1132–1142, 2006.

[41] P. B. Jacobson, S. L. Kuchera, A. Metz, C. Schachtele, K. Imre, and D. J. Schrier, "Anti-inflammatory properties of Go 6850: a selective inhibitor of protein kinase C," *Journal of Pharmacology and Experimental Therapeutics*, vol. 275, no. 2, pp. 995–1002, 1995.

[42] S. A. Matthews, M. N. Navarro, L. V. Sinclair, E. Emslie, C. Feijoo-Carnero, and D. A. Cantrell, "Unique functions for protein kinase D1 and protein kinase D2 in mammalian cells," *Biochemical Journal*, vol. 432, no. 1, pp. 153–163, 2010.

[43] R. Corriden and P. A. Insel, "New insights regarding the regulation of chemotaxis by nucleotides, adenosine, and their receptors," *Purinergic Signalling*, vol. 8, no. 3, pp. 587–598, 2012.

[44] S. Koizumi, Y. Shigemoto-Mogami, K. Nasu-Tada et al., "UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis," *Nature*, vol. 446, no. 7139, pp. 1091–1095, 2007.

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Research Article

$P2Y_{12}$ Receptor on the Verge of a Neuroinflammatory Breakdown

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In the CNS, neuroinflammation occurring during pathologies as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) is the consequence of an intricate interplay orchestrated by various cell phenotypes. Among the molecular cues having a role in this process, extracellular nucleotides are responsible for intercellular communication and propagation of inflammatory stimuli. This occurs by binding to several receptor subtypes, defined P2X/P2Y, which are widespread in different tissues and simultaneously localized on multiple cells. For instance, the metabotropic $P2Y_{12}$ subtype is found in the CNS on microglia, affecting activation and chemotaxis, on oligodendrocytes, possessing a hypothesized role in myelination, and on astrocytes. By comparative analysis, we have established here that $P2Y_{12}$ receptor immunolabelled by antibodies against C-terminus or second intracellular loop, is, respectively, distributed and modulated under neuroinflammatory conditions on ramified microglia or myelinated fibers, in primary organotypic cerebellar cultures, tissue slices from rat striatum and cerebellum, spinal cord sections from symptomatic/end stage SOD1-G93A ALS mice, and finally autoptic cortical tissue from progressive MS donors. We suggest that modulation of $P2Y_{12}$ expression might play a dual role as analytic marker of branched/surveillant microglia and demyelinating lesions, thus potentially acquiring a predictive value under neuroinflammatory conditions as those found in ALS and MS.

1. Introduction

A basic set of proteins and mRNAs are differentially expressed among cell types, temporally and spatially, generating a vast assortment of cell phenotypes and/or activation states within a single tissue. Outlining this protein/mRNA portrait is thus crucial for understanding not only the uniqueness characterizing cells, but especially their distinguished functions [1]. This becomes of major relevance when the balance between cell-intrinsic properties and identity cues received and provided by each cell to its neighboring cells then shapes the cell-to-cell cross talk during physiopathological conditions. In the CNS, neuroinflammation is the typical consequence of the interchange among different cell types, particularly neurons, astrocytes, oligodendrocytes and microglia, of a variety of cues as neurotransmitters, cytokines, chemokines, toxic metabolites that condition the final protein/mRNA profiles of cells, their activation states and functional outcomes [2, 3]. Since neuroinflammation accompanies a large variety of neurodegenerative diseases, there is increasing interest in determining how the different cell phenotypes and cellular interconnectivity might contribute to reduce inflammation and reverse neurodegeneration.

Microglia actively participate to the context-dependent, neuroprotective/neurotoxic molecular network that is triggered during neuroinflammation [4]. Among the molecular cues having a key role in this process, extracellular nucleotides are major responsible for intercellular communication and propagation of inflammatory stimuli [5–7]. This occurs by specific binding to various receptor subtypes, termed ionotropic P2X and metabotropic P2Y, which are simultaneously localized on several different cell phenotypes. Among these, the $P2Y_{12}$ receptor subtype [8] belonging to the Gi class of G protein-coupled receptors is activated by ADP. Two transcript variants apparently encoding the same protein isoform have been identified so far for $P2RY_{12}$ gene [8],

but the determinants for cell specificity of $P2Y_{12}$ protein expression are still unknown.

$P2Y_{12}$ is found on the surface mainly, but not exclusively, of blood platelets, where it acts as blood clotting regulator and target for the treatment of thromboembolisms [9, 10]. In the nervous system, the tissue- and cellular-selective expression of $P2Y_{12}$ exhibits a pattern throughout white and gray matter consistent with astrocyte expression [8], although it has not been found colocalization between $P2Y_{12}$ and GFAP-positive astrocytes in rat brain cortex and nucleus accumbens, despite the abundant presence of $P2Y_{12}$ mRNA [11]. Moreover, we previously established *in vivo* the expression of $P2Y_{12}$ in oligodendrocytes and myelin sheaths of rat cerebral cortex, subcortical areas, and periventricular white matter. This localization is confirmed throughout the corticospinal tract, therefore suggesting high conserved tissue-homogeneity and phenotype-specificity, and a hypothesized role in myelination [12–15]. $P2Y_{12}$ is finally observed in brain and spinal cord resident microglia, where it affects activation, chemotaxis [16–19] and neuropathic pain [20], but it is not observed, for example, in peripheral macrophages in spleen [18, 20]. $P2Y_{12}$ expression in primary microglia is variable with postnatal development and shows sexually dimorphic behavior [21].

Through the use of all the available $P2Y_{12}$-immunoreactive antibodies recognizing the C-terminus or the second intracellular loop of the receptor, the aim of the present work is to provide comparative evidence about $P2Y_{12}$ cell specificity in microglia versus oligodendrocytes particularly from the healthy and diseased CNS under neuroinflammatory conditions as those sustained during amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS).

2. Materials and Methods

2.1. Animals. Adult B6.Cg-Tg (SOD1-G93A)1Gur/J mice expressing high copy number of mutant human SOD1 with a Gly93Ala substitution (SOD1-G93A) were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in our indoor animal facility as described in Apolloni and collaborators, [22]. The animals were euthanized, according to the guidelines for preclinical testing and colony management [23]. Also neonatal Wistar and adult Lewis rats (from Charles River Laboratories, LC, Italy) were housed in our indoor animal facility.

Animal procedures were performed according to European Guidelines for the use of animals in research (86/609/CEE) and the requirements of Italian laws (D.L. 116/92). Ethical procedures were approved by Animal Welfare Office, Department of Public Health and Veterinary, Nutrition and Food Safety, General Management of Animal Care and Veterinary Drugs of the Italian Ministry of Health. All efforts were made to minimize animal suffering and number of animals necessary to produce reliable results.

2.2. Mouse Microglia Primary Cultures. Microglia primary cultures prepared from mouse cortex as previously described [24] were about 98% pure, as verified by immunofluorescence with CD11b (for microglia), glial fibrillary acidic protein (GFAP, for astrocytes), neuronal nuclei (NeuN, for neurons), and SMI94 (for oligodendrocytes).

2.3. Rat Microglia and Oligodendrocyte Primary Cultures. Purified cultures of oligodendrocytes were prepared from forebrain of postnatal day 1-2 Wistar rats, according to minor modifications from a previously described method [25]. After removing the meninges, cortices were minced, digested, and dissociated by passage through 70 μm nylon cell strainer (BD Biosciences, Europe). Cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 4 mM glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin in T75 poly-D-lysine-coated flasks, at about 10 million cells/flask. The cultures were maintained at 37°C in a 5% CO_2 and 95% air atmosphere for 14 days. Mixed glial cultures were then shaken at 200 rpm at 37°C for 1 h and 98% pure microglia collected from the supernatant of each flask, as verified by immunofluorescence with GFAP, NeuN, Neural/Glial antigen 2 (NG2, for oligodendroglial precursor cells), myelin basic protein (MBP, for mature oligodendrocytes), and CD11b. After further shaking at 200 rpm and 37°C for 15–18 h, the detached cell suspension was finally incubated for 1 h at 37°C for differential adhesion of contaminating cells. The non-adherent cells were filtered through 40 μm nylon cell strainer (BD Biosciences), spun for 10 min at 100 g, resuspended in oligodendroglial precursor cells medium (basal chemically defined medium: DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1% bovine serum albumin (BSA), 50 $\mu g\,mL^{-1}$ apo-transferrin, 5 $\mu g\ mL^{-1}$ insulin, 30 nM sodium selenite, 10 nM D-biotin, and 10 nM hydrocortisone) containing 10 $ng\,mL^{-1}$ platelet derived growth factor-AA (PDGF-AA) and 10 $ng\,mL^{-1}$ basic fibroblast growth factor (bFGF), and seeded at the density of 1×10^4 cells/cm^2 into poly D,L-ornithine-coated plates. The cells were then induced to differentiate into mature oligodendrocytes when the basal chemically defined medium was added with 15 nM triiodothyronine, 10 $ng\,mL^{-1}$ ciliary neurotrophic factor (CNTF) and 0,05 mg 10 mL^{-1} N-acetyl-L-cysteine (NAC), for 4–7 days. A 98% pure population of oligodendrocytes was thus obtained, as verified by immunofluorescence with NG2, MBP, GFAP, NeuN, and CD11b antibodies.

2.4. Cerebellar Organotypic Cultures. Organotypic cerebellar slice cultures were prepared with modifications from previously published protocols [26, 27]. Briefly, cerebella were excised from neonatal Wistar rat (8–10 days old), cut on a Mc Ilwain tissue chopper (400 μm) and parasagittal slices separated into cold Hank's balanced salt solution (HBSS). Two to three slices were plated on each Millicell CM culture inserts (Millipore, Italy) and kept in organotypic maintenance medium (50% Basal Medium Eagle-BME, 25% HBSS, 25% heat-inactivated horse serum, supplemented with 5 mg/mL glucose, 1 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin) at 37°C in a 5% CO_2 and 95% air atmosphere. The medium was changed every 2 days and double immunofluorescence was performed in free floating

after 7–10 days *in vitro*. Organotypic cultures were washed three times with PBS, fixed with 4% paraformaldehyde for 1 h, rinsed, and blocked for 1 h in Phosphate buffered saline (PBS)/10% normal donkey serum (NDS)/0.4% Triton X-100. Primary antibodies were incubated for 24 h in PBS/2% NDS/0.4% Triton X-100 (Table 1). The secondary antibodies used for double labelling are Cy3-conjugated donkey anti-rabbit IgG (1 : 100, Jackson Immunoresearch, Europe) or Alexa Fluor 488-AffiniPure donkey anti-mouse IgG (1 : 200, Jackson Immunoresearch). After rinsing, sections were mounted, covered with Fluoromount medium (Sigma-Aldrich, Milan, Italy) and a coverslip, and analyzed by confocal microscopy.

2.5. Immunofluorescence on Mouse Microglia and Rat Oligodendrocytes. Microglia and oligodendrocytes were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min (oligodendrocytes) or 20 min (microglia), washed, permeabilized with 0.05–0.1% Triton X-100 for 10 min, rinsed, and blocked for 30 min in PBS/1% BSA. Microglia were stained for about 3 h at 37°C in 1% PBS/BSA with 5 μg/mL Cy2-phalloidin (Sigma-Aldrich), in combination with primary antibodies against $P2Y_{12}$ receptor, as reported in Table 1. Oligodendrocytes were stained with primary antibodies against $P2Y_{12}$ receptor and MBP or NG2. The secondary antibodies used for double labelling are Cy3-conjugated donkey anti-rabbit IgG (1 : 100, Jackson Immunoresearch) or Alexa Fluor 488-AffiniPure donkey anti-mouse IgG (1 : 200, Jackson Immunoresearch). Cells were extensively washed and stained with the nucleic acid blue dye, Hoechst 33342 (1 : 1000). After rinsing, cells were covered with Fluoromount medium (Sigma-Aldrich) and a coverslip and analyzed by confocal microscopy.

2.6. Histological Procedures and Immunofluorescence on Mouse and Rat Tissue. Animals were anaesthetized by intraperitoneal injection of chloral hydrate (500 mg/Kg) and transcardially perfused with PBS followed by 4% paraformaldehyde at pH 7.4. Tissue samples (mice spinal cord and rat brain) were then postfixed for 1-2 days, and cryoprotected in 30% sucrose in PBS at 4°C. Tissues were stored at −80°C.

Mice spinal cords (L3–L5) were cut at 30 μm thickness with a frozen microtome. Sections were mounted on slide glasses and allowed to air-dry (1-2 h). A rectangle was then drawn around the sections with a PAP pen (Sigma-Aldrich). Rat brains were cut at 40 μm thickness using a cryostat microtome and sections were processed in free-floating. Double immunofluorescence analysis was performed after blocking in PBS containing 10% NDS and 0.3% Triton X-100 for 1 h at room temperature. Sections were incubated with different combinations of primary antibodies (Table 1), in PBS, 0.3% Triton X-100 and 2% NDS, for 24–48 h at 4°C. Finally, sections were washed with PBS and incubated with appropriate fluorescent-conjugated secondary antibodies for 3 h at room temperature. After rinsing, sections were covered with Fluoromount medium (Sigma-Aldrich) and a coverslip and analyzed by confocal microscopy.

2.7. Human Tissue Source, Lesion Detection, Classification and Immunofluorescence. Tissues supplied by UK Multiple Sclerosis Tissue Bank at Imperial College, London, were collected postmortem with fully informed consent from both donors and close relatives. Procedures for retrieval, processing, and storage have gained ethical approval from all appropriate committees. The brain tissues analyzed were from 7 neuropathologically confirmed cases of secondary progressive MS (SPMS). Analysis was performed also on samples from patients who died due to nonneurological diseases (healthy). Cerebral hemispheres were fixed with 4% paraformaldehyde for about two weeks, coronally sliced, and blocked. Individual blocks were cryoprotected in 30% sucrose for one week and frozen by immersion in isopentane precooled on a bed of dry ice. Frozen tissue blocks were stored at −80°C. Cryostat sections (30–40 μm thick) were either stained with Luxol fast blue and cresyl fast violet (Kluver-Barrera staining), in order to detect white matter lesions and their cellularity, or subjected to immunohistochemistry for MBP, in order to distinguish grey matter lesions. Sections were processed in free-floating for double immunofluorescence studies, as described in Amadio and collaborators [13].

2.8. Confocal Analysis. Immunofluorescence was analyzed by means of a confocal laser scanning microscope (Zeiss, LSM700, Germany) equipped with four laser lines: 405, 488, 561, and 639 nm. The brightness and contrast of the digital images were adjusted using the Zen software (Zeiss).

2.9. Plasmid Construction and Transfection. Human $P2Y_{12}$ complete cDNA was obtained by reverse transcription with enhanced avian RT-PCR kit (Sigma-Aldrich) from total human brain RNA (Life Technologies, Paisley, UK). The obtained cDNA was then cloned into the XhoI and XbaI sites of the eukaryotic expression vector CS2 + MT to provide N-terminal 6X c-Myc epitope-tagged mammalian expression plasmids, which has been validated by DNA sequencing. Oligos used for amplification were as follows: *forward* 5′gcCTCGAGatgcaagccgtcgacaacctc3′ and reverse 5′gcTCTAGAgtttacattggagtctcttc designed on human $P2Y_{12}$ mRNA (NM_022788.3).

Human embryonic kidney 293 cells (HEK 293) and Sloan-Kettering neuroblastoma SH-SY5Y clone (SH-SY5Y) cells were grown in DMEM supplemented with 10% FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37°C in atmosphere containing 5% CO_2. One day before transfection, HEK293 or SH-SY5Y cells were plated and transfection of $P2Y_{12}$-CS2 + MT or CS2 + MT empty vector was performed with lipofectamine 2000 (Life Technologies), according to manufacturer instructions.

2.10. RT-PCR. Primary rat microglial and oligodendrocyte cells were lysed with TRIzol (Life Technologies) and total RNA was extracted following the manufacturer's instructions. After DNase treatment (Qiagen, Hilden, Germany), equal amount of total RNA (1 μg) was subjected to retrotranscription by high capacity RNA-to-cDNA kit (Life Technologies) and 50 ng of each cDNA were amplified with rat

Table 1: Primary antibodies employed in the study.

Antigen	Clone	Epitope (aa)	Target	Dilution	Source
MBP	2	119–131	Mature oligodendrocytes/myelin	1 : 100	Chemicon
SMI94	SMI-94	70–89	Mature oligodendrocytes/myelin	1 : 1000	Covance
NG2 chondroitin sulfate proteoglycan	132.39	*	Oligodendrocyte precursor cells	1 : 200	Chemicon
CD11b rat	OX-42	*	Microglia/macrophages	1 : 200	AbD Serotec
CD11b mouse	5C6	*	Microglia/macrophages	1 : 200	AbD Serotec
CD68	FA-11	*	Macrophages/monocytes	1 : 200	AbD Serotec
HLA-DP, DQ, DR (MHC II)	CR3/43	*	Microglia/macrophages	1 : 100	Dako
Integrin αII/β3	(A2A9/6)	Full length	Platelets	1 : 100	Santa Cruz
NeuN	A60	*	Neurons	1 : 200	Millipore
GFAP	G-A-5	*	Astrocytes	1 : 400	Sigma
$P2Y_{12}$ receptor (*intra1*)	Polyclonal	125–142	$P2Y_{12}$ receptor	1 : 200	Sigma
$P2Y_{12}$ receptor (*intra2*)	Polyclonal	125–142	$P2Y_{12}$ receptor	1 : 200–300	Alomone
$P2Y_{12}$ receptor-ATTO-594 (*intra fl*)	Polyclonal	125–142	$P2Y_{12}$ receptor	1 : 50	Alomone
$P2Y_{12}$ receptor mouse/rat (*c-ter*)	Polyclonal	C-terminus [18]	$P2Y_{12}$ receptor	1 : 200	Anaspec
$P2Y_{12}$ receptor human (*c-ter*)	Polyclonal	324–342	$P2Y_{12}$ receptor	1 : 200	Anaspec

CD11b: cluster of differentiation 11b; CD68: cluster of differentiation 68; fl: fluorescent at 594 nm; GFAP: glial fibrillary acidic protein; HLA: human leukocyte antigen; MBP: myelin basic protein; MHC: major histocompatibility complex; NeuN: neuronal nuclei; NG2: neural/glial antigen 2.
*Not specified in the data sheet.

$P2Y_{12}$ specific primers (F: 5′-GATTGATAACCATTGACC-3′; R: 5′-GGTGAGAATCATGTTAGG-3′). The number of cycles was fixed to 35. Amplification products (10 μL of 20) were electrophoresed on 2% agarose gel containing ethidium bromide (1 μg/mL, Sigma Aldrich), photographed under UV light using Kodak Image Station 440CF, with Molecular Imaging Software 4.0.1.

2.11. Protein Extraction, SDS-PAGE and Western Blotting. In order to isolate total protein extracts, microglia and oligodendrocytes were harvested with ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), added with protease inhibitor cocktail (Sigma Aldrich). Lysates were kept for 30 min on ice and then centrifuged for 10 min at 14,000 rpm, at 4°C. Snap-frozen blocks from cases MS114, MS125, and MS163 supplied by UK Multiple Sclerosis Tissue Bank at Imperial College in London were homogenized in RIPA buffer, added with protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 14,000 rpm for 10 min at 4°C. Rat brain and mouse brain and spinal cord were homogenized and sonicated in RIPA buffer, added with protease inhibitor cocktail, kept for 1 h on ice, and centrifuged at 4°C for 10 min at 13,000 rpm. Supernatants were collected and analyzed for protein content by Bradford colorimetric assay (Biorad, Milan, Italy). Analysis of protein components was performed by polyacrylamide gel (SDS-PAGE) separation (BioRad) and transfer onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, IT). After saturation, blots were probed overnight at 4°C, with the specified antibody (Table 1), and finally incubated for 1 h with HRP-conjugated secondary antibodies and detected on X-ray film (Aurogene, Rome, Italy), using ECL Advance Western blotting detection kit (Amersham Biosciences). Quantifications were performed by Kodak Image Station. $P2Y_{12}$ protein was detected with molecular mass comprised between 40 kDa (as predicted by amino acid sequence) and 49-50 kDa (as predicted by the manufacturer data sheet).

2.12. Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM). Analysis was performed with the statistical software package MedCalc (Medcalc Software, Mariakerke, Belgium). Statistical differences between groups were verified by Student's t-test. $^*P < 0.05$ was considered significant.

3. Results

In order to provide wide ranging comparative analysis of $P2Y_{12}$ expression particularly in microglia and oligodendrocytes under neuroinflammatory conditions, we performed immunofluorescence and confocal analysis of receptor expression in primary cortical and organotypic cerebellar cultures, in tissue slices from rat striatum and cerebellum, in

spinal cord sections from symptomatic and end stage SOD1-G93A ALS mouse model, finally in autoptic cortical tissue from progressive MS donors.

3.1. Mapping the Recognition Sites of P2Y$_{12}$ Antibodies on Receptor Protein and Antibodies Validation. P2Y$_{12}$ receptor is formed by two transcript variants that give rise to identical proteins with 342 amino acids, a secondary structure constituted by seven hydrophobic transmembrane domains connected by three extracellular and three intracellular loops, with four extracellular cysteine residues most likely contributing to the nucleotide binding site [28] (Figure 1(a)). The commercially available P2Y$_{12}$ antibodies that we mostly used in our work are raised against the second intracellular loop, and precisely amino acids 125–142 (here named *intra1*, *intra2*, and *intra fl*; see red circle in Figure 1(a)), and against the C-terminus, here named *c-ter* (see green oval in Figure 1(a)) (see also Table 1).

In order to validate the use of these different antibodies for P2Y$_{12}$ receptor, we compared them on recombinant P2Y$_{12}$ receptor protein obtained by cloning the complete cDNA of the human receptor into the eukaryotic expression vector CS2 + MT, to provide the expression of a N-terminal c-Myc tagged fusion protein. After transfection into SH-SY5Y and HEK293 cell lines, we analyzed total protein extracts by SDS-PAGE and Western blotting using *c-ter*, *intra1*, and *intra2* antibodies. Although with different intensities, all the antibodies recognize the myc-P2Y$_{12}$ protein band at the predicted molecular mass of 50 kDa. No signal is detected when transfection is performed with empty vector (control). These results confirm the specificity of the used antibodies toward denatured recombinant P2Y$_{12}$ receptor (Figure 1(b)).

In order to verify the expression of P2Y$_{12}$ mRNA in rat primary oligodendrocytes (OL) and microglia (rMG), RT-PCR was performed using specific primers designed on receptor sequence. As shown in Figure 1(d), RT-PCR on both rMG and OL reveals the presence of the predicted P2Y$_{12}$ mRNA band. The absence of DNA contamination is confirmed in empty control lanes.

Next, we validated the antibodies with protein extracts from dissociated primary cultures or tissues from different species. P2Y$_{12}$ protein is specifically recognized in extracts from human cerebral cortex snap frozen tissue, rat and mouse brain, mouse primary microglia (mMG) and OL (Figure 1(c)). No signals are obtained when the immunoreactions are performed in the presence of P2Y$_{12}$ neutralizing peptides, when available from manufacturer (data not shown).

3.2. Presence of P2Y$_{12}$ Receptor in Dissociated and Organotypic Primary Cultures. In order to establish cell specificity of P2Y$_{12}$ receptor expression, we performed comparative immunofluorescence and confocal analysis in primary dissociated cortical microglia and oligodendrocytes (Figures 2(a) and 2(b)), as well as organotypic cerebellar cultures (Figure 2(c)). P2Y$_{12}$ is strongly recognized by both C-terminus- and second intracellular loop-recognizing antibodies (red, *c-ter*, upper left inset; *intra fl*, upper right inset; *intra1*, lower right inset and *intra2*, lower left inset), specifically distinguishing the very heterogeneous morphological features of mouse primary microglia, as shown by double fluorescence and confocal analysis performed with phalloidin (green), a marker for filamentous actin (Figure 2(a)). Fan-like cells (insets), elongated rod-like cell bodies with short and tiny branches, asymmetrical hairy cells with miniature processes or lamellipodia are simultaneously observed (*c-ter* Phalloidin Hoechst, merged). Likewise, all the P2Y$_{12}$ antibodies (*intra2*, *intra1*, *c-ter*, and *intra fl*) recognize the multibranched morphology of rat mature oligodendrocytes (Figure 2(b), OL, insets, red) in primary cultures, as confirmed by double immunofluorescence and confocal analysis with MBP antibody (OL, *intra1*-MBP, yellow merged image). In addition, both *intra2* and *c-ter* antibodies distinguish the NG2-positive rat oligodendrocyte precursor cells (OPC, merged insets).

P2Y$_{12}$ immunoreactivity is confirmed in the *ex-vivo* system of organotypic rat cerebellar cultures (Figure 2(c)). However, differently from mouse microglia and rat oligodendrocyte primary cultures, the second intracellular loop- (red, *intra1*) and C-terminus-recognizing (red, *c-ter*) antibodies surprisingly immunoreact with different cell phenotypes when the integrity and cytoarchitecture of the tissue is preserved, as in organotypic cultures. While *intra1* antibody (red) exclusively labels MBP-positive fibers (green) and highlights myelin structures, *c-ter* antibody does not immunoreact with myelinated fibers, being present on cells likely resembling microglia (insets), as also confirmed by colocalization with microglial marker CD11b (data not shown). Results similar to those found with *intra1* were also obtained with *intra2* antibodies, lots AN01/02/04 (data not shown).

3.3. Cell-Selective Presence of P2Y$_{12}$ Receptor in Rat Brain Tissue. We next verified if the cell type-specific presence of P2Y$_{12}$ receptor observed in organotypic culture, either in myelin structures or in microglia, respectively, by the use of the second intracellular loop-(*intra1*) or the C-terminus-recognizing (*c-ter*) antibody, is also confirmed in rat cerebellar and striatal slice tissues (Figure 3). All the antibodies raised against the second intracellular loop (red, *intra1*, *intra2*, *intra fl*) identify the abundant presence of P2Y$_{12}$ receptor only on myelinated fibers from both cerebellum (Figures 3(a), 3(b), and 3(c)) and striatum (Figures 3(d), 3(e), and 3(f)), as confirmed by colocalization of signals obtained with *intra fl* and MBP antibodies in cerebellum (+MBP, inset c2, merged, yellow) and striatum (data not shown); colocalization of signals obtained with *intra1* and *intra2* with MBP antibodies in cerebellum and striatum (data not shown); immunoreactivity of *intra1*, *intra2*, and *intra fl* antibodies for structures identical to those observed in cerebellum (data not shown) and striatum with MBP antibody (see for instance MBP, inset e1, green); identification by second intracellular loop P2Y$_{12}$ antibodies of structures totally different from both Bergmann glia and astrocytes recognized by specific GFAP antibody in cerebellum (inset b1, green), and from microglia identified by specific CD11b antibody in cerebellum (+CD11b, inset c1, merged, green) and striatum (+CD11b, inset f1, merged, green). Of notice, all the 2nd intracellular loop

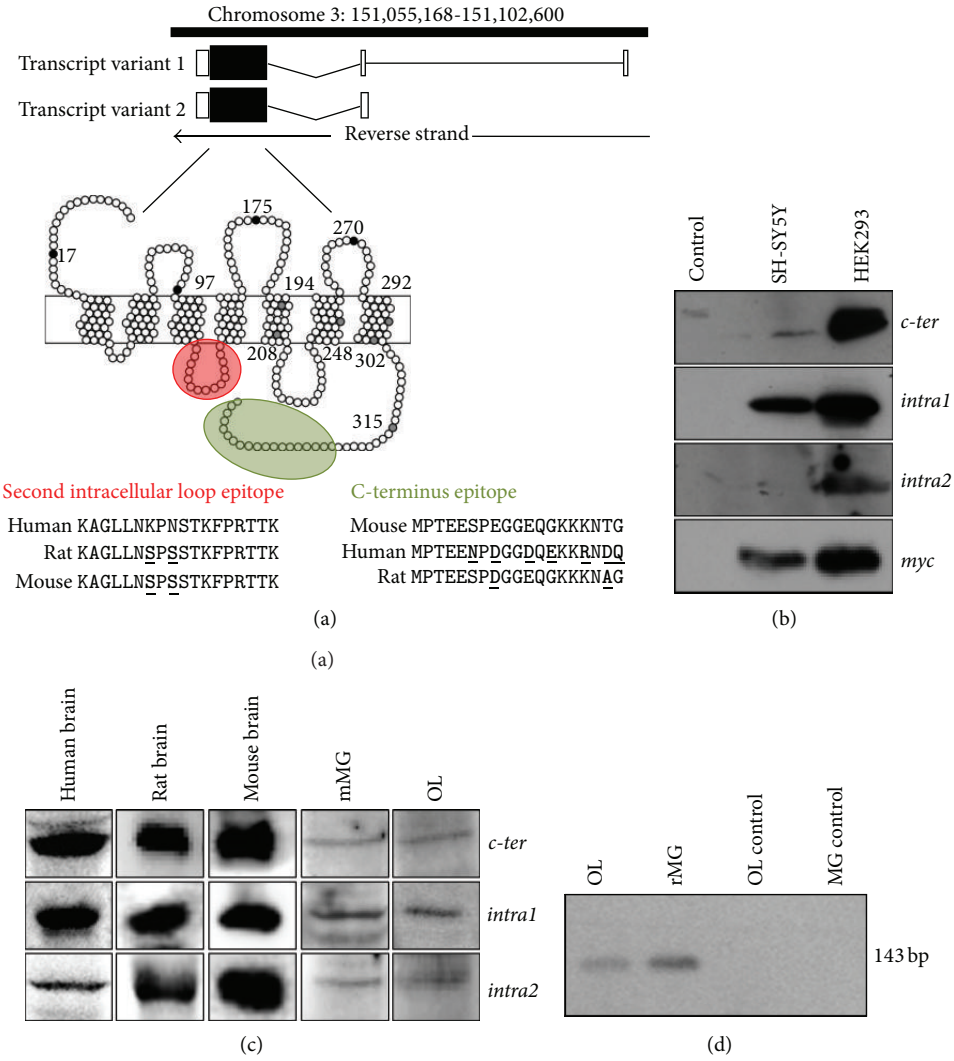

FIGURE 1: $P2Y_{12}$ antibodies validation and RT-PCR analysis. (a) Scheme of human $P2RY_{12}$ gene location, transcript variants [29, 30], and protein structure, with amino acid epitopes recognized by the used antibodies (Table 1) and highlighted in color (*intra1*, *intra2*, and *intra fl*, red circle; *c-ter*, green oval). Species conservation for each epitope was calculated by using BLAT tool of UCSC genome browser [31]. (b) Total protein extracts from SH-SY5Y or HEK293 cells expressing Myc-tagged $P2Y_{12}$ receptor were subjected to Western blot analysis with the indicated antibodies. (c) Total protein extracted from human, rat and mouse brain, from primary mouse microglia (mMG) and rat oligodendrocyte (OL) cultures were subjected to Western blot analysis with the indicated antibodies. For *intra2* antibodies, lots AN01/02/04/0502/0602 were used. (d) RT-PCR using primers specific for $P2Y_{12}$ mRNA was performed on total RNA from rat microglia (rMG) and OL. Control lanes show RT-PCR performed without reverse transcriptase enzyme.

antibodies are able to describe the specific cytoarchitecture of both cerebellum, where radiant and sparse fibers clearly characterize the *lobuli*, and of striatum, where white matter is instead organized in distinct bundles.

As in organotypic cultures, the C-terminus antibody (red, *c-ter*) instead recognizes only microglia in rat cerebellum (Figures 3(g), 3(h), and 3(i), and insets g1, h1, i1), striatum (Figures 3(j), 3(k), and 3(l)) and cerebral cortex slices (data not shown), with a signal more uniformly distributed throughout the whole tissue. By comparing the CD11b and *c-ter* immunolabelling in the striatum, we furthermore observe that the mutual intensity of signals is cell-selective within the microglia population, with some cells exclusively positive for *c-ter* (arrow), and others instead showing different grades of CD11b-*c-ter* colocalization (see for instance orange, yellow and greenish cells in the merged field). Regrettably, double immunofluorescence with *c-ter* and Iba1 microglia marker [32] is not practicable, since both antibodies are raised in the same species. However, no colocalization of signals is ever shown between MBP (green) and *c-ter* antibodies (+MBP, inset j1, merged).

3.4. $P2Y_{12}$ Expression Is Modulated during Neuroinflammation in Spinal Cord Microglia of SOD1-G93A ALS Mouse Model. In order to verify if $P2Y_{12}$ recognized by *c-ter* antibody exclusively in microglia from rat brain slices could be

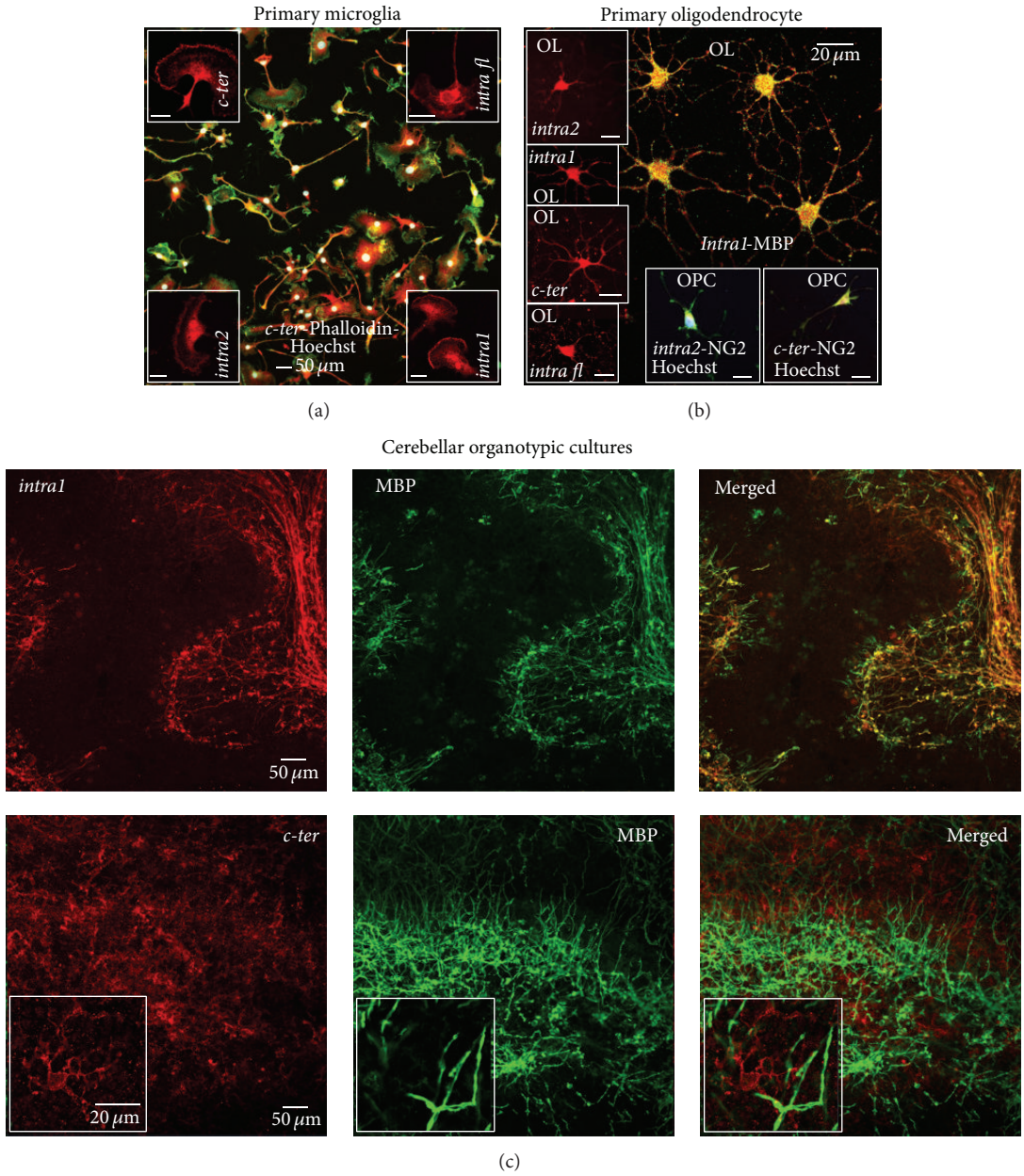

FIGURE 2: $P2Y_{12}$ receptor in dissociated and organotypic primary cultures. (a) Mouse primary cortical microglia were subjected to immunofluorescence and confocal analysis with phalloidin (green, merged field) and $P2Y_{12}$ receptor antibodies (red, insets and merged) and Hoechst (white, merged). Scale bars in insets: 20 μm. (b) Double immunofluorescence and confocal analysis of primary rat mature (OL) and precursor (OPC) oligodendrocytes was performed with antibodies for $P2Y_{12}$ receptor, MBP, NG2 (Table 1). For *intra2* antibodies, lots AN01/02/04/0502/0602 were used. (c) Rat cerebellar organotypic cultures were analyzed by double immunofluorescence and confocal microscopy for *intra1* (red) and *c-ter* (red), highlighting different structures (see also insets), and MBP (green).

used as specific microglia marker during neuroinflammation, we validated its use in a typical neuroinflammatory disease such as ALS and for the first time characterized the presence of $P2Y_{12}$ receptor in SOD1-G93A mouse model (Figure 4). By immunofluorescence and confocal analysis on lumbar spinal cord sections (L3–L5) of wild-type (WT) mice, we first compared the immunoreactive signals obtained with *c-ter* and specific microglia markers CD11b (red, recognizing ramified microglia) and CD68 (red, recognizing roundish activated microglia). In WT mice, *c-ter* (green) is abundantly and strongly immunoreacting with the microglia population and colocalizing with the majority of CD11b-positive cells, in both dorsal (DH) and ventral (VH) horns of spinal cord (Figure 4(a), left panel, merged yellow signal). All CD11b-positive cells share immunoreactivity for *c-ter*, as proved by the absence of red CD11b signal. Conversely, not all *c-ter*-positive cells immunoreact also with CD11b antibody, as proved by the presence of some green *c-ter* signals.

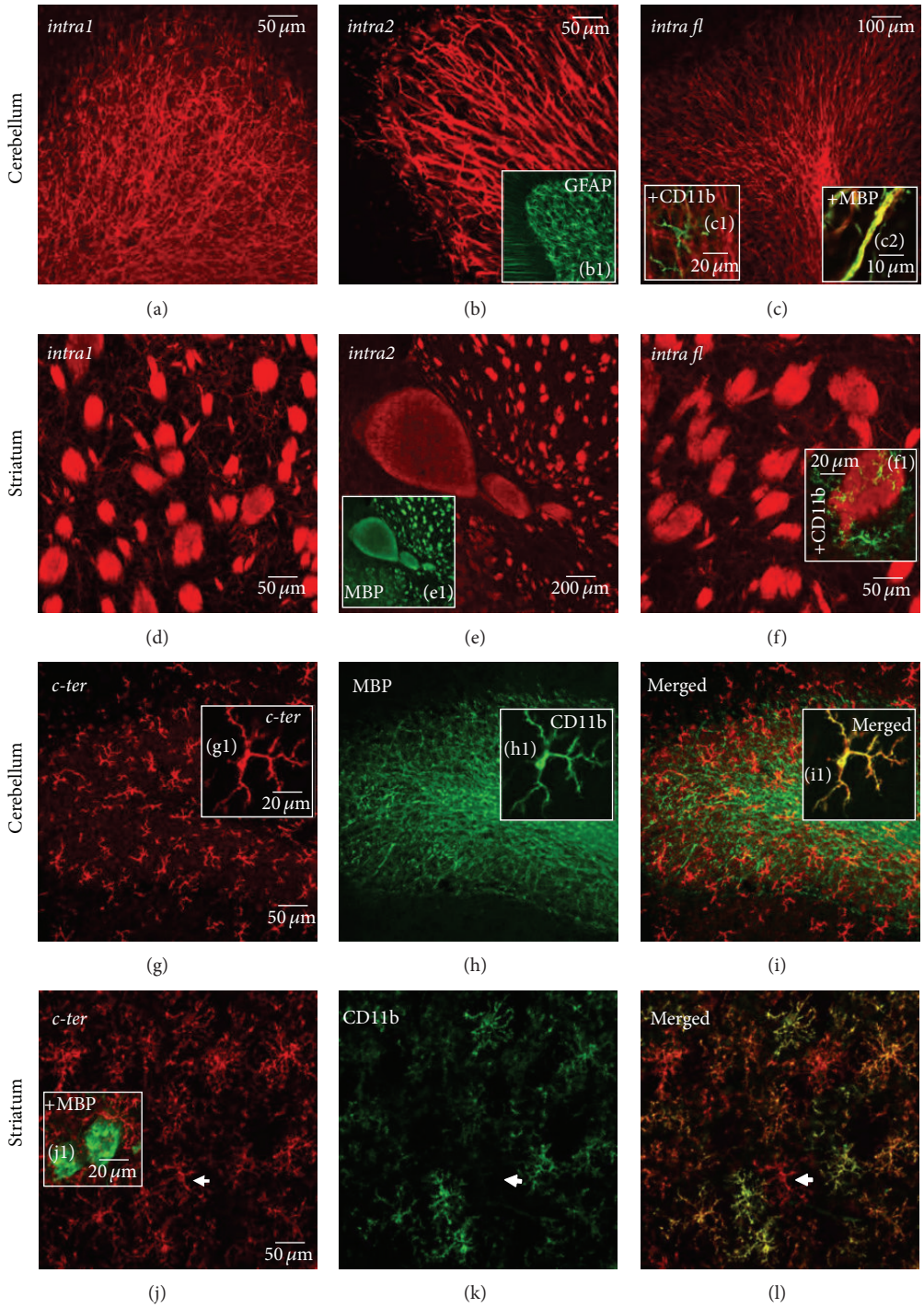

FIGURE 3: $P2Y_{12}$ receptor in rat brain tissue. Double immunofluorescence and confocal analysis was performed on sections from rat cerebellum (panels (a), (b), (c), (g), (h), and (i)) and striatum (panels (d), (e), (f), (j), (k), and (l)) with *intra1*, *intra2*-lots AN01/02/04, *intra fl*, *c-ter* (all red), and GFAP (green, inset b1), CD11b (green, insets c1, f1, h1; yellow merged, inset i1; green, panel (k); merged, panel (l)), MBP (yellow merged, inset c2; green, inset e1; green, panels (h) and (i); green, inset j1) antibodies.

In addition, we never observe colocalization with the rare activated CD68-positive (red) microglia cells present in WT healthy tissue (Figure 4(a), right panel, merged and inset).

To test if *c-ter* antibody can further recognize microglia activation during the progression of ALS, we next examined SOD1-G93A spinal cord sections at two different stages of the disease, that is, 20 weeks, a phase when the disease accelerates, and end stage, that is, 23 weeks, when the animals reach the score of 1 accordingly to a behavioral score system [22]. At both stages, SOD1-G93A mice show a significant increase in

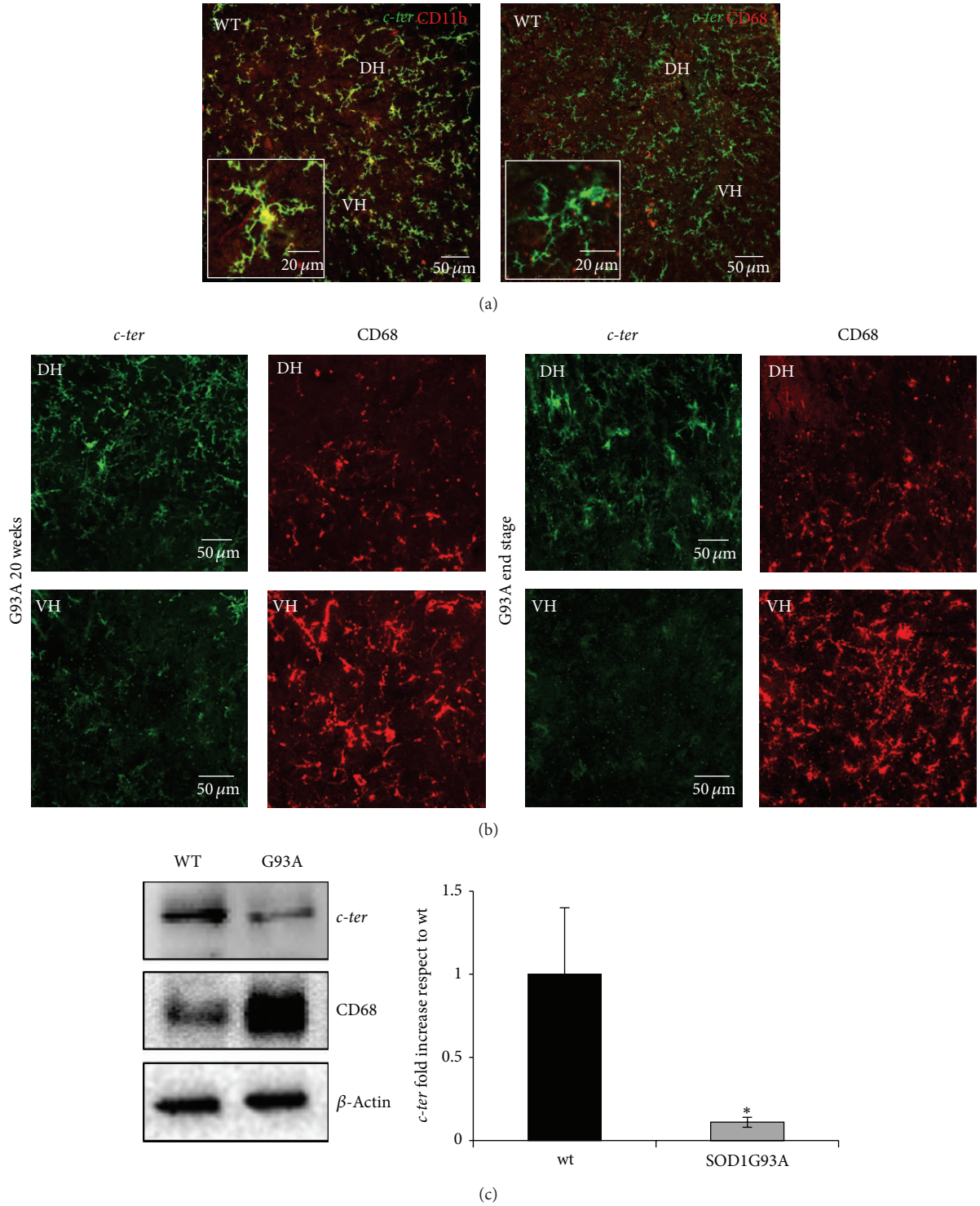

FIGURE 4: Temporal and regional pattern of $P2Y_{12}$ expression in SOD1-G93A ALS spinal microglia. (a) Double immunofluorescence and confocal analysis on lumbar spinal cord sections (L3–L5) of wild-type (WT) mice was performed with *c-ter* antibody (green and yellow, merged and insets), CD11b (left panel, yellow, merged and inset), and CD68 (right panel, red, merged and inset), in both dorsal (DH) and ventral (VH) horns of spinal cord. (b) Double immunofluorescence and confocal analysis on SOD1-G93A lumbar spinal cord sections (L3–L5) at two different stages of ALS disease, that is, 20 weeks, and end stage, was performed with *c-ter* (green) and CD68 (red) antibodies. (c) Equal amount of total lumbar spinal cord lysates (L3–L5) from WT and SOD1-G93A ($n = 4$ for each group) were subjected to Western blotting and immunoreactions with *c-ter* and CD68 antibodies; anti-β-actin was used for protein normalization. Data represent means ± SEM. Statistical significance was calculated by Student's t-test, $^{*}P < 0.05$.

CD68 immunostaining not only when compared to WT mice (Figure 4(a)) [22], but also in VH with respect to DH, and this is even more evident at end stage with respect to 20 weeks (red, Figure 4(b)). Remarkably, the immunoreactive signal of *c-ter* (green, Figure 4(b)) decreases during disease progression and the effect is pronounced especially in VH, where motor neuron loss, tissue damage, and microglia activation are known to be increased [2]. Particularly at 20 weeks, *c-ter*-expressing microglia are still ramified in DH, where few CD68-positive cells are present, but start to disappear in VH, where instead CD68-positive cells are increased. At end stage, *c-ter* staining decreases in DH with respect to 20 weeks and disappears almost completely in VH, concomitantly with a robust increase in CD68 staining. A parallel decrease in *c-ter* and increase of CD68 immunoreactivities is also confirmed by Western blot analysis performed on SOD1-G93A lumbar spinal cord homogenates at end stage (Figure 4(c)).

3.5. $P2Y_{12}$ Expression Is Modulated during Neuroinflammation in MS Brain Lesions. As in rat organotypic cerebellar cultures (Figure 2(b)) and rat cerebellar, striatal, cortical, or mouse spinal cord tissue slices (Figures 3 and 4), a similar pattern of $P2Y_{12}$ receptor expression is shown in human frontal cortex autoptic tissue by using *c-ter* antibody that highlights only microglia uniformly distributed throughout the entire healthy tissue (red), but not MBP-positive myelinated structures (green), as detected by the absence of overlapping immunofluorescent signals (Figures 5(a), 5(b), and 5(c), merged and insets a1, c1).

On the contrary, *intra1* (red, Figures 5(d), 5(e), and 5(f)), *intra2* [13], and *intra fl* (data not shown) antibodies recognize exclusively myelinated fibers in tissue from healthy (insets d1, e1, and f1) and progressive MS donors (Figures 5(d), 5(e), and 5(f)), as it is found with rat tissue (Figures 2 and 3). Moreover, we confirm a decrease of $P2Y_{12}$ receptor signal in proximity to the demyelinating lesion, as detected by loss of MBP-positive fibers (see asterisks) [13]. No colocalization of signals is shown with MHC II-positive microglia (+ MHC II, inset d3, merged).

Importantly, *c-ter*, *intra1* (red, insets a-f2) and *intra2*, and *intra fl* (data not shown) antibodies all recognize the presence of $P2Y_{12}$ receptor in integrin αII/β3-positive platelets (green) contained in the blood vessels of the analyzed tissues.

As observed in ALS mouse spinal cord where $P2Y_{12}$ receptor detected by *c-ter* antibody is shown to temporally and regionally decrease in microglia as a function of increasing inflammatory damage (Figure 4), we notice that microglia gradually lose immunoreactivity for *c-ter* antibody (Figure 6) in proximity to the demyelinating active cortical lesions of MS expressing augmented positivity for MHC II [33–35]. Moreover, by comparing MHC II and *c-ter* signals, we recognize four areas ("a–d") inside and around the lesion, where microglia express different amount of these proteins. In zone "a," at the edge of the lesion, we observe a predominance of *c-ter* immunoreactivity (red) compared to that of MHC II (green), as depicted in the merged field by a major occurrence of red signal. In zone "b," closer to the lesion, we notice a prevalence of active MHC II-positive green cells, but still the presence of few red and yellow signals. Finally, the extreme conditions are represented in zones "c" and "d," apparently in the core or outside the lesion, respectively, where microglia express either almost exclusively MHC II protein ("c") or $P2Y_{12}$ receptor on ramified microglia ("d").

4. Discussion

The interchange among different cell types of molecular cues that condition the cell specificity and the protein profile of each cell characterizes the morphological and functional heterogeneity in particular of microglia within various CNS regions [36], developmental stages [37, 38] and, even more, states of activation during pathological conditions [19]. In the case, for instance, of ALS, the release of signals from motor neurons apparently denotes one of the earliest phase of the disease, with microglia behaving as an M2 phenotype producing neuroprotective factors to repair motor neurons and preventing them against further injury [39]. As disease rises, motor neurons start releasing "alarm signals" that in turn convert microglia from beneficial M2 to cytotoxic M1 phenotype, with consequent release of proinflammatory cytokines. These often induce astrocytic dysfunction and further motor neuron degeneration [40]. In recent years, a dual functional phenotype of microglia has been identified also in MS. For instance, M1 markers are abundantly expressed in normal appearing white matter and throughout active demyelinating MS lesions by activated microglia and macrophages, although in human active MS lesions microglia show an intermediate activation status [41]. In addition, M2 microglia appear fundamental to guide oligodendrocyte remyelination in mice, and a switch from M1- to M2-dominant response occurs in microglia and peripherally derived macrophages when remyelination starts [42]. Only therapeutic procedures that both down-regulate the harmful responses and up-regulate the beneficial responses may hopefully slow pathological progression and provide meaningful hope for treatment. At the same time, the identification of clear markers involved in the M2/M1 microglia transition becomes mandatory for presymptomatic diagnosis, monitoring of disease progression, and efficacy of therapies.

Under this perspective, and consistently with previous findings establishing the role of purinergic receptors in the pathogenesis of both ALS and MS [14, 43], our present work serves this aim, by highlighting the gradual loss of $P2Y_{12}$ immunoreactivity as an early marker of neuroinflammation and microglia metamorphosis. We have indeed demonstrated here that $P2Y_{12}$ receptor protein identified in primary cultures of both microglia (Figure 2(a)) [18, 44] and oligodendrocytes (Figure 2(b)) [15] by different but $P2Y_{12}$-selective antibodies can be instead recognized in branched microglia exclusively by the use of *c-ter* antibody (Figures 3–6). This occurs only when the integrity and cytoarchitecture of the tissue is typically preserved in the presence of the least experimental manipulations, that is, in organotypic cultures (Figure 2(c)) and tissues slices for instance from rat striatum and cerebellum (Figure 3), mouse spinal cord (Figure 4), and human cerebral cortex (Figures 5 and 6). A similar difference in primary cultured cell versus tissue distribution of a

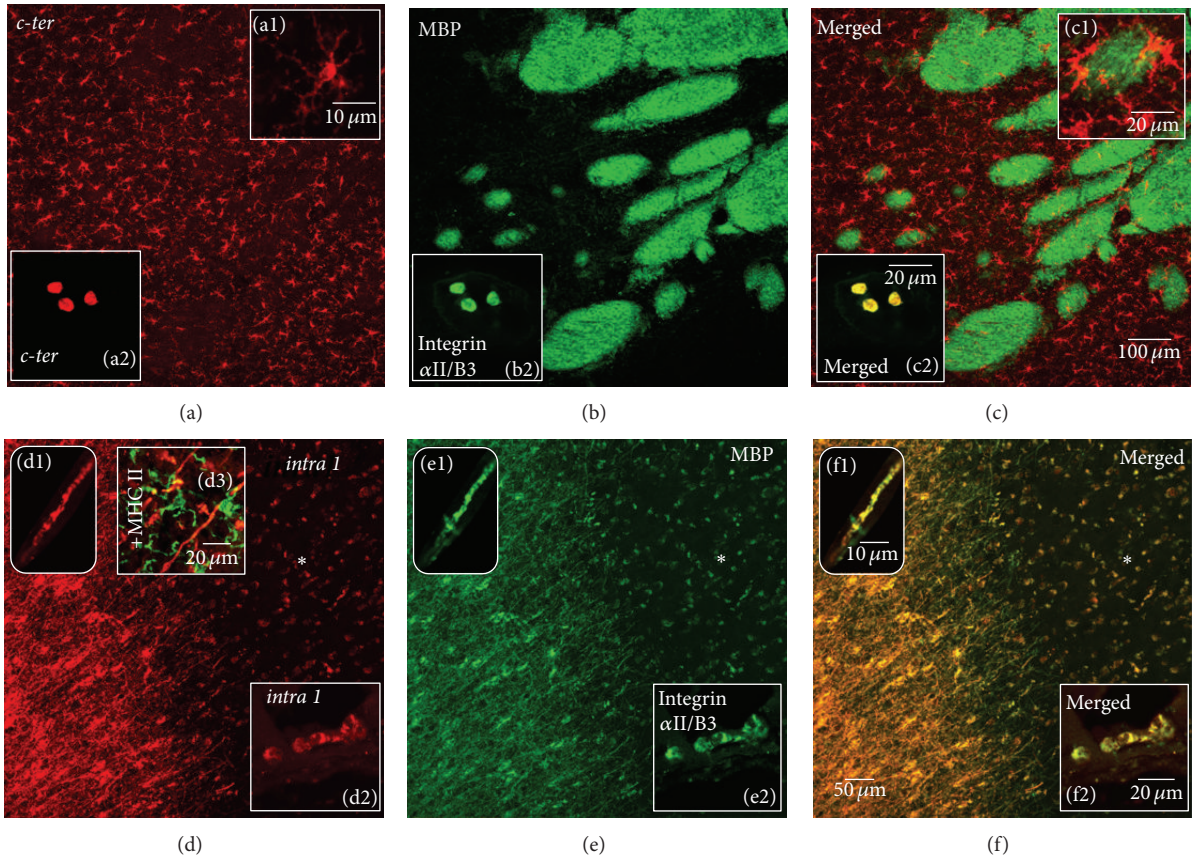

FIGURE 5: $P2Y_{12}$ receptor in human cortex. Sections from human healthy and SPMS frontal cortex were analyzed by double immunofluorescence and confocal microscopy for the immunoreactive markers *c-ter* (red, panels (a), (c); insets a1, a2, c1; yellow merged, inset c2), *intra1* (red, panels (d), insets d1, d2, d3; yellow merged, panel f, insets f1, f2), MBP (green, panels (b), (c), (e), insets c1, e1; yellow merged, panel (f), inset f1), MHC II (green, inset d3), and integrin αII/β3 (green, insets b2, e2; yellow merged, insets c2, f2). The asterisks show decreased $P2Y_{12}$ immunoreactivity in proximity to MS lesion.

protein was previously demonstrated with large-conductance calcium-activated potassium channel expression, in vascular endothelium [45]. A first implication emerging from these results is that a reliable evidence about selective $P2Y_{12}$ expression in cells of healthy or neuroinflammatory states is genuine only when cell connectivity and tissue architecture are fully preserved. We have indeed shown this, by proving that the antibodies used for $P2Y_{12}$, recognizing either the C-terminus or the second intracellular loop of the receptor (Figure 1 and Table 1) and immunolabelling, respectively, microglia or myelinated fibers in the CNS, are all still able to immunoreact for instance with platelets (Figure 5), where the receptor was originally described to be present and to have a role in the processes of activation, aggregation [46–49], primary hemostasis, and arterial thrombosis [50–55]. A possible explanation for the microglia versus oligodendrocyte selectivity of the $P2Y_{12}$ antibodies might be that Gi-coupling, and/or quaternary structure, post-transcriptional modifications, and subcellular localization of $P2Y_{12}$ that remain strictly preserved in platelets, are instead divergent in microglia with respect to oligodendrocytes/myelinated fibers. In this case, a cell-specific network of $P2Y_{12}$ oligomeric interactions and/or a distinct subcellular partitioning might simply mask the recognition sites of the different antibodies on $P2Y_{12}$ protein in different cell types. To support this hypothesis, we know that in platelets $P2Y_{12}$ indeed resides in subcellular lipid raft structures and its partitioning out from rafts causes for instance inactivation [56] and that also the presence of another purinergic receptor, the ionotropic $P2X_3$, in lipid rafts has cell-specific properties shared in cerebellar granule neurons and total brain tissue but not in neuroblastoma cells and dorsal root ganglia [57] and that the specific antagonist clopidogrel inhibits $P2Y_{12}$ by breaking down the homoligomeric complex to single monomers [58] and finally, that hetero-oligomerization of $P2Y_{12}$ is demonstrated with $P2Y_1$, $P2Y_2$, $P2Y_{13}$, and with adenosine A_1, A_{2A} receptors in different cellular backgrounds [59]. All these features might very well explain also why it has not been found colocalization between $P2Y_{12}$ and GFAP-positive astrocytes in rat brain cortex and nucleus accumbens, despite the abundant presence of $P2Y_{12}$ mRNA [11] and, moreover, why $P2Y_{12}$ is specifically observed in brain and spinal cord resident microglia but is not observed, for example, in peripheral macrophages in spleen [18, 20].

A second implication that emerges from our results is that the morphological metamorphosis that microglia undergo

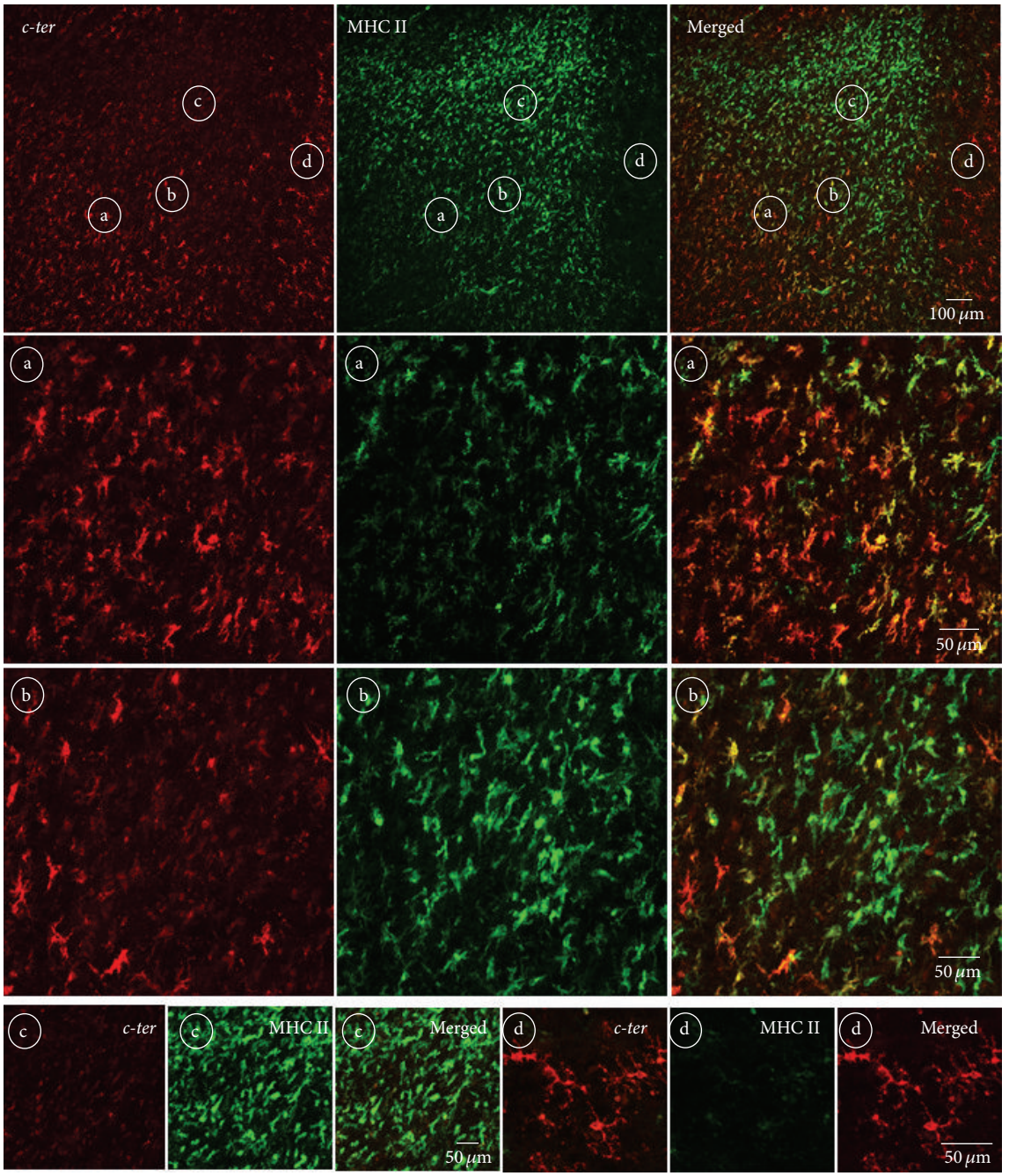

Figure 6: Regional distribution of $P2Y_{12}$ in proximity to MS lesions. Sections from SPMS frontal cortex were analyzed by double immunofluorescence and confocal microscopy for *c-ter* (red) and MHC II (green) immunoreactivity. In proximity to the demyelinating active cortical lesion expressing augmented positivity for MHC II, microglia gradually lose immunoreactivity for *c-ter* antibody. Microglia express differential immunoreactivity in the four chosen areas which are found inside (circled b-c) and around (circled a–d) a lesion.

under neuroinflammatory conditions as those triggered during ALS and MS, can be remarkably highlighted by the progressive reduction of $P2Y_{12}$ immunostaining obtained with *c-ter* antibody that reacts, also in this case, exclusively with multibranched microglia still present in the tissue (Figures 4 and 6). This closely reflects the expression of $P2Y_{12}$ that is robust in the resting/surveillant branched state but dramatically decreased after morphological transition and activation of microglia [18]. Our observations are also in line with the central role played by $P2Y_{12}$ in branched microglia membrane ruffling and inspection of the environment [60]. On the other hand, they depict a morphological/functional state of microglia that only partially overlaps with CD11b and MHC II immunoreactivities, which are furthermore known to increase during activation but instead highly contrasts with CD68 immunostaining that is totally absent in ramified microglia [19]. In parallel to our results, these last antibodies actually accentuate the progressive transition

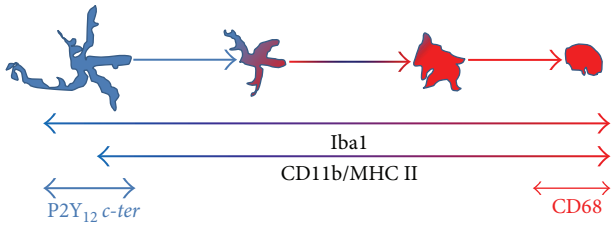

Figure 7: Draw of microglial marker expression as a function of activation. Branched microglia are represented in blue and activated microglia in red. Iba1 [22], CD11b, and MHC II are mostly expressed in microglia throughout the different morphological states and their expression increases during activation (light blue to red color). $P2Y_{12}$*c-ter* (light blue) and CD68 (red) are expressed, respectively, in branched or roundish/activated microglia.

of microglia from a lesser ramified shape to a significantly more activated amoeboid phenotype (Figures 4, 6, and 7), thus suggesting the dual use of *c-ter* and CD68 antibodies as markers, respectively, for branched resting/surveillant versus roundish/activated microglia. A reduction of *c-ter*-$P2Y_{12}$ immunostaining that is concomitant to an increase, for instance, of MHC II/CD11b/CD68 immunoreactivity, could thus become a feasible approach to detect an increasing neuroinflammatory condition. Indeed, $P2Y_{12}$ is considered an essential component and primary site at which nucleotides such as ADP act to promote directional microglia movement or chemotaxis at early stages of CNS injury [17]. In particular, microglia from mice lacking $P2Y_{12}$ exhibit normal baseline motility but are unable to polarize and to elicit directional branch extension and migration toward nucleotides *in vitro*, or sites of cortical damage *in vivo* [18]. These notions are consistent with our results that fail to describe *c-ter*-$P2Y_{12}$ immunoreactivity on roundish phagocytizing, or polarized migratory microglia. However, we still do not know if reduction/absence of $P2Y_{12}$ *c-ter*-immunolabelling on activated microglia might be a cause or a consequence of morphological/functional transition or might simply reflect a cell-selective hindrance and lack of access to the immunogenic sites by the antibody. Further work will clarify this issue. Anyhow, we can assert that the distinctive recognition of multibranched microglia renders the *c-ter* antibody a novel and useful tool to discriminate among microglia morphological states, thus making $P2Y_{12}$ receptor a selective and early marker for the ramified phase.

In parallel to this, we have also proven that all the several antibodies raised against the second intracellular loop of $P2Y_{12}$ (*intra1*, *intra2*, and *intra fl*) can likely be employed as markers for the presence of MS lesions. Although with different intensities, they not only recognize the receptor specifically on myelinated fibers of organotypic cultures (Figure 2(c)), tissues slices from rat striatum or cerebellum (Figure 3) and human cerebral cortex, but also furthermore highlight the reduction of $P2Y_{12}$ signal that occurs for instance in MS tissue (Figure 5) in correlation to the extent of demyelination found in all types of grey matter cortical plaques (I–III) and subcortical white matter [13].

In brief, we have shown here that the presence of $P2Y_{12}$ receptor can be simultaneously identified by the C-terminus and the second intracellular loop antibodies. When this occurs, a condition of intact myelinated fibers and branched/surveillant microglia is represented at once that perhaps signifies a "healthy" state of the analyzed tissue. Any deviance from this picture likely characterizes a neuroinflammatory condition. For instance in MS, a decrease in the second intracellular loop immunoreactions accompanied by an increase of C-terminus immunoreactivity will possibly depict the loss of myelin and replacement by ramified microglia that often occur in an inactive plaque. On the contrary, a decrease in C-terminus immunolabelling in the abundant presence of second intracellular loop-positive myelinated fibers would indicate an early active plaque where M1/M2 microglia reactivity starts to take place.

5. Conclusion

By comparative analysis of all the available $P2Y_{12}$-immunoreactive antibodies recognizing the C-terminus or the second intracellular loop of the receptor, we have established here that, under experimental conditions of well-preserved cytoarchitecture and tissue integrity, $P2Y_{12}$ receptor expressed by both ramified microglia or oligodendrocytes/myelinated fibers might serve a dual function as specific marker, respectively, of branched/surveillant microglia as well as demyelinating lesions. We believe that $P2Y_{12}$ identification and modulation might potentially acquire an important predictive value under neuroinflammatory conditions, as those found in ALS and MS. $P2Y_{12}$ is likely to deserve a key role in the verge of a neuroinflammatory breakdown.

Abbreviations

ALS: Amyotrophic lateral sclerosis
CD11b: Cluster of differentiation 11b
CD68: Cluster of differentiation 68
DH: Dorsal horn
GFAP: Glial fibrillary acidic protein
MHC II: Major histocompatibility complex II
MBP: Myelin basic protein
MS: Multiple sclerosis
NeuN: Neuronal nuclei
NG2: Neural/glial antigen 2
NDS: Normal donkey serum
SPMS: Secondary progressive multiple sclerosis
PBS: Phosphate buffer saline
VH: Ventral horn.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Susanna Amadio and Cinzia Volonté conceived and designed the study. Chiara Parisi performed heterologous $P2Y_{12}$ expression and molecular analysis. Cinzia Montilli and

Alberto Savio Carrubba performed primary oligodendrocyte purification, cerebellar organotypic cultures, and immunofluorescence studies on cells and rat and human tissue. Savina Apolloni was responsible for experiments with ALS mouse tissue and primary microglia. Susanna Amadio performed confocal fluorescence analysis. Cinzia Volonté and Susanna Amadio wrote the paper. All authors read and approved the final version of the paper.

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References

[1] N. Guérout, X. Li, and F. Barnabé-Heider, "Cell fate control in the developing central nervous system," *Experimental Cell Research*, vol. 321, no. 1, pp. 77–83, 2014.

[2] T. Philips and W. Robberecht, "Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease," *The Lancet Neurology*, vol. 10, no. 3, pp. 253–263, 2011.

[3] E. Ellwardt and F. Zipp, "Molecular mechanisms linking neuroinflammation and neurodegeneration in MS," *Experimental Neurology*, 2014.

[4] J. G. Walsh, D. A. Muruve, and C. Power, "Inflammasomes in the CNS," *Nature Reviews Neuroscience*, vol. 15, no. 2, pp. 84–97, 2014.

[5] F. di Virgilio, S. Ceruti, P. Bramanti, and M. P. Abbracchio, "Purinergic signalling in inflammation of the central nervous system," *Trends in Neurosciences*, vol. 32, no. 2, pp. 79–87, 2009.

[6] C. Volonté, S. Apolloni, S. D. Skaper, and G. Burnstock, "P2X7 Receptors: channels, pores and more," *CNS and Neurological Disorders-Drug Targets*, vol. 11, no. 6, pp. 705–721, 2012.

[7] G. A. Weisman, J. M. Camden, T. S. Peterson, D. Ajit, L. T. Woods, and L. Erb, "P2 receptors for extracellular nucleotides in the central nervous system: role of P2X7 and P2Y2 receptor interactions in neuroinflammation," *Molecular Neurobiology*, vol. 46, no. 1, pp. 96–113, 2012.

[8] G. Hollopeter, H. Jantzen, D. Vincent et al., "Identification of the platelet ADP receptor targeted by antithrombotic drugs," *Nature*, vol. 409, no. 6817, pp. 202–207, 2001.

[9] C. Gachet, "The platelet P2 receptors as molecular targets for old and new antiplatelet drugs," *Pharmacology and Therapeutics*, vol. 108, no. 2, pp. 180–192, 2005.

[10] C. Gachet, "$P2Y_{12}$ receptors in platelets and other hematopoietic and non-hematopoietic cells," *Purinergic Signalling*, vol. 8, no. 3, pp. 609–619, 2012.

[11] H. Franke, U. Krügel, J. Grosche et al., "P2Y receptor expression on astrocytes in the nucleus accumbens of rats," *Neuroscience*, vol. 127, no. 2, pp. 431–441, 2004.

[12] S. Amadio, G. Tramini, A. Martorana et al., "Oligodendrocytes express P2Y12 metabotropic receptor in adult rat brain," *Neuroscience*, vol. 141, no. 3, pp. 1171–1180, 2006.

[13] S. Amadio, C. Montilli, R. Magliozzi, G. Bernardi, R. Reynolds, and C. Volonté, "P2Y12 receptor protein in cortical gray matter lesions in multiple sclerosis," *Cerebral Cortex*, vol. 20, no. 6, pp. 1263–1273, 2010.

[14] S. Amadio, S. Apolloni, N. D'Ambrosi, and C. Volonté, "Purinergic signalling at the plasma membrane: a multipurpose and multidirectional mode to deal with amyotrophic lateral sclerosis and multiple sclerosis," *Journal of Neurochemistry*, vol. 116, no. 5, pp. 796–805, 2011.

[15] J. Dennis, M. K. Morgan, M. R. Graf, and B. Fuss, "P2Y 12 receptor expression is a critical determinant of functional responsiveness to ATX's MORFO domain," *Purinergic Signalling*, vol. 8, no. 2, pp. 181–190, 2012.

[16] Y. Sasaki, M. Hoshi, C. Akazawa et al., "Selective Expression of Gi/o-Coupled ATP Receptor $P2Y_{12}$ in Microglia in Rat Brain," *Glia*, vol. 44, no. 3, pp. 242–250, 2003.

[17] K. Nasu-Tada, S. Koizumi, and K. Inoue, "Involvement of β1 integrin in microglial chemotaxis and proliferation on fibronectin: different regulations by ADP through PKA," *GLIA*, vol. 52, no. 2, pp. 98–107, 2005.

[18] S. E. Haynes, G. Hollopeter, G. Yang et al., "The P2Y12 receptor regulates microglial activation by extracellular nucleotides," *Nature Neuroscience*, vol. 9, no. 12, pp. 1512–1519, 2006.

[19] H. Kettenmann, U. K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[20] K. Kobayashi, H. Yamanaka, T. Fukuoka, Y. Dai, K. Obata, and K. Noguchi, "P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain," *Journal of Neuroscience*, vol. 28, no. 11, pp. 2892–2902, 2008.

[21] J. M. Crain, M. Nikodemova, and J. J. Watters, "Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia," *Journal of Neuroinflammation*, vol. 6, article 24, 2009.

[22] S. Apolloni, S. Amadio, C. Montilli, C. Volonté, and N. D'Ambrosi, "Ablation of P2X7 receptor exacerbates gliosis and motoneuron death in the SOD1-G93A mouse model of amyotrophic lateral sclerosis," *Human Molecular Genetics*, vol. 22, no. 20, pp. 4102–4116, 2013.

[23] A. C. Ludolph, C. Bendotti, E. Blaugrund et al., "Guidelines for preclinical animal research in ALS/MND: a consensus meeting," *Amyotrophic Lateral Sclerosis*, vol. 11, no. 1-2, pp. 38–45, 2010.

[24] S. Apolloni, C. Parisi, M. G. Pesaresi et al., "The NADPH oxidase pathway is dysregulated by the P2X7 receptor in the sod1-g93a microglia model of amyotrophic lateral sclerosis," *Journal of Immunology*, vol. 190, no. 10, pp. 5187–5195, 2013.

[25] Y. Chen, V. Balasubramaniyan, J. Peng et al., "Isolation and culture of rat and mouse oligodendrocyte precursor cells," *Nature Protocols*, vol. 2, no. 5, pp. 1044–1051, 2007.

[26] L. Stoppini, P.-A. Buchs, and D. Muller, "A simple method for organotypic cultures of nervous tissue," *Journal of Neuroscience Methods*, vol. 37, no. 2, pp. 173–182, 1991.

[27] I. Dusart, M. S. Airaksinen, and C. Sotelo, "Purkinje cell survival and axonal regeneration are age dependent: an in vitro study," *Journal of Neuroscience*, vol. 17, no. 10, pp. 3710–3726, 1997.

[28] I. von Kügelgen, "Pharmacological profiles of cloned mammalian P2Y-receptor subtypes," *Pharmacology & Therapeutics*, vol. 110, no. 3, pp. 415–432, 2006.

[29] P. J. Kersey, D. M. Staines, D. Lawson et al., "Ensembl Genomes: an integrative resource for genome-scale data from non-vertebrate species," *Nucleic Acids Research*, vol. 40, no. 1, pp. D91–D97, 2012.

[30] K. D. Pruitt, G. R. Brown, S. M. Hiatt et al., "RefSeq: an update on mammalian reference sequences," *Nucleic Acids Research*, vol. 42, pp. D756–D763, 2014.

[31] W. J. Kent, "BLAT—the BLAST-like alignment tool," *Genome Research*, vol. 12, no. 4, pp. 656–664, 2002.

[32] D. Ito, Y. Imai, K. Ohsawa, K. Nakajima, Y. Fukuuchi, and S. Kohsaka, "Microglia-specific localisation of a novel calcium binding protein, Iba1," *Molecular Brain Research*, vol. 57, no. 1, pp. 1–9, 1998.

[33] G. M. Hayes, M. N. Woodroofe, and M. L. Cuzner, "Microglia express MHC class II in normal and demyelinating human white matter," *Annals of the New York Academy of Sciences*, vol. 540, pp. 501–503, 1988.

[34] L. Bö, S. Mörk, P. A. Kong, H. Nyland, C. A. Pardo, and B. D. Trapp, "Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions," *Journal of Neuroimmunology*, vol. 51, no. 2, pp. 135–146, 1994.

[35] B. D. Trapp, J. Peterson, R. M. Ransohoff, R. Rudick, S. Mörk, and L. Bö, "Axonal transection in the lesions of multiple sclerosis," *TheNew England Journal of Medicine*, vol. 338, no. 5, pp. 278–285, 1998.

[36] M. Olah, K. Biber, J. Vinet, and H. W. G. M. Boddeke, "Microglia phenotype diversity," *CNS and Neurological Disorders—Drug Targets*, vol. 10, no. 1, pp. 108–118, 2011.

[37] L. Pont-Lezica, C. Béchade, Y. Belarif-Cantaut, O. Pascual, and A. Bessis, "Physiological roles of microglia during development," *Journal of Neurochemistry*, vol. 119, no. 5, pp. 901–908, 2011.

[38] G. J. Harry, "Microglia during development and aging," *Pharmacology & Therapeutics*, vol. 139, no. 3, pp. 313–326, 2013.

[39] J. S. Henkel, D. R. Beers, W. Zhao, and S. H. Appel, "Microglia in ALS: the good, the bad, and the resting," *Journal of Neuroimmune Pharmacology*, vol. 4, no. 4, pp. 389–398, 2009.

[40] M. C. Evans, Y. Couch, N. Sibson, and M. R. Turner, "Inflammation and neurovascular changes in amyotrophic lateral sclerosis," *Molecular and Cellular Neuroscience*, vol. 53, pp. 34–41, 2013.

[41] D. Y. Vogel, E. J. Vereyken, J. E. Glim et al., "Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status," *Journal of Neuroinflammation*, vol. 10, article 35, 2013.

[42] V. E. Miron, A. Boyd, J. W. Zhao et al., "M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination," *Nature Neuroscience*, vol. 16, no. 9, pp. 1211–1218, 2013.

[43] C. Volonté, S. Apolloni, M. T. Carrì, and N. D'Ambrosi, "ALS: focus on purinergic signalling," *Pharmacology & Therapeutics*, vol. 132, no. 1, pp. 111–122, 2011.

[44] S. Amadio, A. de Ninno, C. Montilli, L. Businaro, A. Gerardino, and C. Volonté, "Plasticity of primary microglia on micropatterned geometries and spontaneous long-distance migration in microfluidic channels," *BMC Neuroscience*, vol. 14, article 121, 2013.

[45] S. L. Sandow and T. H. Grayson, "Limits of isolation and culture: Intact vascular endothelium and BK_{Ca}," *The American Journal of Physiology: Heart and Circulatory Physiology*, vol. 297, no. 1, pp. H1–H7, 2009.

[46] S. M. O. Hourani and D. A. Hall, "P2T purinoceptors: ADP receptors on platelets," *CIBA Foundation Symposia*, vol. 198, pp. 53–70, 1996.

[47] J. L. Daniel, C. Dangelmaier, J. Jin, B. Ashby, J. B. Smith, and S. P. Kunapuli, "Molecular basis for ADP-induced platelet activation I. Evidence for three distinct ADP receptors on human platelets," *The Journal of Biological Chemistry*, vol. 273, no. 4, pp. 2024–2029, 1998.

[48] G. E. Jarvis, R. G. Humphries, M. J. Robertson, and P. Leff, "ADP can induce aggregation of human platelets via both $P2Y_1$ and P_{2T} receptors," *British Journal of Pharmacology*, vol. 129, no. 2, pp. 275–282, 2000.

[49] P. André, S. M. Delaney, T. La Rocca et al., "P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries," *Journal of Clinical Investigation*, vol. 112, no. 3, pp. 398–406, 2003.

[50] D. C. B. Mills, R. Puri, C.-J. Hu et al., "Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 12, no. 4, pp. 430–436, 1992.

[51] C. Gachet, P. Savi, P. Ohlmann, J.-P. Maffrand, K. H. Jakobs, and J.-P. Cazenave, "ADP receptor induced activation of guanine nucleotide binding proteins in rat platelet membranes: an effect selectively blocked by the thienopyridine clopidogrel," *Journal of Thrombosis and Haemostasis*, vol. 68, no. 1, pp. 79–83, 1992.

[52] K. Schror, "The basic pharmacology of ticlopidine and clopidogrel," *Platelets*, vol. 4, no. 5, pp. 252–261, 1993.

[53] P. Savi, J. Combalbert, C. Gaich et al., "The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450-1A," *Thrombosis and Haemostasis*, vol. 72, no. 2, pp. 313–317, 1994.

[54] I. Shalito, O. Kopyleva, and V. Serebruany, "Novel antiplatelet agents in development: prasugrel, ticagrelor, and cangrelor and beyond," *American Journal of Therapeutics*, vol. 16, no. 5, pp. 451–458, 2009.

[55] M. Cattaneo, "New P2Y12 blockers," *Journal of Thrombosis and Haemostasis*, vol. 7, supplement 1, pp. 262–265, 2009.

[56] T. M. Quinton, S. Kim, J. Jin, and S. P. Kunapuli, "Lipid rafts are required in $G\alpha_i$ signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation," *Journal of Thrombosis and Haemostasis*, vol. 3, no. 5, pp. 1036–1041, 2005.

[57] F. Vacca, S. Amadio, G. Sancesario, G. Bernardi, and C. Volonté, "P2X3 receptor localizes into lipid rafts in neuronal cells," *Journal of Neuroscience Research*, vol. 76, no. 5, pp. 653–661, 2004.

[58] P. Savi, J. Zachayus, N. Delesque-Touchard et al., "The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 29, pp. 11069–11074, 2006.

[59] K. Schicker, S. Hussl, G. K. Chandaka et al., "A membrane network of receptors and enzymes for adenine nucleotides and nucleosides," *Biochimica et Biophysica Acta*, vol. 1793, no. 2, pp. 325–334, 2009.

[60] S. Honda, Y. Sasaki, K. Ohsawa et al., "Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors," *Journal of Neuroscience*, vol. 21, no. 6, pp. 1975–1982, 2001.

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Review Article

Adenosine A_{2A} Receptors Modulate Acute Injury and Neuroinflammation in Brain Ischemia

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The extracellular concentration of adenosine in the brain increases dramatically during ischemia. Adenosine A_{2A} receptor is expressed in neurons and glial cells and in inflammatory cells (lymphocytes and granulocytes). Recently, adenosine A_{2A} receptor emerged as a potential therapeutic attractive target in ischemia. Ischemia is a multifactorial pathology characterized by different events evolving in the time. After ischemia the early massive increase of extracellular glutamate is followed by activation of resident immune cells, that is, microglia, and production or activation of inflammation mediators. Proinflammatory cytokines, which upregulate cell adhesion molecules, exert an important role in promoting recruitment of leukocytes that in turn promote expansion of the inflammatory response in ischemic tissue. Protracted neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression. A_{2A} receptors present on central cells and on blood cells account for important effects depending on the time-related evolution of the pathological condition. Evidence suggests that A_{2A} receptor antagonists provide early protection via centrally mediated control of excessive excitotoxicity, while A_{2A} receptor agonists provide protracted protection by controlling massive blood cell infiltration in the hours and days after ischemia. Focus on inflammatory responses provides for adenosine A_{2A} receptor agonists a wide therapeutic time-window of hours and even days after stroke.

1. Introduction

Ischemic stroke is the second leading cause of death in major industrialized countries, with a mortality rate of around 30%, and the major cause of long-lasting disabilities [1]. Ischemic stroke results from a transient or permanent reduction in cerebral blood flow which is, in most cases, caused by the occlusion of a major brain artery, either by an embolus or by local thrombosis. Currently, there is no promising pharmacotherapy for acute ischemic stroke aside from intravenous or intra-arterial thrombolysis. Yet, because of the narrow therapeutic time-window involved, thrombolytic application is very restricted in clinical settings [2]. Neuroprotective drugs such as glutamate receptor antagonists have shown therapeutic potential in animal stroke trials but have failed to be efficacious during clinical trials [3, 4]. Death-signaling proteins involved in the progression from N-methyl-D-aspartic acid (NMDA) receptor stimulation to excitotoxic neuronal death emerged as possible novel targets for neuroprotection. In particular, inhibition of activation of transcription factors and related proteins, including p38, JNK, and SREBP1, is neuroprotective in animal models of stroke [5]. On the other hand, ischemia is a multifactorial pathology characterized by different events evolving in the time. After ischemia the early massive increase of extracellular glutamate is followed by activation of resident immune cells, that is, microglia, and production or activation of inflammation mediators [6]. Proinflammatory cytokines, which upregulate cell adhesion molecules, exert an important role in promoting neutrophil infiltration and accumulation in brain parenchyma [7, 8]. Although after ischemia precocious activation of immune cells may be neuroprotective and

supportive for regeneration, protracted neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression.

The extracellular adenosine concentration increases dramatically during *in vivo* ischemia as demonstrated first by the cortical cup technique [9, 10] and later on by the microdialysis technique [11–15]. The increase of adenosine extracellular level is attributable to different reasons. Early after ischemia, the increase of adenosine is mainly attributable to extracellularly released ATP [16] that is hydrolysed by ectonucleotidases (NTPDases 1, 2, and 3 that convert ATP to ADP and AMP) and ecto-5′-nucleotidase that converts AMP to adenosine [17, 18]. Thereafter adenosine *per se* is mainly released from cells likely by the equilibrative nucleoside transporter (ENT) 2 [16]. Inhibition of adenosine-uptake processes due to downregulation of concentrative nucleoside transporters (CNT) 2 and 3 and of the ENT1 also contributes to the extracellular adenosine increase after stroke [19].

Numerous authors have indicated adenosine and its receptors as a target for therapeutic implementation in the treatment of stroke. Extracellular adenosine acts through multiple *G*-protein coupled receptors (adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3) to exert a variety of physiological effects [20]. Adenosine receptors are expressed at significant levels in neurons and glial cells and in inflammatory cells (such as lymphocytes and granulocytes) [21–26] (Figure 1). The wide distribution is consistent with the multifaceted neurochemical and molecular effects of adenosine receptor activation and suggests that the role of adenosine in ischemia is the consequence of an interplay among different receptor activation in neuronal, glial, and inflammatory cells, which changes depending on the time-related development of the pathological condition.

During ischemia, adenosine has long been known to act predominantly as a neuroprotectant endogenous agent [27–32]. Adenosine infusion into the ischemic striatum has been shown to significantly ameliorate neurological outcome and reduce infarct volume after transient focal cerebral ischemia [33]. Protective effects are greatly attributed to A_1 receptor activation due to reduced Ca^{2+} influx, thus lowering presynaptic release of excitatory neurotransmitters [33–38] and in particular of glutamate which exerts an excitotoxic effect during ischemia mainly by overstimulation of NMDA receptors [39]. In addition, by directly increasing the K^+ and Cl^- ion conductances, adenosine stabilises the neuronal membrane potentials, thus reducing neuronal excitability [39]. Consequent reduction in cellular metabolism and energy consumption [40] and moderate lowering of the body/brain temperature [41] protect against ischemia.

Although data demonstrate a neuroprotective effect of adenosine through A_1 receptors during ischemia, the use of selective A_1 agonists is hampered by undesirable effects such as sedation, bradycardia, and hypotension [42, 43]. More recently adenosine A_{2A} receptors emerged as an interesting target in ischemia.

We largely limit our overview to the A_{2A} adenosine receptor subtype in brain whose new insights are into control of excitotoxicity and neuroinflammation phenomena in ischemia. In this paper, we summarize recent developments that have contributed to the understanding of how this adenosine receptor subtype modulates tissue damage in brain ischemia models. A list of A_{2A} receptor ligands used in different "*in vitro*" and "*in vivo*" hypoxia/ischemia models is provided in Table 1.

2. Adenosine A_{2A} Receptor Antagonists Protect against Primary Ischemic Injury

2.1. A_{2A} Receptor Antagonists Are Protective against Ischemic Damage. Gao and Phillis [50] demonstrated for the first time that the nonselective A_{2A} receptor antagonist, 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]quinazolin-5-amine (CGS15943), reduced cerebral ischemic injury in the gerbil following global forebrain ischemia. Thereafter many reports have confirmed the neuroprotective role of A_{2A} receptor antagonists in different models of ischemia. The selective A_{2A} receptor antagonist, 8-(3-chlorostyryl) caffeine (CSC), as well as the less selective antagonists, CGS15943 and 4-amino [1,2,4] triazolo [4,3a] quinoxalines (CP66713), both administered before ischemia, protected against hippocampal cell injury during global forebrain ischemia in gerbils [49, 52]. The selective A_{2A} receptor antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl) phenol (ZM241385), administered before ischemia, reduced hippocampal injury and improved performance in the Morris water maze in hyperglycemic four-vessel occluded rats [54]. In all the mentioned studies, adenosine A_{2A} receptor antagonists were administered before ischemia. Relevantly to a possible clinical use of drugs in stroke, in subsequent studies, A_{2A} antagonists were administered after ischemia. The selective A_{2A} receptor antagonist, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4,triazolo[1,5-c]pyrimidine (SCH58261), acutely administered after hypoxia/ischemia in neonatal rats [57] and soon after focal ischemia in adult rats [58, 59] reduced brain damage 24 hours thereafter. The same antagonist, administered subchronically, was protective against brain damage, neurological deficit [60, 61, 67], and disorganization of myelin [61] 24 hours after focal cerebral ischemia in the adult rat. In the model of global ischemia (i.e., 7 min asphyxic cardiac arrest) in newborn piglets, posttreatment with SCH58261, infused soon after resuscitation and for 6 hours, improved neurologic recovery and protected striatopallidal neurons after 4 days from ischemia [63]. SCH58261 behaves as a significant protective agent at a dose (0.01 mg/kg) that does not have cardiovascular effects. This low dose does not affect motor activity in naive animals but decreases contralateral turning behaviour after monolateral middle cerebral artery occlusion (MCAo) induced by the monofilament technique [59, 60]. At a higher dose, in the range that is effective in different models of Parkinson's Disease (PD), the same drug significantly increases motility and rearing in the rat [68]. A noxious role of A_{2A} receptors during ischemia is supported by the observation that A_{2A} receptor knock-out (KO) mice show significantly decreased infarct volumes after focal cerebral ischemia when compared with their wild-type littermates [69, 70].

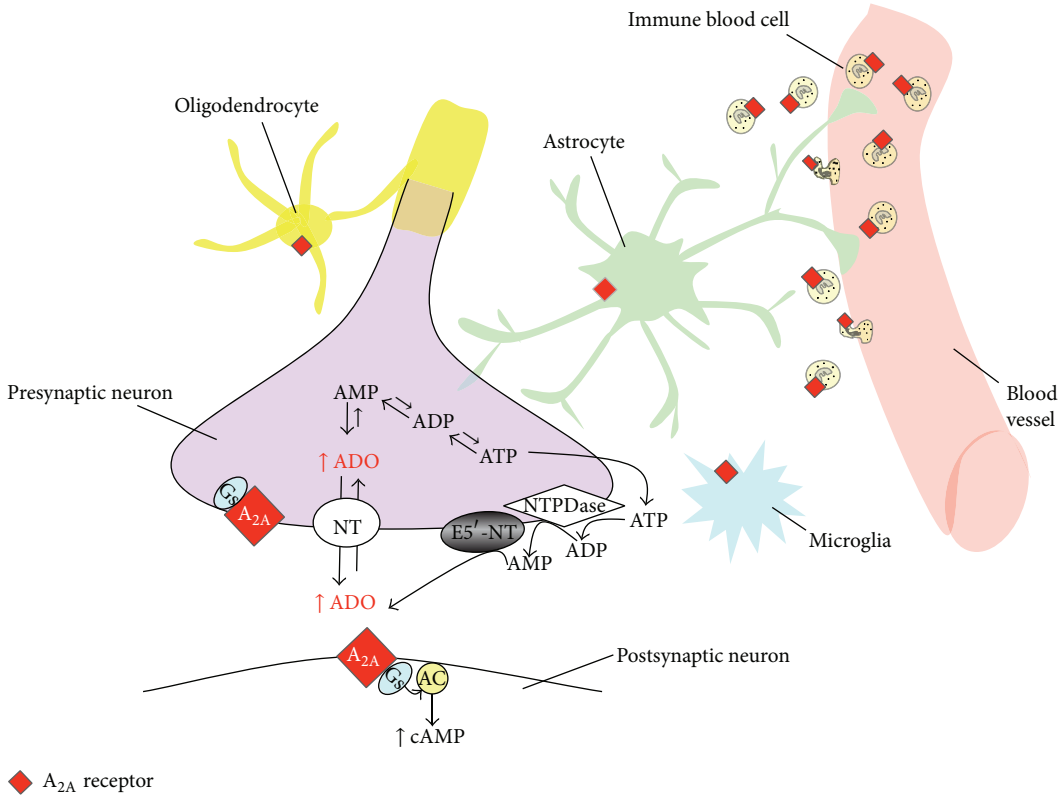

FIGURE 1: Schematic drawing of adenosine A_{2A} receptor localization on different cell types. Adenosine A_{2A} receptors are expressed at central level on presynaptic and postsynaptic neurons, on astrocytes, on microglia, and on oligodendrocytes. A_{2A} receptors are present also at peripheral level on leukocytes and vasculature. After cerebral ischemia, leukocytes infiltrate into ischemic tissue due to increased permeability of blood-brain barrier (BBB). During ischemia, extracellular adenosine level increases mainly due to (i) extracellular ATP degradation by NTPDases; (ii) release of adenosine *per se* from cells likely by the equilibrative nucleoside transporter (ENT); (iii) inhibition of adenosine-uptake processes due to downregulation of concentrative nucleoside transporters (CNT) 2 and 3 and of ENT. AC: adenylate cyclase; ADO: adenosine; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; E5′-NT: ecto-5′-nucleotidase; NT: nucleoside transporter; Gs: stimulatory *G*-protein; NTDPase: ecto-nucleoside triphosphate diphosphohydrolases. The proportions of the various components of the nervous tissue have not been kept.

Most recently, the question has been raised if A_{2A} receptor continuous blockade over an extended time-window after ischemia is protective. CSC continuously administered over 72 hours, using subcutaneously implanted osmotic minipumps, after permanent MCAo in spontaneously hypertensive rats, did not decrease brain infarct volume determined by magnetic resonance imaging 3 days after induction of ischemia [53]. Authors attributed the lack of protection to high hepatic metabolism and elimination of CSC [53]. Consistently, Melani and coworkers (unpublished observation) found a lack of protection on infarct volume by SCH58261 administered subchronically (three times in the first day) or chronically (twice/day for 7 days) 7 days after 1 hour transient MCAo.

2.2. A_{2A} Receptor Antagonism Protects from the Increase of Glutamate Extracellular Concentrations and NMDA Receptor Function. A_{2A} receptors are expressed on neurons at high levels in the striatum [71] and at lower levels in all other brain regions as detected by autoradiography [72] and real time PCR [73]. A_{2A} receptors in the striatum are mostly present on GABA-enkephalin neurons [74] but are also located presynaptically [25, 75, 76] on glutamatergic terminals [77] where they can directly regulate glutamate outflow under normoxic [78, 79] and ischemic conditions [65, 66]. Adenosine, by A_{2A} receptor stimulation, promotes glutamate release under normoxic and ischemic conditions *in vivo* [44, 51, 80–82]. Consistently, A_{2A} receptors play an important modulation of synaptic transmission [83, 84] as mostly demonstrated in the hippocampus [85–87]. In the CA1 area of the rat hippocampus, which is the most sensitive region to ischemia, the selective A_{2A} receptor agonist, CGS21680, clearly reduces the depression of synaptic activity brought about by OGD [47]. Following A_{2A} receptor stimulation the increase of extracellular glutamate concentration counteracts depression brought about by adenosine A_1 receptors. In agreement, the selective A_{2A} receptor antagonists, ZM241385 and SCH58261, delay the appearance of anoxic depolarization (AD), a phenomenon strictly related to cell damage and death [88], protect from the synaptic activity depression brought about by a severe (7 min) OGD period, and protect CA1 neuron and astrocyte from injury [55]. Same effects of ZM241385 were observed

Table 1: Adenosine A_{2A} receptor ligands used in brain ischemia "*in vivo*" and "*in vitro*" models.

	Brain ischemia model	References
Adenosine A_{2A} receptor agonists		
CGS21680	Global ischemia in rat	[44]
	Global ischemia in gerbil	[45]
	Focal ischemia in rat	[46]
	OGD hippocampal slices	[47, 48]
APEC	Global ischemia in gerbil	[49]
Adenosine A_{2A} receptor antagonists		
CGS15943	Global ischemia in gerbil	[50]
	Global ischemia in rat	[51]
CSC	Global ischemia in gerbil	[49, 52]
	Focal ischemia in hypertensive rat	[53]
CP66713	Global ischemia in gerbil	[52]
ZM241385	Global ischemia in rat	[54]
	OGD hippocampal slices	[55, 56]
	Hypoxia/ischemia in neonatal rat	[57]
SCH58261	Focal ischemia in rat	[58–62]
	Global ischemia in newborn piglet	[63]
	OGD hippocampal slices	[55, 64]
	OGD cerebrocortical slices	[65, 66]
DMPX	OGD hippocampal slices	[48]

APEC: 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5′-N-ethylcarboxoamidoadenosine; CGS15943: 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine; CGS21680: 2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; CP66713: 4-amino[1,2,4]triazolo[4,3a]quinoxalines; CSC: 8-(3-chlorostyryl)caffeine; DMPX: 3,7-dimethyl-1-propargylxanthine; OGD: oxygen and glucose deprivation; SCH58261: 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-[1,2,4]triazolo[1,5-c]pyrimidine; ZM241385: 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol.

after a severe 9 min OGD period in the gyrus dentatus of the hippocampus [56]. The time-window of the protective effects of the A_{2A} receptor antagonists in the hippocampus overlaps with the delay obtained by treating the slices with glutamate receptor antagonists [89, 90], indicating that their effects are attributable to reduced glutamate excitotoxicity.

Several mechanisms contribute to the A_{2A} receptor regulation of extracellular glutamate concentrations. A_{2A} receptor stimulation might regulate extracellular glutamate not only by reducing release from glutamatergic terminals but also by modulation of glutamate uptake transporter. In the brain, adenosine A_{2A} receptors are expressed on both neurons and glia [21, 71]. In particular, A_{2A} receptors located on astrocytes mediate inhibition of glutamate uptake by glutamate transporter-1 (GLT-1) [91–93]. Recent data show that while acute exposure to the selective A_{2A} receptor agonist, CGS21680, reduces glutamate uptake, prolonged exposure to the same agonist inhibits GLT-1 and glutamate-aspartate transporter mRNA and protein levels from astrocytes [94]. Such inhibition is exerted through modulation of Na^+/K^+-ATPase [95]. An imbalance of A_1/A_{2A} receptor expression might also contribute to inhibition of excitatory synaptic transmission under ischemia. Short periods of global ischemia decrease A_1 adenosine receptor density in the brain likely due to an internalization of A_1 adenosine receptors in nerve terminals [96]. Moreover tight A_1/A_{2A} receptor interaction exists. In hippocampal and cortical nerve terminals A_{2A} receptors might increase glutamate outflow by a protein kinase C-mediated decrease of the affinity of A_1 receptors [97]. A heteromerization of adenosines A_1 and A_{2A} receptors in striatal glutamatergic nerve terminals might allow adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission. A main biochemical characteristic of the A_1/A_2 receptor heteromer is the ability of A_{2A} receptor activation to reduce the affinity of the A_1 receptor for agonists with an ultimate switch mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamate release [98].

Adenosine acting on A_{2A} receptors is such an important modulatory substance by controlling synaptic transmission and also by regulating AMPA [99] and NMDA receptor function [100]. In striatal membranes, the NMDA-mediated excitation, leading to a depolarized plateau potential and spike firing, is regulated by dopamine and adenosine acting at D_2 and A_{2A} receptor heteromers that regulate Ca^{++} channel activity through mechanisms relying upon specific protein-protein interactions [101]. A_{2A} receptor chronic blockade by treatment with SCH58261 induces a remodeling of NR1 and NR2A/NR2B subunit expression of NMDA receptors in the striatum of Huntington transgenic mice [102]. Moreover, given that mGlu5 receptors "set the tone" of NMDA receptor-mediated neurotransmission [103], it appears important that mGlu5 receptors are under the tight control of A_{2A} receptors [100]. In the hippocampus A_{2A} and mGlu5 receptors are colocated and A_{2A} receptors play a permissive role in mGlu5 receptor-mediated potentiation of NMDA effects

[104]. Such modulations by A_{2A} receptors might be relevant in pathological conditions such as ischemia. By the use of SCH58261, it was demonstrated that A_{2A} receptors support the expression and recruitment of calcium-permeable AMPA receptors during LTP induced by OGD in rat hippocampal slices [64]. In a model of global ischemia in newborn piglets (7 min Asphyxic Cardiac Arrest), inhibition of phosphorylation of NMDA receptor NR1 subunit and inhibition of Na^+/K^+-ATPase and of cAMP-regulated phosphoprotein 32 kDa (DARPP32) might also account for protective effect of the selective A_{2A} receptor antagonist SCH58261 [63]. The ability of adenosine A_{2A} receptors in controlling glutamate receptor functions might represent an attractive mechanism in protecting against acute excitoxicity after ischemia. In fact, in a number of *in vitro* and *in vivo* experimental models of ischemia, glutamate receptor antagonists, acting either on NMDA receptor or on group I metabotropic receptors, are effective neuroprotective agents; none of the glutamate receptor antagonists tested in clinical trials showed positive results or had an acceptable benefit/side effects ratio [105].

In vivo, a definite overexpression of A_{2A} receptors was found in neurons of the striatum and cortex 24 hours after focal ischemia [106] and, in *in vivo* experiments, the low dose of SCH58261 that protects against tissue damage induced by MCAo or quinolinic acid (QA) excitotoxicity also reduces glutamate extracellular concentrations estimated by microdialysis [59, 107]. This supports that protective effects of low doses of A_{2A} receptor antagonists administered early after brain ischemia are largely due to reduced excitotoxicity and to the ensuing excitotoxic cascade attributable to stimulation of NMDA receptors [59]. The robust protection by A_{2A} receptor antagonism is consistent with the observation that adenosine A_{2A} receptor KO mice are protected from an excess of striatal glutamate outflow and damage induced by transient MCAo [69, 70].

A further protective effect of A_{2A} receptor antagonism may be attributed to the capability of increasing GABA outflow during ischemia. The major part of excitatory glutamatergic innervation is modulated by inhibitory GABA-releasing interneurons. Potentiation of GABAergic synaptic transmission has neuroprotective effects in several experimental models of cerebral ischemia [108]. GABA is strongly increased in the cortex and striatum during ischemia [15, 109] and evidence shows that selective A_{2A} receptor stimulation decreases ischemia-evoked GABA outflow [109, 110] and enhances GABA transport into nerve terminals by restraining PKC inhibition of GAT-1 [111].

The neuroprotective properties of A_{2A} receptor antagonists largely reside in effects mediated by A_{2A} receptors located on brain cells, in particular in control of excitotoxicity as demonstrated by the observation that the A_{2A} receptor selective antagonist, ZM241385, injected peripherally or directly intra-hippocampus is protective against excitotoxicity induced by kainate [48] and by the combinations of quinolinic acid and IL-1β [112].

2.3. A_{2A} Receptor Antagonists Protect from Ischemia–Induced Activation of Mitogen-Activated Protein Kinases (MAPKs) and c-fos Expression.

Several data indicate that regulation of proteins involved in transcriptional or post-translational mechanisms plays an important role in the neuroprotective effect of A_{2A} receptor antagonism in ischemia.

All members of the MAPKs family are activated up to 24 hours after ischemia [113, 114]. p38 and ERK1/2 are activated in neurons and in microglia [60, 113, 115, 116]. A definite overexpression of A_{2A} receptors was found not only in neurons but also on microglia of the ischemic tissue 24 hours after focal ischemia [106]. Subchronic administration of the A_{2A} receptor antagonist, SCH58261, reduced phospho-p38 in microglia while it did not affect ERK1/2 activation [60]. It is known that soon after excitotoxic phenomena, resident microglial cells initiate a rapid change in their phenotype that is referred to as microglial cell activation [117] and, by producing cytotoxic substances and cytokines, start an inflammatory response that exacerbate brain damage [6]. Since inhibition of p38 activation has direct neuroprotective effects in hippocampal brain slices after OGD [118], a control of p38 activation by A_{2A} receptor antagonism [60] might account for protection after ischemia. Such results are in agreement with the result that intracerebroventricular injection of SCH58261 prevents the recruitment of activated microglial cells and the increase in IL-1β evaluated 4 hours after intraperitoneal administration of lipopolysaccharide (LPS) [119]. It is also important to consider that A_{2A} receptor antagonists are effective in preventing neurotoxicity in isolated glia. A_{2A} receptor stimulation is known in fact to cause activation of microglia [120] and A_{2A} receptor antagonists have been shown to suppress microglia activation in murine N9 microglial cells exposed to an inflammatory stimulus such as LPS [121]. A_{2A} receptor antagonist suppresses the CGS21680-induced potentiation of LPS-induced NO release from mixed glial cultures as well [122]. Overall results indicate that A_{2A} receptors present on microglial cells are pivotal in mediating a secondary damage consisting in neuroinflammation (see later in the paper) after ischemia.

Twenty-four hours after MCAo, subchronic administration of the A_{2A} receptor antagonist, SCH58261, also reduces phospho-JNK, that is expressed in few neurons, but mainly in mature oligodendrocytes and in oligodendrocyte precursors (OPCs) (stained by Olig2 and NG2 antibodies) [61, 123]. Phospho-JNK is a factor involved in oligodendrocyte death [124, 125]. Interestingly activation of JNK has been described in oligodendrocytes in multiple sclerosis lesions where oligodendrocytes are major targets of the disease [126]. A specific peptide inhibitor of JNK protects against cell death induced by OGD *in vitro* [127] and by MCAo *in vivo* [127, 128]. JNK2/3 KO mice are protected from damage following cerebral ischemia [129, 130]. Therefore we must assume that JNK activation in oligodendrocytes and neurons represents a noxious event after ischemia that can damage oligodendrocytes bringing to myelin damage and disorganization [61]. A_{2A} receptor antagonism also reduces Olig2 [61] that is a transcription factor expressed mostly by OPC while mature oligodendrocytes are characterized by lower levels of Olig2 [131]. Data have suggested that A_{2A} antagonism stimulates OPC differentiation to mature cells after ischemia. In agreement we have recently reported

that, in primary OPC culture, selective stimulation of A_{2A} receptors by CGS21680 inhibits maturation of OPC in the firsts 10 days of *in vitro* differentiation [132]. The drug also inhibits K^+ "delayed rectifier" channels (KDR) [132] that are known to inhibit proliferation and differentiation of OPC to mature oligodendrocytes, thus preventing myelin deposition [133, 134].

Besides a direct effect of the A_{2A} receptor antagonists on A_{2A} receptors located on oligodendrocytes or microglia, we must consider that the reduced MAPK activation by SCH58261, in the initial hours after *in vivo* ischemia, is secondary, to overall reduction in the excitotoxic cascade that in turn primes MAPK activation [59]. In fact, oligodendroglia are extremely sensitive to glutamate receptor overactivation and ensuing oxidative stress [135–137] as well as to cytokines [138] and p38 activation is definitely induced by NMDA receptor stimulation in cerebellar granule cells [139] and in spinal cord cultures [140].

It is of note that, twenty-four hours after permanent MCAo, the A_{2A} antagonist, SCH58261, also reduces gene c-fos expression in glial cells [62]. Products of the Fos family are players in inducing inflammatory gene expression in glial cells [141].

3. Adenosine A_{2A} Receptor Agonists Protect against Secondary Injury

3.1. A_{2A} Receptor Agonists Are Protective against Ischemic Damage. While many data support that A_{2A} receptor antagonists protect against central excitotoxicity, the protective effect of A_{2A} receptor agonists appears attributable to different mechanisms. The A_{2A} receptor antagonist ZM241385 administered repeatedly (1 mg/kg i.p.) in the 12 hours after traumatic brain injury was protective 15 min after trauma when cerebro spinal fluid (CSF) glutamate concentration rose; conversely, the A_{2A} receptor agonist, CGS21680, administered repeatedly (0.1 mg/kg i.p.) in the 12 hours after trauma was protective 3 hours after trauma when CSF glutamate concentrations were down [142].

A protective role of adenosine A_{2A} receptor in hypoxia/ischemia was demonstrated in newborn rodents. A_{2A} receptor KO neonatal mice show aggravated hypoxic/ischemic injury in comparison to wild-type mice [143] and, in immature brain forebrain slices, it was demonstrated that cannabinoids induce robust neuroprotection through both CB(2) and A_{2A} adenosine receptors [144]. Most recently it was demonstrated that A_{2A} receptor KO mice subjected to chronic cerebral hypoperfusion by permanent stenosis of bilateral common carotid artery show impairment in working memory, increased demyelination, proliferation of glia, and increased levels of proinflammatory cytokines [145]. In adult gerbil, a protective effect of adenosine A_{2A} receptor agonists was reported by Von Lubitz et al. [49] who demonstrated that the A_{2A} receptor agonist, APEC, administered systemically before a global 10 min ischemia, ameliorated recovery of blood flow and animal and neuron survival. Moreover Sheardown and Knutsen [45] demonstrated that a high dose of the selective A_{2A} receptor agonist, CGS21680 (10 mg/kg i.p.), administered after 5 min of global ischemia in gerbil, exhibited highly significant protection against neuronal loss, but was inactive at 3 mg/kg. In these two works in adult gerbils, adenosine agonists were administered before ischemia or at a high dose. In considering translation to clinic, a main problem of A_{2A} receptor agonists is their cardiovascular effect: adenosine A_{2A} receptors located on vase smooth muscle and endotelial cells exert a vasodilatory effect [146]. Consistently A_{2A} receptor agonists might induce hypotension and increase hearth rate. Schindler and coworkers [147, 148] reported that the decrease of blood pressure induced by 0.5 mg/kg i.p. CGS21680 in conscious rats is most probably mediated in the periphery, while the increase of heart rate is mediated at central level. We recently demonstrated that the selective A_{2A} receptor agonist, CGS21680, at dose of 0.1 mg/kg i.p., increased heart rate only in the first hour after administration, but no effect on blood pressure or on heart rate was observed at the lower dose of 0.01 mg/kg [46]. Relevantly our recent experiments have demonstrated that the A_{2A} receptor agonist, CGS21680, administered twice/day for 7 days (chronic protocol) at dose of 0.01 and 0.1 mg/kg, starting 4 hours after transient (1 hour) MCAo, induced protection from neurological deficit, weight loss, cortical infarct volume, myelin disorganization and glial activation [46]. Protective effect is exerted only when CGS21680 is chronically administered. In fact the A_{2A} receptor agonist administered at the same dose (0.1 mg/kg) but in a shorter therapeutic window (4 and 20 hours after induction of MCAo, subchronic protocol) has not reduced the infarct volume 24 hours after permanent MCAo nor 7 days after transient MCAo (unpublished data; see Table 2). The protective effects of chronic administration of CGS21680 at dose of 0.01 and 0.1 mg/kg neither can be attributed to changes in the cardiovascular parameters either at peripheral or central level nor can be attributed to direct effects on motility because CGS21680 at these low doses does not affect motor behavior of rats [149].

Several mechanisms might account for protection by A_{2A} receptor stimulation by direct effects on brain cells. In a rat model of intracerebral hemorrhage, CGS21680 administered directly into the striatum immediately prior to the induction of intracerebral hemorrhage reduces parenchymal neutrophile infiltration and tissue damage: an effect that might be mediated by inhibition of TNF-α expression [150]. Moreover, activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors [151] as NGF in microglia [152], BDNF in mice hippocampus [153], in rat cortical neurons [154], and in primary cultures of microglia [121], and GDNF in striatal neurons [155]. Consistently it was recently demonstrated that *in vivo* chronic oral administration of the A_{2A} receptor antagonist, KW-6002, decreases both mRNA and protein levels of BDNF receptor (TrkB-FL) and its signaling in the hippocampal CA1 area [156]. The increase in neurotrophic factor expression by adenosine A_{2A} receptor stimulation may contribute to restore neurological functions and cerebral damage after brain ischemia. We must also remember that adenosine is implicated in cerebral blood flow regulation as a vasodilator

Table 2: Effect of A_{2A} receptor agonist, CGS21680, in acute and delayed phase of stroke.

Drug	24 h after pMCAo Infarct volume (mm^3)		Treatment	7 days after tMCAo Infarct volume (mm^3)	
	Cortex	Striatum		Cortex	Striatum
Vehicle	69.43 ± 1.87	46.03 ± 2.7	Subchronic	75.1 ± 5.1	28.4 ± 2.2
			Chronic	76.2 ± 4.3	31.3 ± 2.6
CGS21680	61.37 ± 8.26	45.68 ± 2.6	Subchronic 0.1	62.7 ± 5.8	30.5 ± 2.5
			Chronic 0.1	48.6 ± 9.5$^{\#}$	27.3 ± 1.7
			Chronic 0.01	51.9 ± 10.4$^{\#}$	20.9 ± 3.8

Data are the mean ± S.E.M. of $n = 6$–8 animals. In the model of permanent MCAo (pMCAo), CGS21680 was administered at the dose of 0.1 mg/kg (i.p.) after 4 h and 20 h from ischemia induction. The infarct volume was evaluated 24 h thereafter.
In the model of transient MCAo (tMCAo), CGS21680 was administered *in subchronic protocol* 4 h and 20 h after ischemia at the dose of 0.1 mg/kg (i.p.) and *in chronic protocol* starting 4 h after ischemia, at the dose of 0.01 or 0.1 mg/kg (i.p.), twice/day for 7 days. The infarct volume was evaluated 7 days after MCAo.
One-way ANOVA: $^{\#}P < 0.05$ versus chronic vehicle-treated rats.
Effects of CGS21680 chronically administered are published [46].

agent acting on A_{2A} receptors on endothelial cells of brain vessels, thus favouring brain perfusion [146].

Several lines of evidence in excitotoxicity and spinal cord trauma *in vivo* models do not support, however, that protection by A_{2A} receptor agonists is exerted at A_{2A} receptors located on CNS cells. Jones and coworkers [157] showed that peripheral administration of the A_{2A} receptor agonist, CGS21680, protected the hippocampus against kainate-induced excitotoxicity while the direct injection of CGS21680 into the hippocampus failed to afford protection [157]. Similar results were obtained after spinal cord trauma where CGS21680 protected from damage when injected systemically but not when centrally injected into the injured spinal cord [158].

3.2. A_{2A} Receptor and Neuroinflammation. Minutes to hours after onset of cerebral ischemia, a cascade of inflammatory events is initiated through activation of resident cells [159]. The early massive increase in extracellular glutamate after ischemia has a main role in activating resident immune cells and producing mediators of inflammation [6]. Immunity and inflammation are key elements of the pathology of stroke. Recent developments have revealed that stroke engaged both innate and adaptative immunity. Molecules generated by cerebral ischemic tissue activate components of innate immunity, promote inflammatory signaling, and contribute to tissue damage. The A_{2A} adenosine receptors are expressed both on cells of innate (microglia, macrophages, mast cells, monocytes, dendritic cells, and neutrophils) and on cells of adaptive (lymphocytes) immunity [160, 161]. Soon after excitotoxic phenomena, microglial cells initiate a rapid change in their phenotype [60, 119] that is referred to as microglial cell activation [117]. Microglia typically respond with proliferation, migration, and production of inflammatory substances to viral or bacterial stimuli or to cell damage and degeneration [121, 162] and, by producing cytotoxic substances, cytokines (TNF-α, IL-1β) [119, 120, 163], and chemokines, contribute to the inflammatory response that follows ischemic insult, further exacerbating brain damage [6]. Proinflammatory mediators and oxidative stress contribute to the endothelial expression of cellular adhesion molecules [7, 8] and to an altered permeability of the blood-brain barrier (BBB) that promotes the infiltration of leukocytes (neutrophils, lymphocytes, and monocytes) [164] in the brain ischemic tissue.

In a model of transient focal cerebral ischemia induced by MCAo, definite microglial activation is present after 12 hours [165]. After ischemia, although reperfusion is necessary for tissue survival, it also contributes to additional tissue damage. Under reperfusion, there is an initial increase of BBB permeability (see [166]) followed by a biphasic increase at 5 and 72 hours [167]. Changes in BBB permeability are responsible for cell infiltration. The nature of BBB permeability is dependent on the duration of ischemia, the degree of reperfusion, and the animal stroke model. Studies in the human brain after ischemic stroke confirm that neutrophils intensively accumulate in the regions of cerebral infarction [6, 168]. Selective immunostaining for granulocytes, by anti-HIS-48 antibody, shows numerous infiltrated cells in ischemic striatal and cortical core two days after tMCAo, while seven days thereafter infiltrated blood cells were not anymore observed [46]. Three days after tMCAo the majority of immune cells are neutrophils and at less extent lymphocytes [165, 169]. After tMCAo, a peak of neutrophil infiltration occurs at 6 and 48 hours thereafter [169]. Infiltrated neutrophils expressing cytokines and chemotactic factors promote expansion of the inflammatory response in ischemic tissue [160]. Correlations among neutrophil accumulation, severity of brain tissue damage, and neurological outcome have been reported by Akopov et al. [168]. Neuroinflammation is now recognized as a predominant mechanism of secondary progression of brain injury after ischemia.

Two days after MCAo, chronic treatment with the A_{2A} adenosine receptor agonist, CGS21680, has definitely reduced the number of infiltrated blood cells in the ischemic areas [46]. These results are in agreement with previous observations that A_{2A} receptor agonists systemically administered after spinal cord injury in mice protect from neurological and tissue damage, reduce inflammation parameters and blood cell infiltration [170–172]. An unequivocal role of A_{2A} receptor in controlling blood cell infiltration was demonstrated also in a model of autoimmune encephalomyelitis:

A_{2A} receptor KO mice displayed increased inflammatory cell infiltration, higher neurological deficit scores and increase of different neuroinflammation parameters [173].

A bulk of evidence indicate that bone marrow-derived cells (BMDCs) are targets of A_{2A} receptor agonist protective effects. Li et al. [174] demonstrated that the protective effect against motor deficits of A_{2A} receptor agonists, systemically administered after spinal trauma, is lost in mice lacking A_{2A} receptors on BMDCs, but is restored in A_{2A} receptor KO mice reconstituted with A_{2A} receptors on BMDCs. Many studies have reported that selective activation of A_{2A} receptors directly on blood cells, including platelets, monocytes, some mast cells, neutrophils, and T cells, inhibits proinflammatory responses [175–177], reduces production of adhesion cell factors, and reduces neutrophil activation, thereby exerting antioxidant and anti-inflammatory effects [178]. A_{2A} receptor activation is known to reduce ischemia-induced rolling, adhesion, and transmigration of various peripheral inflammatory cells (such as lymphocytes, neutrophils) [160]. Overall results suggest that protection due to A_{2A} receptor agonists systemically and repeatedly administered after brain ischemia is strongly exerted at peripheral BMDCs resulting ultimately in reduced leukocyte infiltration and reduced inflammatory cascade at the central level. Consistent with its anti-inflammatory and immunosuppressive role, the protective effect of adenosine A_{2A} receptor stimulation has been observed in different pathologies where inflammatory process has an important role in tissue damage [124, 172, 179, 180] such as ischemia/reperfusion liver injury [181], spinal cord trauma [158], rheumatoid arthritis (RA) [182], acute lung inflammation [183], intestine ischemia/reperfusion injury [184], and experimental autoimmune encephalomyelitis [185].

By controlling brain neuroinflammation and BDNF signalling [186, 187], A_{2A} receptors might also have a potential for synaptic plasticity and neurogenetic processes after ischemia. Neuroinflammation in fact is known to result in inhibition of adult neurogenesis [188].

The notion that A_{2A} receptors on BMDCs are the target of the protective effects of A_{2A} receptor agonists should be reconciled with the information that selective inactivation of A_{2A} receptors on BMDCs (wild-type mice transplanted with A_{2A} receptor KO bone marrow cells) attenuates ischemic brain injury, inhibits inflammatory cytokines production, and increases the expression of anti-inflammatory cytokines in the ischemic brain 22 hours after 2 hours of focal ischemia induced by MCAo. This neuroprotection however cannot be explained by altered infiltration of the major inflammatory cells, neutrophils and microglial cells, in the ischemic brain and remains to be clarified [189].

4. Caffeine Consumption and Stroke Incidence

It has been reported that acute coffee consumption is associated with increased risk of ischemic stroke in the subsequent hour in infrequent coffee drinkers (<1 cup) [190]. The increased risk might be related within hours after consumption to acute deleterious effects of the unselective A_1/A_{2A} receptor antagonist, caffeine, that increases circulating norepinephrine [191], rises mean blood pressure [192], increases arterial stiffness [193], and impairs endothelium-dependent vasodilation [194].

More studies have instead investigated the effect of habitual consumption of caffeine on the risk of stroke. Controversial results, mainly in relation to the dose intake, were obtained [195]. A study showed that the long-term moderate consumption of coffee can provide protective effects (reducing the risk of both coronary heart disease and stroke by 10%–20%) in healthy individuals yet detrimental effects when intake was high [196]. In agreement, Larsson and Orsini [197] reported that it is the moderate coffee consumption (3-4 cups/day) that reduces the risk of stroke. Additionally, one study showed that coffee consumption (more than 4 cups/day) in men was not associated with increased risk of stroke [198] while studies performed in Swedish and USA women have indicated that habitual intake of coffee (from 1 to 5 or more cups/day) was associated with a statistically significant lower risk of total stroke [199], cerebral infarction, and subarachnoid hemorrhage but not intracerebral hemorrhage [200]. In contrast, an epidemiological study showed that neither the high (more than 4 cups/day) nor the low doses (less than 2 cups/day) have the most dangerous effect but it is the intermediate consumption (2–4 cups/day) of coffee which can be the most harmful [201]. Thus the effect of different consumption of caffeine in reducing the risk of ischemic stroke still demand further study.

5. A_{2A} Receptor Based Therapies in Cerebral Ischemia

Evidence reported up to now indicate that antagonism or stimulation of A_{2A} receptors might be a protective strategy secondary to the time-related development of phenomena typical of ischemia. After ischemia, extracellular glutamate concentrations remain elevated at least up to 4 hours after permanent MCAo [15, 59] and up to 12 hours after brain trauma [142]. The massive increase of glutamate excitotoxicity triggers acute tissue injury and the start of an inflammatory cascade that is stressed by blood cell infiltration. While central A_{2A} receptors in the first hours after ischemia are critical in increasing glutamate extracellular concentrations, A_{2A} receptors on blood cells are critical hours and days after ischemia in decreasing activation, adhesion, and infiltration of blood cells in brain parenchyma. Altogether, evidence suggests that A_{2A} receptor antagonists provide protection centrally by reducing excitotoxicity, while A_{2A} receptor agonists provide protection by acting on blood cells controlling massive infiltration and neuroinflammation in the hours after brain ischemia. In agreement the lack of detecting a protection by A_{2A} receptor antagonism at later time after stroke [53, our unpublished observation] might be attributable to the fact that protection is overwhelmed by subsequent damage brought about by blood cell infiltration that starts 6 hours after ischemia and peaks at 2 days thereafter [46, 165, 169].

These observations highlight that a therapeutic strategy with adenosine A_{2A} receptor antagonists/agonists should be

carefully evaluated in terms of time after ischemia. When considering use of adenosine A_{2A} receptor active drugs to protect against brain ischemia, attention should be given to administration time after injury and to the dose used. In fact A_{2A} receptors located on endothelial cells mediate important effect on systemic blood pressure and heart frequency. However both A_{2A} receptor antagonists [58, 59, 61] and agonists [46] are protective in ischemia models at doses that do not modify blood pressure nor the heart frequency.

The design and development of new adenosine A_{2A} receptor ligands is an area of intense research activity [202, 203].

6. Conclusions

Under neurodegenerative conditions involving ischemia, excitotoxicity is a first phenomenon. Thereafter, the interplay of resident glial cells with infiltrating peripheral BMDCs produces neuroinflammation. On the light that the role of adenosine A_{2A} receptors in ischemia is not univocal, it is important to clarify the windows in which A_{2A} receptors play a noxious or protective role after ischemia. This will be important to devise a correct therapeutic strategy with antagonists and/or agonists at this receptor. Considering translation to clinical practice, a very short time-window of minutes/few hours would be available for A_{2A} receptor antagonists after stroke, while a focus on inflammatory responses to stroke provides a wide therapeutic time-window of hours and even days after stroke for adenosine A_{2A} receptor agonists. A novel therapeutic strategy could involve, when possible, early treatment with A_{2A} receptor antagonists to reduce excitotoxicity followed by adenosine A_{2A} receptor agonist treatment for the control of later secondary injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] E. P. Soler and V. C. Ruiz, "Epidemiology and risk factors of cerebral ischemia and ischemic heart diseases: similarities and differences," *Current Cardiology Reviews*, vol. 6, no. 3, pp. 138–149, 2010.

[2] F. Chen, Z. Qi, Y. Luo et al., "Non-pharmaceutical therapies for stroke: mechanisms and clinical implications," *Progress in Neurobiology*, vol. 115C, pp. 246–269, 2014.

[3] J. de Keyser, G. Sulter, and P. G. Luiten, "Clinical trials with neuroprotective drugs in acute ischaemic stroke: are we doing the right thing?" *Trends in Neurosciences*, vol. 22, no. 12, pp. 535–540, 1999.

[4] Z. O'Bryant, K. T. Vann, and Z. G. Xiong, "Translational strategies for neuroprotection in ischemic stroke—focusing on acid-sensing ion channel 1a," *Translational Stroke Research*, vol. 5, no. 1, pp. 59–68, 2014.

[5] T. W. Lai, S. Zhang, and Y. T. Wang, "Excitotoxicity and stroke: identifying novel targets for neuroprotection," *Progress in Neurobiology C*, vol. 115, pp. 157–188, 2014.

[6] U. Dirnagl, C. Iadecola, and M. A. Moskowitz, "Pathobiology of ischaemic stroke: an integrated view," *Trends in Neurosciences*, vol. 22, no. 9, pp. 391–397, 1999.

[7] J. Huang, U. M. Upadhyay, and R. J. Tamargo, "Inflammation in stroke and focal cerebral ischemia," *Surgical Neurology*, vol. 66, no. 3, pp. 232–245, 2006.

[8] G. Stoll, S. Jander, and M. Schroeter, "Inflammation and glial responses in ischemic brain lesions," *Progress in Neurobiology*, vol. 56, no. 2, pp. 149–171, 1998.

[9] J. W. Phillis, M. Smith-Barbour, M. H. O'Regan, and L. M. Perkins, "Amino acid and purine release in rat brain following temporary middle cerebral artery occlusion," *Neurochemical Research*, vol. 19, no. 9, pp. 1125–1130, 1994.

[10] J. W. Phillis, M. Smith-Barbour, and M. H. O'Regan, "Changes in extracellular amino acid neurotransmitters and purines during and following ischemias of different durations in the rat cerebral cortex," *Neurochemistry International*, vol. 29, no. 2, pp. 115–120, 1996.

[11] H. Hagberg, P. Andersson, J. Lacarewicz, I. Jacobson, S. Butcher, and M. Sandberg, "Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia," *Journal of Neurochemistry*, vol. 49, no. 1, pp. 227–231, 1987.

[12] E. Dux, J. Fastbom, U. Ungerstedt, K. Rudolphi, and B. B. Fredholm, "Protective effect of adenosine and a novel xanthine derivative propentofylline on the cell damage after bilateral carotid occlusion in the gerbil hippocampus," *Brain Research*, vol. 516, no. 2, pp. 248–256, 1990.

[13] K. Matsumoto, R. Graf, G. Rosner, N. Shimada, and W.-D. Heiss, "Flow thresholds for extracellular purine catabolite elevation in cat focal ischemia," *Brain Research*, vol. 579, no. 2, pp. 309–314, 1992.

[14] V. M. Sciotti, F. M. Roche, M. C. Grabb, and D. G. L. van Wylen, "Adenosine receptor blockade augments interstitial fluid levels of excitatory amino acids during cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 12, no. 4, pp. 646–655, 1992.

[15] A. Melani, L. Pantoni, C. Corsi et al., "Striatal outflow of adenosine, excitatory amino acids, γ-aminobutyric acid, and taurine in awake freely moving rats after middle cerebral artery occlusion: correlations with neurological deficit and histopathological damage," *Stroke*, vol. 30, no. 11, pp. 2448–2455, 1999.

[16] A. Melani, F. Corti, H. Stephan et al., "Ecto-ATPase inhibition: ATP and adenosine release under physiological and ischemic in vivo conditions in the rat striatum," *Experimental Neurology*, vol. 233, no. 1, pp. 193–204, 2012.

[17] H. Zimmermann, "Extracellular metabolism of ATP and other nucleotides," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 362, no. 4-5, pp. 299–309, 2000.

[18] M. Fausther, J. Lecka, E. Soliman et al., "Coexpression of ecto-5'-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 302, no. 4, pp. G447–G459, 2012.

[19] L. Medina-Pulido, M. Molina-Arcas, C. Justicia et al., "Hypoxia and P1 receptor activation regulate the high-affinity concentrative adenosine transporter CNT2 in differentiated neuronal PC12 cells," *Biochemical Journal*, vol. 454, no. 3, pp. 437–445, 2013.

[20] B. B. Fredholm, K. A. Jacobson, K. N. Klotz, and J. Linden, "International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors," *Pharmacological Reviews*, vol. 53, no. 4, pp. 527–552, 2001.

[21] B. L. Fiebich, K. Biber, K. Lieb et al., "Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_{2a}-receptors," *GLIA*, vol. 18, no. 2, pp. 152–160, 1996.

[22] R. A. Peterfreund, M. MacCollin, J. Gusella, and J. Stephen Fink, "Characterization and expression of the human A_{2a} adenosine receptor gene," *Journal of Neurochemistry*, vol. 66, no. 1, pp. 362–368, 1996.

[23] C. Brodie, P. M. Blumberg, and K. A. Jacobson, "Activation of the A_{2A} adenosine receptor inhibits nitric oxide production in glial cells," *FEBS Letters*, vol. 429, no. 2, pp. 139–142, 1998.

[24] P. Svenningsson, C. Le Moine, G. Fisone, and B. B. Fredholm, "Distribution, biochemistry and function of striatal adenosine A_{2A} receptors," *Progress in Neurobiology*, vol. 59, no. 4, pp. 355–396, 1999.

[25] B. D. Hettinger, A. Lee, J. Linden, and D. L. Rosin, "Ultrastructural localization of adenosine A_{2A} receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum," *Journal of Comparative Neurology*, vol. 431, pp. 331–346, 2001.

[26] L. Yu, M. C. Frith, Y. Suzuki et al., "Characterization of genomic organization of the adenosine A_{2A} receptor gene by molecular and bioinformatics analyses," *Brain Research*, vol. 1000, no. 1-2, pp. 156–173, 2004.

[27] E. Ongini, M. Adami, C. Ferri, and R. Bertorelli, "Adenosine A_{2A} receptors and neuroprotection," *Annals of the New York Academy of Sciences*, vol. 825, pp. 30–48, 1997.

[28] R. A. Cunha, "Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors," *Neurochemistry International*, vol. 38, no. 2, pp. 107–125, 2001.

[29] J. A. Ribeiro, A. M. Sebastião, and A. De Mendonça, "Adenosine receptors in the nervous system: pathophysiological implications," *Progress in Neurobiology*, vol. 68, no. 6, pp. 377–392, 2002.

[30] M. A. Schwarzschild, J. Chen, and A. Ascherio, "Caffeinated clues and the promise of adenosine A_{2a} antagonists in PD," *Neurology*, vol. 58, no. 8, pp. 1154–1160, 2002.

[31] B. B. Fredholm, R. A. Cunha, and P. Svenningsson, "Pharmacology of adenosine A_{2A} receptors and therapeutic applications," *Current Topics in Medicinal Chemistry*, vol. 3, no. 4, pp. 413–426, 2003.

[32] F. Pedata, A. M. Pugliese, E. Coppi et al., "Adenosine in the central nervous system: effects on neurotransmission and neuroprotection," *Immunology, Endocrine and Metabolic Agents in Medicinal Chemistry*, vol. 7, no. 4, pp. 304–321, 2007.

[33] H. Kitagawa, A. Mori, J. Shimada, Y. Mitsumoto, and T. Kikuchi, "Intracerebral adenosine infusion improves neurological outcome after transient focal ischemia in rats," *Neurological Research*, vol. 24, no. 3, pp. 317–323, 2002.

[34] R. Corradetti, G. Lo Conte, F. Moroni, M. Beatrice Passani, and G. Pepeu, "Adenosine decreases aspartate and glutamate release from rat hippocampal slices," *European Journal of Pharmacology*, vol. 104, no. 1-2, pp. 19–26, 1984.

[35] T. V. Dunwiddie, "Interactions between the effects of adenosine and calcium on synaptic responses in rat hippocampus in vitro," *Journal of Physiology*, vol. 350, pp. 545–559, 1984.

[36] T. Zetterstrom and M. Fillenz, "Adenosine agonists can both inhibit and enhance in vivo striatal dopamine release," *European Journal of Pharmacology*, vol. 180, no. 1, pp. 137–143, 1990.

[37] F. Pedata, S. Latini, A. M. Pugliese, and G. Pepeu, "Investigations into the adenosine outflow from hippocampal slices evoked by ischemia-like conditions," *Journal of Neurochemistry*, vol. 61, no. 1, pp. 284–289, 1993.

[38] P. Andine, "Involvement of adenosine in ischemic and postischemic calcium regulation," *Molecular and Chemical Neuropathology*, vol. 18, no. 1-2, pp. 35–49, 1993.

[39] D. W. Choi, "Possible mechanisms limiting N-methyl-D-aspartate receptor overactivation and the therapeutic efficacy of N-methyl-D-aspartate antagonists," *Stroke*, vol. 21, no. 11, pp. I-20–I-22, 1990.

[40] R. W. Greene and H. L. Haas, "The electrophysiology of adenosine in the mammalian central nervous system," *Progress in Neurobiology*, vol. 36, no. 4, pp. 329–341, 1991.

[41] A. V. Gourine, N. Dale, V. N. Gourine, and K. M. Spyer, "Fever in systemic inflammation: roles of purines," *Frontiers in Bioscience*, vol. 9, pp. 1011–1022, 2004.

[42] P. J. White, R. B. Rose'Meyer, and W. Hope, "Functional characterization of adenosine receptors in the nucleus tractus solitarius mediating hypotensive responses in the rat," *The British Journal of Pharmacology*, vol. 117, no. 2, pp. 305–308, 1996.

[43] B. B. Fredholm, J. Chen, S. A. Masino, and J. Vaugeois, "Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs," *Annual Review of Pharmacology and Toxicology*, vol. 45, pp. 385–412, 2005.

[44] M. H. O'Regan, R. E. Simpson, L. M. Perkins, and J. W. Phillis, "The selective A_{2A} adenosine receptor agonist CGS21680 enhances excitatory transmitter amino acid release from the ischemic rat cerebral cortex," *Neuroscience Letters*, vol. 138, no. 1, pp. 169–172, 1992.

[45] M. J. Sheardown and L. J. S. Knutsen, "Unexpected neuroprotection observed with the adenosine A_{2A} receptor agonist CGS21680," *Drug Development Research*, vol. 39, pp. 108–114, 1996.

[46] A. Melani, F. Corti, L. Cellai, M. G. Vannucchi, and F. Pedata, "Low doses of the selective adenosine A_{2A} receptor agonist CGS21680 are protective in a rat model of transient cerebral ischemia," *Brain Research*, vol. 1551, pp. 59–72, 2014.

[47] S. Latini, F. Bordoni, R. Corradetti, G. Pepeu, and F. Pedata, "Effect of A_{2A} adenosine receptor stimulation and antagonism on synaptic depression induced by in vitro ischaemia in rat hippocampal slices," *British Journal of Pharmacology*, vol. 128, no. 5, pp. 1035–1044, 1999.

[48] P. A. Jones, R. A. Smith, and T. W. Stone, "Protection against kainate-induced excitotoxicity by adenosine A_{2A} receptor agonists and antagonists," *Neuroscience*, vol. 85, no. 1, pp. 229–237, 1998.

[49] D. K. J. E. Von Lubitz, R. C. S. Lin, and K. A. Jacobson, "Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A_{2A} receptor agonist and antagonist," *European Journal of Pharmacology*, vol. 287, no. 3, pp. 295–302, 1995.

[50] Y. Gao and J. W. Phillis, "CGS 15943, An adenosine A2 receptor antagonist, reduces cerebral ischemic injury in the mongolian gerbil," *Life Sciences*, vol. 55, no. 3, pp. PL61–PL65, 1994.

[51] R. E. Simpson, M. H. O'Regan, L. M. Perkins, and J. W. Phillis, "Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: effects of adenosine receptor agonists and antagonists," *Journal of Neurochemistry*, vol. 58, no. 5, pp. 1683–1690, 1992.

[52] J. W. Phillis, "The effects of selective A_1 and A_{2a} adenosine receptor antagonists on cerebral ischemic injury in the gerbil," *Brain Research*, vol. 705, no. 1-2, pp. 79–84, 1995.

[53] U. Fronz, A. Deten, F. Baumann et al., "Continuous adenosine A_{2A} receptor antagonism after focal cerebral ischemia in spontaneously hypertensive rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 387, pp. 165–173, 2014.

[54] H. Higashi, J. R. Meno, A. S. Marwaha, and H. R. Winn, "Hippocampal injury and neurobehavioral deficits following hyperglycemic cerebral ischemia: effect of theophylline and ZM 241385," *Journal of Neurosurgery*, vol. 96, no. 1, pp. 117–126, 2002.

[55] A. M. Pugliese, C. Traini, S. Cipriani et al., "The adenosine A_{2A} receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices," *British Journal of Pharmacology*, vol. 157, no. 5, pp. 818–830, 2009.

[56] G. Maraula, C. Traini, T. Mello et al., "Effects of oxygen and glucose deprivation on synaptic transmission in rat dentate gyrus: role of A_{2A} adenosine receptors," *Neuropharmacology*, vol. 67, pp. 511–520, 2013.

[57] E. Bona, U. Ådén, E. Gilland, B. B. Fredholm, and H. Hagberg, "Neonatal cerebral hypoxia-ischemia: the effect of adenosine receptor antagonists," *Neuropharmacology*, vol. 36, no. 9, pp. 1327–1338, 1997.

[58] A. Monopoli, G. Lozza, A. Forlani, A. Mattavelli, and E. Ongini, "Blockade of adenosine A_{2A} receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats," *NeuroReport*, vol. 9, no. 17, pp. 3955–3959, 1998.

[59] A. Melani, L. Pantoni, F. Bordoni et al., "The selective A_{2A} receptor antagonist SCH 58261 reduces striatal transmitter outflow, turning behavior and ischemic brain damage induced by permanent focal ischemia in the rat," *Brain Research*, vol. 959, no. 2, pp. 243–250, 2003.

[60] A. Melani, M. Gianfriddo, M. G. Vannucchi et al., "The selective A_{2A} receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia," *Brain Research*, vol. 1073-1074, no. 1, pp. 470–480, 2006.

[61] A. Melani, S. Cipriani, M. G. Vannucchi et al., "Selective adenosine A_{2A} receptor antagonism reduces JNK activation in oligodendrocytes after cerebral ischaemia," *Brain*, vol. 132, no. 6, pp. 1480–1495, 2009.

[62] A. Petroni, N. Papini, M. Blasevich, and C. Galli, "Blockade of A_{2A} adenosine receptors leads to *c-fos* inhibition in a rat model of brain ischemia," *Pharmacological Research*, vol. 45, no. 2, pp. 125–128, 2002.

[63] Z. J. Yang, B. Wang, H. Kwansa et al., "Adenosine A_{2A} receptor contributes to ischemic brain damage in newborn piglet," *Journal of Cerebral Blood Flow and Metabolism*, vol. 33, pp. 1612–1620, 2013.

[64] R. B. Dias, D. M. Rombo, J. A. Ribeiro, and A. M. Sebastião, "Ischemia-induced synaptic plasticity drives sustained expression of calcium-permeable AMPA receptors in the hippocampus," *Neuropharmacology*, vol. 65, pp. 114–122, 2013.

[65] M. Marcoli, L. Raiteri, A. Bonfanti et al., "Sensitivity to selective adenosine A_1 and A_{2A} receptor antagonists of the release of glutamate induced by ischemia in rat cerebrocortical slices," *Neuropharmacology*, vol. 45, no. 2, pp. 201–210, 2003.

[66] M. Marcoli, A. Bonfanti, P. Roccatagliata et al., "Glutamate efflux from human cerebrocortical slices during ischemia: vesicular-like mode of glutamate release and sensitivity to A_{2A} adenosine receptor blockade," *Neuropharmacology*, vol. 47, no. 6, pp. 884–891, 2004.

[67] F. Pedata, M. Gianfriddo, D. Turchi, and A. Melani, "The protective effect of adenosine A_{2A} receptor antagonism in cerebral ischemia," *Neurological Research*, vol. 27, no. 2, pp. 169–174, 2005.

[68] P. Svenningsson, G. G. Nomikos, E. Ongini, and B. B. Fredholm, "Antagonism of adenosine A_{2A} receptors underlies the behavioural activating effect of caffeine and is associated with reduced expression of messenger RNA for NGFI-A and NGFI-B in caudate-putamen and nucleus accumbens," *Neuroscience*, vol. 79, no. 3, pp. 753–764, 1997.

[69] J. F. Chen, Z. Huang, J. Ma et al., "A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice," *Journal of Neuroscience*, vol. 19, no. 21, pp. 9192–9200, 1999.

[70] L. Gui, W. Duan, H. Tian et al., "Adenosine A_{2A} receptor deficiency reduces striatal glutamate outflow and attenuates brain injury induced by transient focal cerebral ischemia in mice," *Brain Research*, vol. 1297, pp. 185–193, 2009.

[71] J. S. Fink, D. R. Weaver, S. A. Rivkees et al., "Molecular cloning of the rat A_2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum," *Molecular Brain Research*, vol. 14, no. 3, pp. 186–195, 1992.

[72] B. Johansson and B. B. Fredholm, "Further characterization of the binding of the adenosine receptor agonist [^{3}H]CGS 21680 to rat brain using autoradiography," *Neuropharmacology*, vol. 34, no. 4, pp. 393–403, 1995.

[73] A. K. Dixon, A. K. Gubitz, D. J. S. Sirinathsinghji, P. J. Richardson, and T. C. Freeman, "Tissue distribution of adenosine receptor mRNAs in the rat," *British Journal of Pharmacology*, vol. 118, no. 6, pp. 1461–1468, 1996.

[74] S. N. Schiffmann, O. Jacobs, and J. J. Vanderhaeghen, "Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an in situ hybridization histochemistry study," *Journal of Neurochemistry*, vol. 57, no. 3, pp. 1062–1067, 1991.

[75] D. L. Rosin, B. D. Hettinger, A. Lee, and J. Linden, "Anatomy of adenosine A_{2A} receptors in brain: morphological substrates for integration of striatal function," *Neurology*, vol. 61, no. 11, pp. S12–S18, 2003.

[76] N. Rebola, P. M. Canas, C. R. Oliveira, and R. A. Cunha, "Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat," *Neuroscience*, vol. 132, no. 4, pp. 893–903, 2005.

[77] N. Rebola, R. J. Rodrigues, L. V. Lopes, P. J. Richardson, C. R. Oliveira, and R. A. Cunha, "Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus," *Neuroscience*, vol. 133, no. 1, pp. 79–83, 2005.

[78] L. V. Lopes, R. A. Cunha, B. Kull, B. B. Fredholm, and J. A. Ribeiro, "Adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission is dependent on tonic A_1 receptor inhibition," *Neuroscience*, vol. 112, no. 2, pp. 319–329, 2002.

[79] R. J. Rodrigues, T. M. Alfaro, N. Rebola, C. R. Oliveira, and R. A. Cunha, "Co-localization and functional interaction

between adenosine A_{2A} and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum," *Journal of Neurochemistry*, vol. 92, no. 3, pp. 433–441, 2005.

[80] P. Popoli, P. Betto, R. Reggio, and G. Ricciarello, "Adenosine A_{2A} receptor stimulation enhances striatal extracellular glutamate levels in rats," *European Journal of Pharmacology*, vol. 287, no. 2, pp. 215–217, 1995.

[81] C. Corsi, A. Melani, L. Bianchi, G. Pepeu, and F. Pedata, "Striatal A_{2A} adenosine receptors differentially regulate spontaneous and K+-evoked glutamate release in vivo in young and aged rats," *NeuroReport*, vol. 10, no. 4, pp. 687–691, 1999.

[82] C. Corsi, A. Melani, L. Bianchi, and F. Pedata, "Striatal A_{2A} adenosine receptor antagonism differentially modifies striatal glutamate outflow in vivo in young and aged rats," *NeuroReport*, vol. 11, no. 11, pp. 2591–2595, 2000.

[83] A. M. Sebastião and J. A. Ribeiro, "Adenosine A_2 receptor-mediated excitatory actions on the nervous system," *Progress in Neurobiology*, vol. 48, no. 3, pp. 167–189, 1996.

[84] L. V. Lopes, A. M. Sebastião, and J. A. Ribeiro, "Adenosine and related drugs in brain diseases: present and future in clinical trials," *Current Topics in Medicinal Chemistry*, vol. 11, no. 8, pp. 1087–1101, 2011.

[85] R. A. Cunha, B. Johansson, I. van der Ploeg, A. M. Sebastião, J. A. Ribeiro, and B. B. Fredholm, "Evidence for functionally important adenosine A_{2A} receptors in the rat hippocampus," *Brain Research*, vol. 649, no. 1-2, pp. 208–216, 1994.

[86] E. Martin O'Kane and T. W. Stone, "Interaction between adenosine A_1 and A_2 receptor-mediated responses in the rat hippocampus in vitro," *European Journal of Pharmacology*, vol. 362, no. 1, pp. 17–25, 1998.

[87] L. V. Lopes, R. A. Cunha, and J. A. Ribeiro, "ZM 241385, an adenosine A_{2A} receptor antagonist, inhibits hippocampal A_1 receptor responses," *European Journal of Pharmacology*, vol. 383, no. 3, pp. 395–398, 1999.

[88] G. G. Somjen, "Mechanisms of spreading depression and hypoxic spreading depression-like depolarization," *Physiological Reviews*, vol. 81, no. 3, pp. 1065–1096, 2001.

[89] E. Tanaka, S. Yamamoto, Y. Kudo, S. Mihara, and H. Higashi, "Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro," *Journal of Neurophysiology*, vol. 78, no. 2, pp. 891–902, 1997.

[90] S. Yamamoto, E. Tanaka, Y. Shoji, Y. Kudo, H. Inokuchi, and H. Higashi, "Factors that reverse the persistent depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro," *Journal of Neurophysiology*, vol. 78, no. 2, pp. 903–911, 1997.

[91] T. Nishizaki, K. Nagai, T. Nomura et al., "A new neuromodulatory pathway with a glial contribution mediated via A_{2A} adenosine receptors," *GLIA*, vol. 39, no. 2, pp. 133–147, 2002.

[92] A. Pintor, M. Galluzzo, R. Grieco, A. Pèzzola, R. Reggio, and P. Popoli, "Adenosine A_{2A} receptor antagonists prevent the increase in striatal glutamate levels induced by glutamate uptake inhibitors," *Journal of Neurochemistry*, vol. 89, no. 1, pp. 152–156, 2004.

[93] A. Pinto-Duarte, J. E. Coelho, R. A. Cunha, J. A. Ribeiro, and A. M. Sebastião, "Adenosine A_{2A} receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus," *Journal of Neurochemistry*, vol. 93, no. 3, pp. 595–604, 2005.

[94] M. Matos, E. Augusto, A. D. Santos-Rodrigues et al., "Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes," *GLIA*, vol. 60, no. 5, pp. 702–716, 2012.

[95] M. Matos, E. Augusto, P. Agostinho, R. A. Cunha, and J. F. Chen, "Antagonistic interaction between adenosine A_{2A} receptors and Na^+/K^+-ATPase-$\alpha 2$ controlling glutamate uptake in astrocytes," *Journal of Neuroscience*, vol. 33, pp. 18492–18502, 2013.

[96] J. E. Coelho, N. Rebola, I. Fragata, J. A. Ribeiro, A. de Mendonça, and R. A. Cunha, "Hypoxia-induced desensitization and internalization of adenosine A_1 receptors in the rat hippocampus," *Neuroscience*, vol. 138, no. 4, pp. 1195–1203, 2006.

[97] L. V. Lopes, R. A. Cunha, and J. A. Ribeiro, "Cross talk between A_1 and A_{2A} adenosine receptors in the hippocampus and cortex of young adult and old rats," *Journal of Neurophysiology*, vol. 82, no. 6, pp. 3196–3203, 1999.

[98] F. Ciruela, V. Casadó, R. J. Rodrigues et al., "Presynaptic control of striatal glutamatergic neurotransmission by adenosine A_1-A_{2A} receptor heteromers," *Journal of Neuroscience*, vol. 26, no. 7, pp. 2080–2087, 2006.

[99] R. B. Dias, J. A. Ribeiro, and A. M. Sebastião, "Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A_{2A} receptors," *Hippocampus*, vol. 22, no. 2, pp. 276–291, 2012.

[100] N. Rebola, R. Lujan, R. A. Cunha, and C. Mulle, "Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses," *Neuron*, vol. 57, no. 1, pp. 121–134, 2008.

[101] K. Azdad, D. Gall, A. S. Woods, C. Ledent, S. Ferré, and S. N. Schiffmann, "Dopamine D_2 and adenosine A_{2A} receptors regulate NMDA-mediated excitation in accumbens neurons through A_{2A}-D_2 receptor heteromerization," *Neuropsychopharmacology*, vol. 34, no. 4, pp. 972–986, 2009.

[102] A. Martire, A. Ferrante, R. L. Potenza et al., "Remodeling of striatal NMDA receptors by chronic A_{2A} receptor blockade in Huntington's disease mice," *Neurobiology of Disease*, vol. 37, no. 1, pp. 99–105, 2010.

[103] S. Alagarsamy, S. T. Rouse, R. W. Gereau IV, S. F. Heinemann, Y. Smith, and P. J. Conn, "Activation of N-methyl-D-aspartate receptors reverses desensitization of metabotropic glutamate receptor, mGluR5, in native and recombinant systems," *Annals of the New York Academy of Sciences*, vol. 868, pp. 526–530, 1999.

[104] M. T. Tebano, A. Martire, N. Rebola et al., "Adenosine A_{2A} receptors and metabotropic glutamate 5 receptors are co-localized and functionally interact in the hippocampus: a possible key mechanism in the modulation of N-methyl-D-aspartate effects," *Journal of Neurochemistry*, vol. 95, no. 4, pp. 1188–1200, 2005.

[105] F. Moroni and A. Chiarugi, "Post-ischemic brain damage: targeting PARP-1 within the ischemic neurovascular units as a realistic avenue to stroke treatment," *The FEBS Journal*, vol. 276, no. 1, pp. 36–45, 2009.

[106] M. L. Trincavelli, A. Melani, S. Guidi et al., "Regulation of A_{2A} adenosine receptor expression and functioning following permanent focal ischemia in rat brain," *Journal of Neurochemistry*, vol. 104, no. 2, pp. 479–490, 2008.

[107] P. Popoli, A. Pintor, M. R. Domenici et al., "Blockade of striatal adenosine A_{2A} receptor reduces, through a presynaptic mechanism, quinolinic acid-induced excitotoxicity: possible relevance to neuroprotective interventions in neurodegenerative diseases of the striatum," *Journal of Neuroscience*, vol. 22, no. 5, pp. 1967–1975, 2002.

[108] R. D. Schwartz-Bloom and R. Sah, "γ-aminobutyric acid$_A$ neurotransmission and cerebral ischemia," *Journal of Neurochemistry*, vol. 77, no. 2, pp. 353–371, 2001.

[109] M. H. O'Regan, R. E. Simpson, L. M. Perkins, and J. W. Phillis, "Adenosine receptor agonists inhibit the release of γ-aminobutyric acid (GABA) from the ischemic rat cerebral cortex," *Brain Research*, vol. 582, no. 1, pp. 22–26, 1992.

[110] P. Saransaari and S. S. Oja, "GABA release modified by adenosine receptors in mouse hippocampal slices under normal and ischemic conditions," *Neurochemical Research*, vol. 30, no. 4, pp. 467–473, 2005.

[111] S. Cristóvão-Ferreira, S. H. Vaz, J. A. Ribeiro, and A. M. Sebastião, "Adenosine A_{2A} receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1," *Journal of Neurochemistry*, vol. 109, no. 2, pp. 336–347, 2009.

[112] T. W. Stone and W. M. H. Behan, "Interleukin-1β but not tumor necrosis factor-α potentiates neuronal damage by quinolinic acid: protection by an adenosine A_{2A} receptor antagonist," *Journal of Neuroscience Research*, vol. 85, no. 5, pp. 1077–1085, 2007.

[113] E. A. Irving, F. C. Barone, A. D. Reith, S. J. Hadingham, and A. A. Parsons, "Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat," *Molecular Brain Research*, vol. 77, no. 1, pp. 65–75, 2000.

[114] D. C. Wu, W. Ye, X. M. Che, and G. Y. Yang, "Activation of mitogen-activated protein kinases after permanent cerebral artery occlusion in mouse brain," *Journal of Cerebral Blood Flow and Metabolism*, vol. 20, no. 9, pp. 1320–1330, 2000.

[115] Y. Takagi, K. Nozaki, T. Sugino, I. Hattori, and N. Hashimoto, "Phosphorylation of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase after transient forebrain ischemia in mice," *Neuroscience Letters*, vol. 294, no. 2, pp. 117–120, 2000.

[116] C. S. Piao, J. B. Kim, P. L. Han, and J. K. Lee, "Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult," *Journal of Neuroscience Research*, vol. 73, no. 4, pp. 537–544, 2003.

[117] A. J. Bruce-Keller, "Microglial-neuronal interactions in synaptic damage and recovery," *Journal of Neuroscience Research*, vol. 58, no. 1, pp. 191–201, 1999.

[118] F. C. Barone, E. A. Irving, A. M. Ray et al., "Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia," *Medicinal Research Reviews*, vol. 21, pp. 129–145, 2001.

[119] N. Rebola, A. P. Simões, P. M. Canas et al., "Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction," *Journal of Neurochemistry*, vol. 117, no. 1, pp. 100–111, 2011.

[120] A. G. Orr, A. L. Orr, X. Li, R. E. Gross, and S. F. Traynelis, "Adenosine A_{2A} receptor mediates microglial process retraction," *Nature Neuroscience*, vol. 12, no. 7, pp. 872–878, 2009.

[121] C. Gomes, R. Ferreira, J. George et al., "Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *Journal of Neuroinflammation*, vol. 10, article 16, 2013.

[122] J. Saura, E. Angulo, A. Ejarque et al., "Adenosine A_{2A} receptor stimulation potentiates nitric oxide release by activated microglia," *Journal of Neurochemistry*, vol. 95, no. 4, pp. 919–929, 2005.

[123] A. Melani, F. Corti, M. G. Vannucchi, D. Nosi, M. G. Giovannini, and F. Pedata, "Role of A_{2A} receptors on modulation of oligodendroglia in cerebral ischemia," *Shock*, vol. 36, supplement 1, p. 24, 2011.

[124] C. L. Howe, A. J. Bieber, A. E. Warrington, L. R. Pease, and M. Rodriguez, "Antiapoptotic signaling by a remyelination-promoting human antimyelin antibody," *Neurobiology of Disease*, vol. 15, no. 1, pp. 120–131, 2004.

[125] A. Jurewicz, M. Matysiak, S. Andrzejak, and K. Selmaj, "TRAIL-induced death of human adult oligodendrocytes is mediated by JNK pathway," *GLIA*, vol. 53, no. 2, pp. 158–166, 2006.

[126] B. Bonetti, C. Stegagno, B. Cannella, N. Rizzuto, G. Moretto, and C. S. Raine, "Activation of NF-κB and c-jun transcription factors in multiple sclerosis lesions: implications for oligodendrocyte pathology," *The American Journal of Pathology*, vol. 155, no. 5, pp. 1433–1438, 1999.

[127] L. Hirt, J. Badaut, J. Thevenet et al., "D-JNKI1, a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia," *Stroke*, vol. 35, no. 7, pp. 1738–1743, 2004.

[128] T. Borsellol, P. G. H. Clarkel, L. Hirt et al., "A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia," *Nature Medicine*, vol. 9, no. 9, pp. 1180–1186, 2003.

[129] C. Y. Kuan, A. J. Whitmarsh, D. D. Yang et al., "A critical role of neural-specific JNK3 for ischemic apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 25, pp. 15184–15189, 2003.

[130] M. Gelderblom, S. Eminel, T. Herdegen, and V. Waetzig, "c-Jun N-terminal kinases (JNKs) and the cytoskeleton—functions beyond neurodegeneration," *International Journal of Developmental Neuroscience*, vol. 22, no. 7, pp. 559–564, 2004.

[131] M. Kitada and D. H. Rowitch, "Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord," *GLIA*, vol. 54, no. 1, pp. 35–46, 2006.

[132] E. Coppi, L. Cellai, G. Maraula, A. M. Pugliese, and F. Pedata, "Adenosine A_{2A} receptors inhibit delayed rectifier potassium currents and cell differentiation in primary purified oligodendrocyte cultures," *Neuropharmacology*, vol. 73, pp. 301–310, 2013.

[133] P. Shrager and S. D. Novakovic, "Control of myelination, axonal growth, and synapse formation in spinal cord explants by ion channels and electrical activity," *Developmental Brain Research*, vol. 88, no. 1, pp. 68–78, 1995.

[134] B. Attali, N. Wang, A. Kolot, A. Sobko, V. Cherepanov, and B. Soliven, "Characterization of delayed rectifier Kv channels in oligodendrocytes and progenitor cells," *Journal of Neuroscience*, vol. 17, no. 21, pp. 8234–8245, 1997.

[135] C. Matute, M. V. Sánchez-Gómez, L. Martínez-Millán, and R. Miledi, "Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 16, pp. 8830–8835, 1997.

[136] C. Matute, E. Alberdi, G. Ibarretxe, and M. V. Sánchez-Gómez, "Excitotoxicity in glial cells," *European Journal of Pharmacology*, vol. 447, no. 2-3, pp. 239–246, 2002.

[137] J. W. Mcdonald, S. P. Althomsons, K. L. Hyrc, D. W. Choi, and M. P. Goldberg, "Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity," *Nature Medicine*, vol. 4, no. 3, pp. 291–297, 1998.

[138] S. A. Back, "Perinatal white matter injury: the changing spectrum of pathology and emerging insights into pathogenetic

mechanisms," *Mental Retardation and Developmental Disabilities Research Reviews*, vol. 12, no. 2, pp. 129–140, 2006.

[139] H. Kawasaki, T. Morooka, S. Shimohama et al., "Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells," *Journal of Biological Chemistry*, vol. 272, no. 30, pp. 18518–18521, 1997.

[140] T. M. Tikka and J. E. Koistinaho, "Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia," *Journal of Immunology*, vol. 166, no. 12, pp. 7527–7533, 2001.

[141] A. J. Lewis and A. M. Manning, "New targets for anti-inflammatory drugs," *Current Opinion in Chemical Biology*, vol. 3, no. 4, pp. 489–494, 1999.

[142] S. S. Dai, Y. G. Zhou, W. Li et al., "Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury," *Journal of Neuroscience*, vol. 30, no. 16, pp. 5802–5810, 2010.

[143] U. Ådén, L. Halldner, H. Lagercrantz, I. Dalmau, C. Ledent, and B. B. Fredholm, "Aggravated brain damage after hypoxic ischemia in immature adenosine A_{2A} knockout mice," *Stroke*, vol. 34, no. 3, pp. 739–744, 2003.

[144] A. Castillo, M. R. Tolón, J. Fernández-Ruiz, J. Romero, and J. Martinez-Orgado, "The neuroprotective effect of cannabidiol in an *in vitro* model of newborn hypoxic-ischemic brain damage in mice is mediated by CB_2 and adenosine receptors," *Neurobiology of Disease*, vol. 37, no. 2, pp. 434–440, 2010.

[145] W. Duan, L. Gui, Z. Zhou et al., "Adenosine A_{2A} receptor deficiency exacerbates white matter lesions and cognitive deficits induced by chronic cerebral hypoperfusion in mice," *Journal of the Neurological Sciences*, vol. 285, no. 1-2, pp. 39–45, 2009.

[146] J. W. Phillis, "Adenosine and adenine nucleotides as regulators of cerebral blood flow: roles of acidosis, cell swelling, and KATP channels," *Critical Reviews in Neurobiology*, vol. 16, no. 4, pp. 237–270, 2004.

[147] C. W. Schindler, M. Karcz-Kubicha, E. B. Thorndike et al., "Lack of adenosine A_1 and dopamine D_2 receptor-mediated modulation of the cardiovascular effects of the adenosine A_{2A} receptor agonist CGS 21680," *European Journal of Pharmacology*, vol. 484, no. 2-3, pp. 269–275, 2004.

[148] C. W. Schindler, M. Karcz-Kubicha, E. B. Thorndike et al., "Role of central and peripheral adenosine receptors in the cardiovascular responses to intraperitoneal injections of adenosine A_1 and A_{2A} subtype receptor agonists," *British Journal of Pharmacology*, vol. 144, no. 5, pp. 642–650, 2005.

[149] J. Wardas, J. Konieczny, and M. Pietraszek, "Influence of CGS 21680, a selective adenosine A_{2A} agonist, on the phencyclidine-induced sensorimotor gating deficit and motor behaviour in rats," *Psychopharmacology*, vol. 168, no. 3, pp. 299–306, 2003.

[150] M. Mayne, J. Fotheringham, H. J. Yan et al., "Adenosine A_{2A} receptor activation reduces proinflammatory events and decreases cell death following intracerebral hemorrhage," *Annals of Neurology*, vol. 49, no. 6, pp. 727–735, 2001.

[151] A. M. Sebastião and J. A. Ribeiro, "Triggering neurotrophic factor actions through adenosine A_{2A} receptor activation: implications for neuroprotection," *British Journal of Pharmacology*, vol. 158, no. 1, pp. 15–22, 2009.

[152] K. Heese, B. L. Fiebich, J. Bauer, and U. Otten, "Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A_{2A}-receptors," *Neuroscience Letters*, vol. 231, no. 2, pp. 83–86, 1997.

[153] M. T. Tebano, A. Martire, R. L. Potenza et al., "Adenosine A_{2A} receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus," *Journal of Neurochemistry*, vol. 104, no. 1, pp. 279–286, 2008.

[154] S. J. Jeon, S. Y. Rhee, J. H. Ryu et al., "Activation of adenosine A_{2A} receptor up-regulates BDNF expression in rat primary cortical neurons," *Neurochemical Research*, vol. 36, no. 12, pp. 2259–2269, 2011.

[155] C. A. R. V. Gomes, S. H. Vaz, J. A. Ribeiro, and A. M. Sebastião, "Glial cell line-derived neurotrophic factor (GDNF) enhances dopamine release from striatal nerve endings in an adenosine A_{2A} receptor-dependent manner," *Brain Research*, vol. 1113, no. 1, pp. 129–136, 2006.

[156] A. Jerónimo-Santos, V. L. Batalha, C. E. Müller et al., "Impact of in vivo chronic blockade of adenosine A_{2A} receptors on the BDNF-mediated facilitation of LTP," *Neuropharmacology*, vol. 83, pp. 99–106, 2014.

[157] P. A. Jones, R. A. Smith, and T. W. Stone, "Protection against hippocampal kainate excitotoxicity by intracerebral administration of an adenosine A_{2A} receptor antagonist," *Brain Research*, vol. 800, no. 2, pp. 328–335, 1998.

[158] I. Paterniti, A. Melani, S. Cipriani et al., "Selective adenosine A_{2A} receptor agonists and antagonists protect against spinal cord injury through peripheral and central effects," *Journal of Neuroinflammation*, vol. 8, article 31, 2011.

[159] R. Macrez, C. Ali, O. Toutirais et al., "Stroke and the immune system: from pathophysiology to new therapeutic strategies," *The Lancet Neurology*, vol. 10, no. 5, pp. 471–480, 2011.

[160] G. Haskó, J. Linden, B. Cronstein, and P. Pacher, "Adenosine receptors: therapeutic aspects for inflammatory and immune diseases," *Nature Reviews Drug Discovery*, vol. 7, no. 9, pp. 759–770, 2008.

[161] L. Antonioli, B. Csóka, M. Fornai et al., "Adenosine and inflammation: what's new on the horizon?" *Drug Discovery Today*, 2014.

[162] P. J. Gebicke-Haerter, F. Christoffel, J. Timmer, H. Northoff, M. Berger, and D. Van Calker, "Both adenosine A_1- and A_2-receptors are required to stimulate microglial proliferation," *Neurochemistry International*, vol. 29, no. 1, pp. 37–42, 1996.

[163] M. Buttini, K. Appel, A. Sauter, P.-J. Gebicke-Haerter, and H. W. G. M. Boddeke, "Expression of tumor necrosis factor alpha after focal cerebral ischaemia in the rat," *Neuroscience*, vol. 71, no. 1, pp. 1–16, 1996.

[164] C. Iadecola and J. Anrather, "Stroke research at a crossroad: asking the brain for directions," *Nature Neuroscience*, vol. 14, no. 11, pp. 1363–1368, 2011.

[165] M. Gelderblom, F. Leypoldt, K. Steinbach et al., "Temporal and spatial dynamics of cerebral immune cell accumulation in stroke," *Stroke*, vol. 40, no. 5, pp. 1849–1857, 2009.

[166] K. E. Sandoval and K. A. Witt, "Blood-brain barrier tight junction permeability and ischemic stroke," *Neurobiology of Disease*, vol. 32, no. 2, pp. 200–219, 2008.

[167] T. Kuroiwa, P. Ting, H. Martinez, and I. Klatzo, "The biphasic opening of the blood-brain barrier to proteins following temporary middle cerebral artery occlusion," *Acta Neuropathologica*, vol. 68, no. 2, pp. 122–129, 1985.

[168] S. E. Akopov, N. A. Simonian, and G. S. Grigorian, "Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage," *Stroke*, vol. 27, no. 10, pp. 1739–1743, 1996.

[169] R. L. Zhang, M. Chopp, H. Chen, and J. H. Garcia, "Temporal profile of ischemic tissue damage, neutrophil response, and vascular plugging following permanent and transient (2H) middle cerebral artery occlusion in the rat," *Journal of the Neurological Sciences*, vol. 125, pp. 3–10, 1994.

[170] D. C. Cassada, C. G. Tribble, J. S. Young et al., "Adenosine A_{2A} analogue improves neurologic outcome after spinal cord trauma in the rabbit," *Journal of Trauma-Injury Infection & Critical Care*, vol. 53, no. 2, pp. 225–231, 2002.

[171] T. Genovese, A. Melani, E. Esposito et al., "The selective adenosine A_{2A} receptor agonist CGS 21680 reduces JNK MAPK activation in oligodendrocytes in injured spinal cord," *Shock*, vol. 32, no. 6, pp. 578–585, 2009.

[172] T. Genovese, A. Melani, E. Esposito et al., "Selective adenosine A_{2A} receptor agonists reduce the apoptosis in an experimental model of spinal cord trauma," *Journal of Biological Regulators and Homeostatic Agents*, vol. 24, no. 1, pp. 73–86, 2010.

[173] S. Yao, Z. Li, Q. Huang et al., "Genetic inactivation of the adenosine A_{2A} receptor exacerbates brain damage in mice with experimental autoimmune encephalomyelitis," *Journal of Neurochemistry*, vol. 123, no. 1, pp. 100–112, 2012.

[174] Y. Li, R. J. Oskouian, Y.-J. Day et al., "Mouse spinal cord compression injury is reduced by either activation of the adenosine A_{2A} receptor on bone marrow-derived cells or deletion of the A_{2A} receptor on non-bone marrow-derived cells," *Neuroscience*, vol. 141, no. 4, pp. 2029–2039, 2006.

[175] G. Haskó, D. G. Kuhel, J. Chen et al., "Adenosine inhibits IL-12 and TNF-α production via adenosine A_{2A} receptor-dependent and independent mechanism," *The FASEB Journal*, vol. 14, no. 13, pp. 2065–2074, 2000.

[176] C. M. Lappas, Y. J. Day, M. A. Marshall, V. H. Engelhard, and J. Linden, "Adenosine A_{2A} receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation," *Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2639–2648, 2006.

[177] M. V. Sitkovsky, "Use of the A_{2A} adenosine receptor as a physiological immunosuppressor and to engineer inflammation in vivo," *Biochemical Pharmacology*, vol. 65, no. 4, pp. 493–501, 2003.

[178] M. V. Sitkovsky, D. Lukashev, S. Apasov et al., "Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A_{2A} receptors," *Annual Review of Immunology*, vol. 22, pp. 657–682, 2004.

[179] M. Odashima, G. Bamias, J. Rivera-Nieves et al., "Activation of A_{2A} adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease," *Gastroenterology*, vol. 129, no. 1, pp. 26–33, 2005.

[180] A. Choukèr, M. Thiel, D. Lukashev et al., "Critical role of hypoxia and A_{2A} adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway," *Molecular Medicine*, vol. 14, no. 3-4, pp. 116–123, 2008.

[181] Y. J. Day, M. A. Marshall, L. Huang, M. J. McDuffie, M. D. Okusa, and J. Linden, "Protection from ischemic liver injury by activation of A_{2A} adenosine receptors during reperfusion: inhibition of chemokine induction," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 286, no. 2, pp. G285–G293, 2004.

[182] E. Mazzon, E. Esposito, D. Impellizzeri et al., "CGS 21680, an Agonist of the Adenosine (A_{2A}) receptor, reduces progression of murine type II collagen-induced arthritis," *Journal of Rheumatology*, vol. 38, no. 10, pp. 2119–2129, 2011.

[183] D. Impellizzeri, R. di Paola, E. Esposito et al., "CGS 21680, an agonist of the adenosine (A_{2A}) receptor, decreases acute lung inflammation," *European Journal of Pharmacology*, vol. 668, no. 1-2, pp. 305–316, 2011.

[184] R. Di Paola, A. Melani, E. Esposito et al., "Adenosine A_{2A} receptor-selective stimulation reduces signaling pathways involved in the development of intestine ischemia and reperfusion injury," *Shock*, vol. 33, no. 5, pp. 541–551, 2010.

[185] J. Xu, S. Guo, Z. Jia, S. Ma, Z. Li, and R. Xue, "Additive effect of prostaglandin E_2 and adenosine in mouse experimental autoimmune encephalomyelitis," *Prostaglandins and Other Lipid Mediators*, vol. 100-101, no. 1, pp. 30–35, 2013.

[186] M. J. Diógenes, C. C. Fernandes, A. M. Sebastião, and J. A. Ribeiro, "Activation of adenosine A_{2A} receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices," *The Journal of Neuroscience*, vol. 24, no. 12, pp. 2905–2913, 2004.

[187] M. J. Diógenes, N. Assaife-Lopes, A. Pinto-Duarte, J. A. Ribeiro, and A. M. Sebastião, "Influence of age on BDNF modulation of hippocampal synaptic transmission: interplay with adenosine A_{2A} receptors," *Hippocampus*, vol. 17, no. 7, pp. 577–585, 2007.

[188] C. T. Ekdahl, J. Claasen, S. Bonde, Z. Kokaia, and O. Lindvall, "Inflammation is detrimental for neurogenesis in adult brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13632–13637, 2003.

[189] L. Yu, Z. Huang, J. F. Mariani, Y. Wang, M. Moskowitz, and J. Chen, "Selective inactivation or reconstitution of adenosine A_{2A} receptors in bone marrow cells reveals their significant contribution to the development of ischemic brain injury," *Nature Medicine*, vol. 10, no. 10, pp. 1081–1087, 2004.

[190] E. Mostofsky, G. Schlaug, K. J. Mukamal, W. D. Rosamond, and M. A. Mittleman, "Coffee and acute ischemic stroke onset: the stroke onset study," *Neurology*, vol. 75, no. 18, pp. 1583–1588, 2010.

[191] P. Smits, G. Pieters, and T. Thien, "The role of epinephrine in the circulatory effects of coffee," *Clinical Pharmacology and Therapeutics*, vol. 40, no. 4, pp. 431–437, 1986.

[192] M.-L. Nurminen, L. Niittynen, R. Korpela, and H. Vapaatalo, "Coffee, caffeine and blood pressure: a critical review," *European Journal of Clinical Nutrition*, vol. 53, no. 11, pp. 831–839, 1999.

[193] A. Mahmud and J. Feely, "Acute effect of caffeine on arterial stiffness and aortic pressure waveform," *Hypertension*, vol. 38, no. 2, pp. 227–231, 2001.

[194] C. M. Papamichael, K. A. Aznaouridis, E. N. Karatzis et al., "Effect of coffee on endothelial function in healthy subjects: the role of caffeine," *Clinical Science*, vol. 109, no. 1, pp. 55–60, 2005.

[195] M. Rivera-Oliver and M. Díaz-Ríos, "Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review," *Life Sciences*, vol. 101, pp. 1–9, 2014.

[196] S. K. Bøhn, N. C. Ward, J. M. Hodgson, and K. D. Croft, "Effects of tea and coffee on cardiovascular disease risk," *Food & function*, vol. 3, no. 6, pp. 575–591, 2012.

[197] S. C. Larsson and N. Orsini, "Coffee consumption and risk of stroke: a dose-response meta-analysis of prospective studies," *American Journal of Epidemiology*, vol. 174, no. 9, pp. 993–1001, 2011.

[198] D. E. Grobbee, E. B. Rimm, E. Giovannucci, G. Colditz, M. Stampfer, and W. Willett, "Coffee, caffeine, and cardiovascular disease in men," *New England Journal of Medicine*, vol. 323, no. 15, pp. 1026–1032, 1990.

[199] E. Lopez-Garcia, F. Rodriguez-Artalejo, K. M. Rexrode, G. Logroscino, F. B. Hu, and R. M. Van Dam, "Coffee consumption and risk of stroke in women," *Circulation*, vol. 119, no. 8, pp. 1116–1123, 2009.

[200] S. C. Larsson, J. Virtamo, and A. Wolk, "Coffee consumption and risk of stroke in women," *Stroke*, vol. 42, no. 4, pp. 908–912, 2011.

[201] M. Montagnana, E. J. Favaloro, and G. Lippi, "Coffee intake and cardiovascular disease: virtue does not take center stage," *Seminars in Thrombosis & Hemostasis*, vol. 38, no. 2, pp. 164–177, 2012.

[202] G. Cristalli, C. E. Müller, and R. Volpini, "Recent developments in Adenosine A_{2A} receptor ligands," *Handbook of Experimental Pharmacology*, vol. 193, pp. 59–98, 2009.

[203] K. A. Jacobson, "Structure-based approaches to ligands for G-protein-coupled adenosine and P2Y receptors, from small molecules to nanoconjugates," *Journal of Medicinal Chemistry*, vol. 56, no. 10, pp. 3749–3767, 2013.

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Review Article

Role of Microglia Adenosine A_{2A} Receptors in Retinal and Brain Neurodegenerative Diseases

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Neuroinflammation mediated by microglial cells in the brain has been commonly associated with neurodegenerative diseases. Whether this microglia-mediated neuroinflammation is cause or consequence of neurodegeneration is still a matter of controversy. However, it is unequivocal that chronic neuroinflammation plays a role in disease progression and halting that process represents a potential therapeutic strategy. The neuromodulator adenosine emerges as a promising targeting candidate based on its ability to regulate microglial proliferation, chemotaxis, and reactivity through the activation of its G protein coupled A_{2A} receptor ($A_{2A}R$). This is in striking agreement with the ability of $A_{2A}R$ blockade to control several brain diseases. Retinal degenerative diseases have been also associated with microglia-mediated neuroinflammation, but the role of $A_{2A}R$ has been scarcely explored. This review aims to compare inflammatory features of Parkinson's and Alzheimer's diseases with glaucoma and diabetic retinopathy, discussing the therapeutic potential of $A_{2A}R$ in these degenerative conditions.

1. Introduction

1.1. Role of Microglia in Brain Physiology. In the central nervous system (CNS), microglial cells participate in innate immunity; microglia can respond to different types of signals, namely the presence of pathogens (extrinsic signals) or to intrinsic signals, namely diffusible mediators released by stressed neurons, astrocytes or microglia (reviewed in [1]). Although the present review mainly focuses on the contribution of microglia to the pathophysiology of neurodegeneration in the brain and the retina, any attempt to interfere with microglia in pathological conditions also needs to take into account the role of microglia in physiological conditions.

In the healthy brain, the majority of microglial cells exhibit a ramified phenotype, compatible with a surveillance function of the surrounding environment. This crucial sensor ability is supported by the constant extension and retraction of cellular processes [2, 3]. This dynamics is not random but instead instructed by increased neuronal activity, that activates pannexin-1 hemichannels, triggering the diffusion of signals, namely, ATP, that drive process motility towards that specific neuron [4]. The interconversion between the so-called "surveying" phenotype (considered more adequate, as compared to the old terminology "resting" phenotype) and the "alerted" phenotype can be driven either by external stimuli (e.g., pathogens) or by neural signals. The latter is achieved by direct neuron-microglia contact or by diffusible mediators (reviewed, e.g., in [1]). This activation of microglia drives some immediate responses that mainly consist in (1) production/release of rectifier mediators and (2) phagocytosis of neurons or subcellular components (mainly dendritic spines and synapses). Microglial phagocytosis of neurons or neuronal structures has been mostly studied in pathological conditions (e.g., [5–8]), but it also takes place in nonpathological conditions. In fact, it is a process of particular importance during neurodevelopment, as shown by Tremblay

and coworkers [9] in the visual system: light deprivation and the subsequent decrease in the workload of neuronal circuits involved in visual processing lead to the engulfment of synaptic elements by microglia. This physiological process, termed synaptic pruning, is regulated by the immune system; synapses and axons to be phagocytosed are labeled by the complement components C1q and C3, which prompt their selective recognition by microglial cells [10–12]. Synaptic pruning is crucial to normal brain wiring and function and any impairment of this process may impact on neurodevelopment. For instance, this was recently associated with deficits in synaptic transmission, which are paralleled by behavioral abnormalities characteristic of disorders of the autism spectrum and other neuropsychiatric conditions [13]. This process also occurs during adulthood, particularly in neurogenic niches of the brain, such as the hippocampus, where microglia phagocytose apoptotic newborn neurons [14].

Intriguingly, as part of their physiological role, microglia also actively shape their neuronal environment thanks to their ability to trigger neuronal death [15–17]. Again, such a role has a particular relevance during brain development, namely, during the first postnatal week, as heralded by the observation that microglia accumulate in regions of developmental cell death in the embryonic cerebral cortex [18]; furthermore, in the spinal cord, the cell death of motor neurons correlates temporally with the arrival of microglia [19].

In addition to their role in synaptic pruning, microglia also regulate synapse formation [20–22]. This function has been shown to be dependent on the production and release of mediators, such as brain-derived neurotrophic factor [20] or interleukin- (IL-) 10 [22], although other diffusible mediators are likely to be involved. This critical function of microglia must be strictly preserved in order to prevent neurodevelopmental deficits, as suggested by a recent *in vitro* study showing that activation of microglia by an inflammatory stimulus may impact on the presynaptic differentiation of immature neurons [23].

Microglial support to synapse formation/elimination is tightly associated with the newly recognized role of microglia as active partners in the transmission of information within synapses [24]. Thus, recent studies show that microglia also monitor the functional state of synapses and respond to changes in synaptic activity [25, 26]. Accordingly, the highly motile processes of microglia contact with synapses and regulate synaptic transmission in nonpathological conditions [9, 10, 27–30].

1.2. Role of Microglia in Retinal Physiology. In the adult retina, the presence of microglia has been described in several mammals species, including rabbits [31–33], mice [34], rats [31, 35, 36], monkeys [37, 38], and humans [39–41]. Microglial cells in the adult normal retina are mainly located in the inner vascularized regions, that is, the nerve fiber and ganglion cell layers and in plexiform layers, whereas they are scarce in the inner nuclear layer and absent in the outer nuclear layer (Figure 1).

In the healthy retina, microglial cells represent a self-renewing population of innate immune cells, which constantly survey their microenvironment, as occurs in the brain. Retinal microglia can also phagocytose pyknotic cells generated upon neural remodeling of the retina [42]. A more recent study performed in zebrafish showed that microglial cells not only have a "cleaning" role in the developing retina, but also are required for normal retinal growth and neurogenesis [43]. Microglia may also play a role in the formation of blood vessels in the developing retina, since microglia depletion during retinal development reduces vascularization, an effect restored by intravitreal injection of microglia [44]. This is in agreement with the origin of retinal microglial cells that originate from cells of mesodermal lineage [45] and populate the retina before vascularization and along with the onset of vasculogenesis [46].

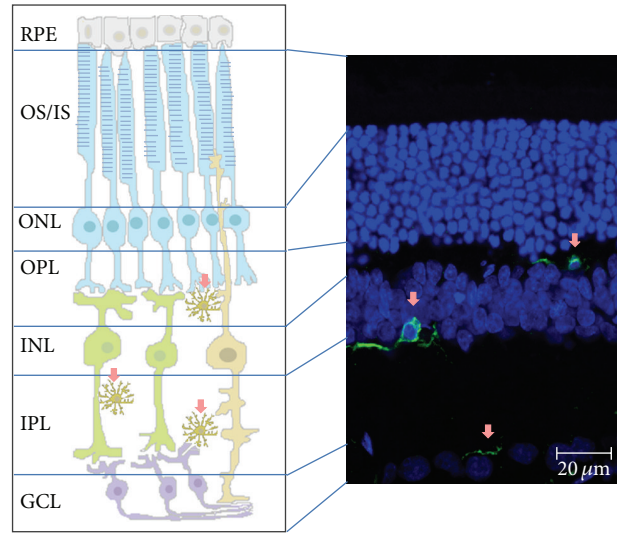

Figure 1: Microglial localization in the retina. Microglial cells in a "surveying" state (pink arrows) in nonpathological conditions are mainly located in the plexiform layers. Retinal layers: OS/IS, outer and inner segments of rods and cones; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Schematic draw of the retinal layers (a) and confocal image from a retinal section where the different layers are depicted (b): nuclear layers (in blue) and microglia cells (in green).

1.3. $A_{2A}R$ Regulation of Microglia Physiology. Adenosine is a neuromodulator, which also exerts important functions in the immune-inflammatory system [47]. Microglial cells express all subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 receptors [48]. Although a large body of evidence highlights the ability of A_1 and A_3 receptors to regulate microglia responses, such as proliferation, morphological phenotype, and release of mediators [49–52], particular attention has been paid to $A_{2A}R$, considered to have a central role in the pathophysiology of degeneration [53–55].

It is claimed that $A_{2A}R$ modulation (both activation and blockade) interferes with microglia-mediated inflammation in degenerative conditions (see below). Of note, in

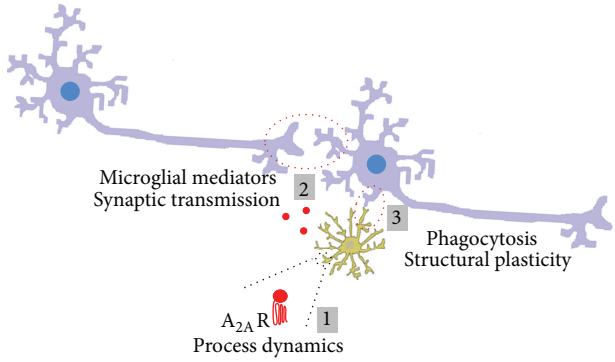

FIGURE 2: Microglia in the healthy brain/retina. Schematic representation of the main functions exerted by microglia (in yellow) under physiological conditions: surveying the environment by constant extension and retraction of processes (it remains to clarify if $A_{2A}R$ regulate this process, as occurs in pathology) (1); regulation of basal synaptic transmission and plasticity through the release of mediators (red circles), some of them being also important mediators of inflammation (2); regulation of spine/synapse structural plasticity, mainly by phagocytosis, a process regulated by inflammatory mediators, according to the neuronal workload (3).

physiological conditions, important functions operated by microglia, namely, the release of mediators, such as trophic factors [56] or nitric oxide (NO) [57], as well as the extension and retraction of processes that govern the surveying activity of microglia [58], are apparently out of $A_{2A}R$ control, until a pathologic insult triggers a gain-of-function of $A_{2A}R$ [56, 57, 59, 60]. However, the milestone study by Davalos et al. [2] shows that the baseline motility of microglial processes in the healthy brain is governed by ATP (and prevented by ATP degradation), as occurs in pathological-like conditions. This observation raises the unanswered question whether the activation of $A_{2A}R$ by ATP-derived adenosine regulates the dynamics of microglial processes in physiological conditions.

1.4. Role of Microglia in Degenerative Conditions of the Brain. The main physiologic roles operated by microglia (release of mediators that control synaptic transmission, synapse formation, and phagocytosis of cells or cellular elements) are strictly dependent upon their sensor ability. Any interference at this functional level may create conditions favoring the development of degenerative processes, which are bolstered by abnormal synaptic transmission, aberrant synapse formation and/or elimination, and abnormal phagocytosis (Figure 2). Therefore, the identification of molecular systems able to modulate microglial functions may help defining new pharmacological targets to interfere with the progression of neurodegenerative diseases. Indeed, microglia-driven neuroinflammation is associated with a broad spectrum of neurodegenerative diseases and has been more detailed in Alzheimer's disease (AD) and Parkinson's disease (PD).

The accumulation of misfolded β-amyloid-containing proteins (Abeta) and alpha-synuclein are histopathological hallmarks of established AD and PD, respectively [61–67]. Protein aggregates can directly exert neurotoxicity [68–70] and can trigger parallel maladaptive changes of glial cells; in fact, animal models of AD and PD and postmortem examination of the brain of AD or PD patients frequently reveal increased numbers of activated microglia in degenerated brain regions [71–76]. Moreover, *in vivo* studies using PET with a radiotracer for activated microglia in AD and PD patients have provided evidence for increased levels of activated microglia in brain regions that are affected by the disease [75–79]. Importantly, protein aggregates may be sufficient causative factors for microglial activation and release of inflammatory mediators [80], which, in turn, amplify neuroinflammation and further exacerbate neurodegeneration [73]. Such a scenario prompts the idea that microglia-induced neuroinflammation may play a critical role in the progression of neurodegenerative conditions [65–67, 81, 82].

Indeed, several microglia-derived inflammatory mediators have been shown to be involved in neuronal damage in neurodegenerative diseases. Thus, one possible causative factor for neuronal death in AD is Aβ-induced NO production by microglia [83]. Furthermore, Aβ and interferon-gamma (IFN-γ) can activate microglia to produce reactive nitrogen intermediates and tumor necrosis factor (TNF), contributing to neuronal degeneration observed in AD [84]. Additional proof-of-concept for the role of microglia in the progression of neuronal damage in AD was derived from the observation that drugs preventing microglial activation indeed delay the emergence of an AD-like phenotype in animal models [85]. Similarly, increased expression of inflammatory mediators is also found in PD animal models [51, 80, 86] and in postmortem PD brains [87, 88], including proinflammatory cytokines, such as IFN-γ, IL-1β, TNF, IL-2, and IL-6, released by microglia [89–91]. The microglial overactivation and the release of proinflammatory cytokines and reactive oxygen species (ROS) are associated with neuronal loss in PD [72, 73]; further evidence for the key role of these microglia-derived mediators in the evolution of neuronal damage in PD was obtained by showing that the inactivation of microglia-derived mediators counteracts neurodegeneration in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) animal model of PD [92–95].

In addition to the direct neurotoxic impact of these microglia-derived inflammatory mediators, the deregulation of the phagocytic activity of microglia also contributes to the progression of neuronal damage. This is heralded by the observations of an increased number of phagocytic microglia close to damaged neurons in PD [96, 97]; furthermore, blocking microglial activation attenuates neurodegeneration, further supporting the role of microglia in the evolution of the pathological process [98]. Increased phagocytosis of neuronal elements seems to be a selective process since *in vitro* studies have suggested that microglia may paradoxically reduce its ability to degrade Aβ-containing aggregates, and their intracellular accumulation leads to dysfunctional/dystrophic microglia [99–101]. In animal models of AD it has been shown in late stages of cerebral amyloidosis that the phagocytic capacity of microglia is impaired [102], and this impairment was described to accelerate pathology progression [103].

In summary, microglial functions, from the release of inflammatory mediators to the ability to phagocytose, are deregulated in neurodegenerative diseases. This implies that

the identification of regulatory systems able to rebalance microglial function may be of therapeutic interest to manage the progression of neurodegenerative diseases.

1.5. Control of Microglia-Driven Neuroinflammation by $A_{2A}R$ in Brain Diseases. The ability of adenosine and $A_{2A}R$ activation to control the activation of different inflammatory cell types has been consistently documented by different groups [47]. Likewise, several *in vitro* and *in vivo* studies clearly demonstrate that $A_{2A}R$ controls several facets of microglia dynamics [56–58, 104, 105], such as (1) the proliferation, (2) the levels of inflammatory enzymes such as cyclooxygenase-2, and (3) the synthesis and release of inflammatory mediators. Furthermore, studies carried out in several models of brain disorders have found that pharmacological blockade or genetic inactivation of $A_{2A}R$ affords a robust neuroprotection [53, 54], and increasing evidence suggests this neuroprotection involves the control of microglia-mediated neuroinflammation [54, 106, 107]. Furthermore, different brain insults triggering neuroinflammation also cause an upregulation of $A_{2A}R$ [56, 60], namely, in microglial cells [56, 57, 59, 108], which is in line with the described ability of cytokines to upregulate $A_{2A}R$ (reviewed by [53]). Finally, $A_{2A}R$ seem to have an additional ability to protect neurons from proinflammatory priming neurodegeneration [109, 110]. This has bolstered the interest to exploit $A_{2A}R$ as a promising pharmacological target to control the neuroinflammatory component of neurodegenerative diseases, allowing the slowdown of their evolution [47, 56, 106, 107].

The clinical interest of the adenosine modulation system in the control of memory dysfunction in AD first arose from epidemiological studies showing an inverse correlation between the consumption of moderate doses of caffeine (a nonselective adenosine receptor antagonist) and the deterioration of memory performance upon aging and AD [111]. This was in notable agreement with animal studies showing that the chronic consumption of caffeine reduces cognitive impairment and decreases Aβ levels in the brain of transgenic mouse models of AD [112–114], as well as in mice exposed to Aβ [104, 115], a purported causative factor of AD [64]. Animal studies were paramount to identify $A_{2A}R$ as the likely targets of caffeine [116], since the pharmacological or genetic blockade of $A_{2A}R$ mimics the neuroprotective effects of caffeine [104, 117]. In accordance with the involvement of neuroinflammatory features in AD, the exposure of rodents to lipopolysaccharide (LPS), which is present in the cell wall of gram-negative bacteria and used as a prototypical activator of microglia, triggers the activation of microglia, a proinflammatory status in the brain parenchyma, and deterioration of synaptic plasticity and memory performance [105]. Notably, this LPS-induced neuroinflammation can be prevented both by the caffeine [118] and by the selective blockade of $A_{2A}R$ [60], which abrogates the LPS-induced dampening of hippocampal synaptic plasticity, the purported neurophysiological basis of learning and memory [119]. Further supporting this role of microglial $A_{2A}R$ in AD, the analysis of postmortem human cortex from AD patients revealed an increased density of $A_{2A}R$ [60] that is more prominent in microglia [120].

As in AD, there is also solid evidence for a role of $A_{2A}R$ in the control of PD, as testified by the recent introduction of $A_{2A}R$ antagonists as coadjuvants in the management of PD [121]. Thus, $A_{2A}R$ antagonists improve PD symptoms in different rodent and primate models of the disease and also in PD patients enrolled in clinical trials (for a review see [122]). Besides the control of motor function, $A_{2A}R$ blockade also dampens microglial activation in the striatum [108] and *substantia nigra* [123] in animal models of PD. Furthermore, caffeine downregulates microglia-driven neuroinflammatory responses and decreases NO production in animal models of PD [124]. Although caffeine acts on both A_1R and $A_{2A}R$, the neuroprotective properties of caffeine in PD are mediated through $A_{2A}R$ blockade [125, 126]. In fact, caffeine consumption has been associated with lower risk of PD in several case-control and cohort studies [127–132]. Interestingly, the association between coffee consumption and PD is strongest among subjects that slowly metabolize caffeine and are homozygous carriers of the CYP1A2 polymorphisms, the gene encoding for cytochrome P450 1A2 [133] which is the main enzyme involved in the metabolism of caffeine.

A recent *ex vivo* study (brain slices from MPTP-treated mice modeling PD) showed that a selective $A_{2A}R$ antagonist restores the ability of microglia to respond to tissue damage [134]. This $A_{2A}R$-mediated control of neuroinflammation is argued to be critical for the neuroprotection afforded by $A_{2A}R$ blockade in PD since the inhibition of microglial function has been shown to be sufficient to decrease the dopaminergic neurodegeneration characteristic of PD.

These two examples of neurodegenerative diseases support the working hypothesis that the beneficial effects resulting from $A_{2A}R$ blockade may involve their ability to attenuate microglial activation and associated chronic neuroinflammatory status, which would interrupt the vicious cross amplifying cycle of degeneration and inflammation leading to a slower development of neurodegenerative disorders (Figure 3).

1.6. Neuroinflammation Is a Common Feature between Retinal and Brain Degenerative Diseases. The combined effect of an ageing population and increasing life expectancy will increase the prevalence of chronic diseases [135], which encompass not only neurodegenerative brain diseases, but also retinal degenerative conditions amongst others. Indeed, the demographic evolution, with an increasing elderly population in western countries, exponentially augments the number of people at risk of age-related visual impairment caused by age-related retinal degenerative diseases [136]. Glaucoma and diabetic retinopathy are leading causes of blindness worldwide. Glaucoma is the second cause of irreversible blindness [137], affecting 70 million people worldwide and approximately 2% of the population over the age of 40 [138]. Diabetic retinopathy is a frequent complication of diabetes and may lead to blindness, making it one of the most feared complications of diabetes. Indeed, diabetic retinopathy is the leading

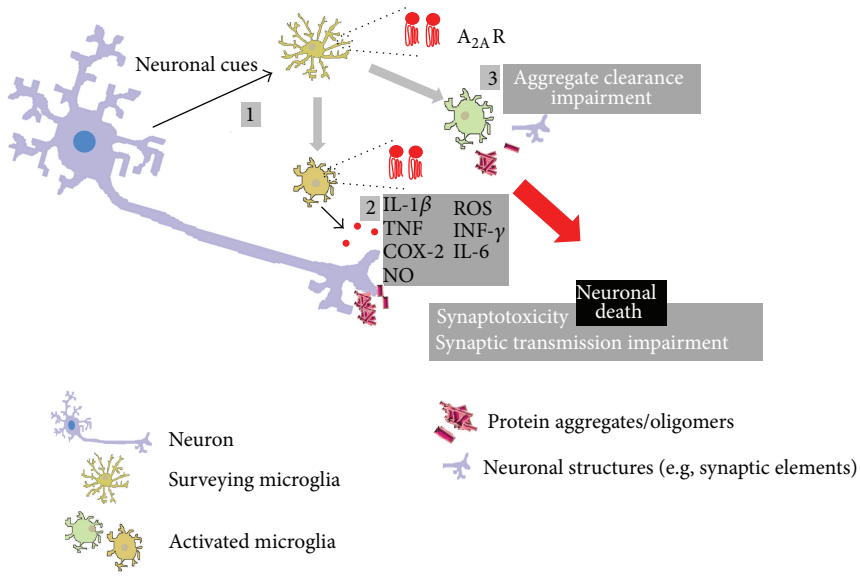

Figure 3: Microglia and neuroinflammation in the brain/retina. Schematic representation of the main inflammatory responses mediated by microglial cells (in yellow) in neurodegenerative conditions. Environment surveillance allows the detection of "pathological" events affecting neurons (in blue-purple); note that appropriate detection of danger signals may also be compromised under these conditions; one of the microglial changes consists in the upregulation of the expression/density of $A_{2A}R$, as described in several degenerative disorders (1), usually paralleled by morphologic changes and by the release of inflammatory mediators (red circles), both anti- and proinflammatory molecules, that may impact on synaptic transmission, ultimately leading to synaptotoxicity (2); the ability of microglia to phagocytose subcellular components of damaged neurons or protein aggregates, typically present in some degenerative diseases, may also be impaired, further amplifying the cascade of events that lead to cell death/degeneration (3).

cause of vision loss in working age adults [139]. Since the number of people affected by diabetes is expected to increase significantly in the next 25 years, from the actual 382 million to beyond 592 million [139], the number of people affected by diabetic retinopathy is expected to greatly expand.

The similarities between AD pathology and retinal degenerative diseases have been described elsewhere [140, 141], and neuroinflammation is a common feature between brain and retinal degenerative diseases. It is, thus, plausible to speculate that therapeutic agents and strategies used for brain neurodegeneration could also be considered for retinal diseases with an underlying chronic inflammation process. Retinal microglia cells express $A_{2A}R$ [142], opening the possibility that the control of microglia-mediated neuroinflammation through $A_{2A}R$ modulation might also be an attractive approach to manage retinal diseases.

1.7. Glaucoma Has a Neuroinflammatory Component. Glaucoma is defined as a group of ocular disorders of multifactorial etiology characterized by progressive optic neuropathy [143] and gradual loss of retinal ganglion cells and optic nerve (retinal ganglion cell axons) damage. Elevated intraocular pressure (IOP) is one of the major risk factors for developing glaucoma or glaucomatous neuropathy [144]. The current therapeutic approach in glaucoma is focused on lowering IOP by pharmacological means, surgically, or with laser treatment. However, patients continue to lose vision despite successful IOP control, and it is becoming clear that the exclusive management of IOP is not sufficient, and neuroprotection of retinal ganglion cells has been proposed as a potential alternative therapy [145].

Several studies have reported that the progressive degeneration of optic nerve axons and retinal ganglion cells in glaucoma is accompanied by chronic alterations in structural and functional characteristics of glial cells in the optic nerve head and retina [146, 147], where an abnormal microglial reactivity and redistribution take place [148]. TNF, IL-6, and IL-18 levels are increased in the retina and optic nerve head in both glaucomatous patients and animal models of glaucoma [149–151] and recent studies demonstrate that microglial activation is an early event in experimental models of glaucoma, which coincides with the onset of RGC death, potentially contributing to disease onset and/or progression [152–154]. Also, the treatment with minocycline, a tetracycline derivative known to reduce microglial activation [155], was able to improve retinal ganglion cell axonal transport and integrity in a mouse model of glaucoma [156].

1.8. Diabetic Retinopathy: A Low-Grade Inflammatory Disease. Diabetic retinopathy is one of the most common complications of diabetes and the most frequent cause of new cases of blindness among adults aged 20–74 years. After 20 years of diabetes, nearly all patients with type 1 and more than 60% of patients with type 2 diabetes have some degree of retinopathy [157]. Diabetic retinopathy has been considered a microvascular disease, but growing evidence demonstrates that retinal neurodegeneration also occurs [158–160], and

diabetic retinopathy is now more accurately defined as a neurovascular disease.

Diabetic retinopathy exhibits characteristics of a chronic inflammatory process: increased levels of cytokines, such as IL-1β, IL-6, and TNF, have been found in the vitreous fluid of diabetic patients [161–163]; retinal TNF levels are also increased in diabetic patients, particularly in those with proliferative diabetic retinopathy [164–166]. The inflammatory profile of diabetic retinopathy has been confirmed in animal models of diabetes, where an increase was found in the levels of IL-1β [167–170] and TNF [170–172] in the retina. Therefore, the role of inflammation is unequivocal in diabetic retinopathy, from the leukocyte adhesion [173, 174] to the increase in inflammatory mediators, such as TNF, which exerts a crucial role in blood retinal barrier breakdown [175], as well as the death of retinal neurons [176]. As occurs in neurodegenerative brain diseases, microglial activation in the retina is also present in different stages of human diabetic retinopathy [177] and further reported in animals models of type 1 [170, 178–180] and type 2 [181] diabetes.

1.9. Is There a Role for $A_{2A}R$ in Retinal Degenerative Diseases? Retinal ischemia is a common cause of visual impairment and blindness (reviewed in [182]). Retinal degeneration after ischemia-reperfusion injury by transient elevation of IOP in rats exhibits an extensive damage at the level of the retinal ganglion cell layer [183], similarly to that reported in human glaucoma [184]. Therefore, IOP-induced retinal ischemia has been extensively used as an animal model of acute glaucoma [185], in which activation of microglia has also been observed [36]. The role of $A_{2A}R$ in retinal ischemia-reperfusion injury is still controversial. On one hand, the treatment with a selective $A_{2A}R$ antagonist protects retinal function and structure in a model of retinal ischemia [186, 187]. On the other hand, it was reported that administration of an $A_{2A}R$ agonist prevents retinal thinning induced by ischemia-reperfusion damage [188].

Traumatic optic neuropathy is an important cause of severe vision loss in 0.5 to 5% of patients with closed head trauma [189]. Trauma is known to cause immediate mechanical damage to the axons of retinal ganglion cells, leading to degeneration. The death of retinal ganglion cells after optic nerve damage seems to be related to the local production of ROS and inflammatory mediators from activated microglial cells [190]. Increased phagocytic and proliferative microglia have been reported after optic nerve injury [191–193]. In the optic nerve crush injury mouse model, an important experimental disease model for traumatic optic neuropathy, a selective $A_{2A}R$ agonist decreased microglial activation, retinal cell death, and release of ROS and proinflammatory cytokines [190]. Moreover, levels of TNF and Iba-1 (a marker of cells from the myeloid lineage, including microglia) are increased in $A_{2A}R$-knockout mice with optic nerve crush. In a different model of retinal degeneration, diabetic retinopathy, it was recently shown that $A_{2A}R$ mRNA transcripts and protein levels increase in the retina of type 1 diabetes models and also in retinal cell cultures exposed to elevated glucose concentration, used to mimic hyperglycemic conditions [194, 195]. $A_{2A}R$-knockout diabetic mice exhibit increased cell death and TNF levels as compared with diabetic wild-type mice [179]. Accordingly, the administration of a selective $A_{2A}R$ agonist resulted in opposite effects upon cell death and TNF levels [179].

Experiments performed *in vitro* emphasize the controversial role played by $A_{2A}R$ in the control of retinal neuroinflammation. While some authors reported that the activation of $A_{2A}R$ attenuates LPS-induced release of TNF in retinal microglia [190], others found that $A_{2A}R$ blockade prevents LPS-induced increase in NO [196]. Moreover, $A_{2A}R$ blockade inhibits the LPS-induced increase in TNF expression and phagocytosis. In a more complex system, the retinal organotypic culture, $A_{2A}R$ blockade inhibits the expression of inducible NO synthase [196].

In summary, it remains to be clarified whether $A_{2A}R$ activation or blockade is the best approach to pharmacologically control neuroinflammation in the retina. This dual neuroprotective ability of $A_{2A}R$ modulation seems to be related with the specific inflammatory profile of different pathologies or pathologic conditions, as well as with the temporal window of neuroinflammation where the exposure to $A_{2A}R$ agonists or antagonists occurs. Although the controversy exists, most studies in brain pathology point towards a neuroprotective effect of $A_{2A}R$ blockade, in line with the ability of selective and nonselective $A_{2A}R$ antagonists to decrease most microglial functions.

2. Concluding Remarks

Brain degenerative diseases, such as AD and PD, are associated with microglial activation and chronic neuroinflammation. In both pathologies, the blockade of $A_{2A}R$ emerges as a candidate mechanism of neuroprotection, through the control of microglial reactivity. Glaucoma and diabetic retinopathy are retinal degenerative diseases, in which neuroinflammation also plays a crucial role. In the retina, microglial cells are also equipped with $A_{2A}R$. Therefore, it is plausible to assume that $A_{2A}R$ modulation may also have a potential protective effect upon inflammation underlying degenerative processes of the retina (Figure 4). It remains to be clarified whether $A_{2A}R$ modulation has a net positive effect in the control of clinical features and progression of retinal degenerative diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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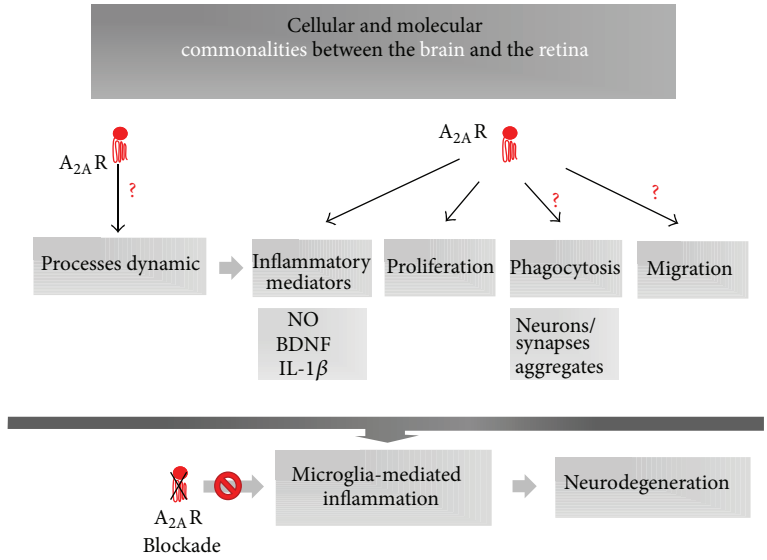

Figure 4: Cellular and molecular commonalities between the brain and the retina. Scheme identifying main microglial functions under the control of $A_{2A}R$: release of inflammatory mediators and cellular proliferation. It remains to clarify if process extension/retraction (which supports the homeostatic surveying role of microglia), phagocytosis, and cellular migration are directly regulated by $A_{2A}R$ modulation (question marks). $A_{2A}R$ modulation is proposed as a promising pharmacological tool in the control of the chronic inflammatory process underlying degenerative conditions of the retina, based on similarities with microglia-mediated inflammation in brain disorders.

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References

[1] K. Saijo and C. K. Glass, "Microglial cell origin and phenotypes in health and disease," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 775–787, 2011.

[2] D. Davalos, J. Grutzendler, G. Yang et al., "ATP mediates rapid microglial response to local brain injury *in vivo*," *Nature Neuroscience*, vol. 8, no. 6, pp. 752–758, 2005.

[3] A. Nimmerjahn, F. Kirchhoff, and F. Helmchen, "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo," *Science*, vol. 308, no. 5726, pp. 1314–1318, 2005.

[4] Y. Li, X. Du, C. Liu, Z. Wen, and J. Du, "Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo," *Developmental Cell*, vol. 23, no. 6, pp. 1189–1202, 2012.

[5] H. Wake, A. J. Moorhouse, S. Jinno, S. Kohsaka, and J. Nabekura, "Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals," *The Journal of Neuroscience*, vol. 29, no. 13, pp. 3974–3980, 2009.

[6] S. M. Lu, M. E. Tremblay, I. L. King et al., "HIV-1 Tat-induced microgliosis and synaptic damage via interactions between peripheral and central myeloid cells," *PLoS ONE*, vol. 6, no. 9, Article ID e23915, 2011.

[7] A. D. Kraft, L. S. Kaltenbach, D. C. Lo, and G. J. Harry, "Activated microglia proliferate at neurites of mutant huntingtin-expressing neurons," *Neurobiology of Aging*, vol. 33, no. 3, pp. 621.e17–621.e33, 2012.

[8] D. Chugh, P. Nilsson, S. A. Afjei, A. Bakochi, and C. T. Ekdahl, "Brain inflammation induces post-synaptic changes during early synapse formation in adult-born hippocampal neurons," *Experimental Neurology*, vol. 250, pp. 176–188, 2013.

[9] M. Tremblay, R. L. Lowery, and A. K. Majewska, "Microglial interactions with synapses are modulated by visual experience," *PLoS Biology*, vol. 8, no. 11, Article ID e1000527, 2010.

[10] R. C. Paolicelli, G. Bolasco, F. Pagani et al., "Synaptic pruning by microglia is necessary for normal brain development," *Science*, vol. 333, no. 6048, pp. 1456–1458, 2011.

[11] B. Linnartz, J. Kopatz, A. J. Tenner, and H. Neumann, "Sialic acid on the neuronal glycocalyx prevents complement c1 binding and complement receptor-3-mediated removal by microglia," *The Journal of Neuroscience*, vol. 32, no. 3, pp. 946–952, 2012.

[12] D. P. Schafer, E. K. Lehrman, A. G. Kautzman et al., "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner," *Neuron*, vol. 74, no. 4, pp. 691–705, 2012.

[13] Y. Zhan, R. C. Paolicelli, F. Sforazzini et al., "Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior," *Nature Neuroscience*, vol. 17, no. 3, pp. 400–406, 2014.

[14] A. Sierra, J. M. Encinas, J. J. P. Deudero et al., "Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis," *Cell Stem Cell*, vol. 7, no. 4, pp. 483–495, 2010.

[15] J. L. Marín-Teva, I. Dusart, C. Colin, A. Gervais, N. Van Rooijen, and M. Mallat, "Microglia promote the death of developing Purkinje cell," *Neuron*, vol. 41, no. 4, pp. 535–547, 2004.

[16] S. Wakselman, C. Béchade, A. Roumier, D. Bernard, A. Triller, and A. Bessis, "Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor," *The Journal of Neuroscience*, vol. 28, no. 32, pp. 8138–8143, 2008.

[17] C. M. Tyler and L. M. Boulanger, "Complement-mediated microglial clearance of developing retinal ganglion cell axons," *Neuron*, vol. 74, no. 4, pp. 597–599, 2012.

[18] N. Swinnen, S. Smolders, A. Avila et al., "Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo," *Glia*, vol. 61, no. 2, pp. 150–163, 2013.

[19] C. Rigato, R. Buckinx, H. Le-Corronc, J. M. Rigo, and P. Legendre, "Pattern of invasion of the embryonic mouse spinal cord by microglial cells at the time of the onset of functional neuronal networks," *GLIA*, vol. 59, no. 4, pp. 675–695, 2011.

[20] C. N. Parkhurst, G. Yang, and I. Ninan, "Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor," *Cell*, vol. 155, no. 7, pp. 1596–1609, 2013.

[21] M. Ueno, Y. Fujita, T. Tanaka et al., "Layer v cortical neurons require microglial support for survival during postnatal development," *Nature Neuroscience*, vol. 16, no. 5, pp. 543–551, 2013.

[22] S. H. Lim, E. Park, B. You et al., "Neuronal synapse formation induced by microglia and interleukin 10," *PLoS One*, vol. 8, no. 11, Article ID e81218, 2013.

[23] G. Cristovao, M. J. Pinto, R. A. Cunha, R. D. Almeida, and C. A. Gomes, "Activation of microglia bolsters synapse formation," *Frontiers in Cellular Neuroscience*, vol. 8, article 153, 2014.

[24] D. P. Schafer, E. K. Lehrman, and B. Stevens, "The "quad-partite" synapse: microglia-synapse interactions in the developing and mature CNS," *Glia*, vol. 61, no. 1, pp. 24–36, 2013.

[25] H. Kettenmann, F. Kirchhoff, and A. Verkhratsky, "Microglia: new roles for the synaptic stripper," *Neuron*, vol. 77, no. 1, pp. 10–18, 2013.

[26] M. E. Tremblay, B. Stevens, A. Sierra, H. Wake, A. Bessis, and A. Nimmerjahn, "The role of microglia in the healthy brain," *Journal of Neuroscience*, vol. 31, no. 45, pp. 16064–16069, 2011.

[27] S. Moriguchi, Y. Mizoguchi, Y. Tomimatsu et al., "Potentiation of NMDA receptor-mediated synaptic responses by microglia," *Molecular Brain Research*, vol. 119, no. 2, pp. 160–169, 2003.

[28] Y. Hayashi, H. Ishibashi, K. Hashimoto, and H. Nakanishi, "Potentiation of the NMDA receptor-mediated responses through the activation of the glycine site by microglia secreting soluble factors," *GLIA*, vol. 53, no. 6, pp. 660–668, 2006.

[29] J. T. Rogers, J. M. Morganti, A. D. Bachstetter et al., "CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity," *The Journal of Neuroscience*, vol. 31, no. 45, pp. 16241–16250, 2011.

[30] K. Ji, J. Miyauchi, and S. E. Tsirka, "Microglia: an active player in the regulation of synaptic activity," *Neural Plasticity*, vol. 2013, Article ID 627325, 9 pages, 2013.

[31] K. W. Ashwell, H. Holländer, W. Streit, and J. Stone, "The appearance and distribution of microglia in the developing retina of the rat," *Visual Neuroscience*, vol. 2, no. 5, pp. 437–448, 1989.

[32] J. Schnitzer, "Enzyme-histochemical demonstration of microglial cells in the adult and postnatal rabbit retina," *Journal of Comparative Neurology*, vol. 282, no. 2, pp. 249–263, 1989.

[33] M. F. Humphrey and S. R. Moore, "Microglial responses to focal lesions of the rabbit retina: correlation with neural and macroglial reactions," *Glia*, vol. 16, no. 4, pp. 325–341, 1996.

[34] C. Zhang, J. K. Shen, T. T. Lam et al., "Activation of microglia and chemokines in light-induced retinal degeneration," *Molecular Vision*, vol. 11, pp. 887–895, 2005.

[35] T. Harada, C. Harada, S. Kohsaka et al., "Microglia-Müller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration," *Journal of Neuroscience*, vol. 22, no. 21, pp. 9228–9236, 2002.

[36] C. Zhang, T. T. Lam, and M. O. Tso, "Heterogeneous populations of microglia/macrophages in the retina and their activation after retinal ischemia and reperfusion injury," *Experimental Eye Research*, vol. 81, no. 6, pp. 700–709, 2005.

[37] F. Vrabec, "Microglia in the monkey and rabbit retina," *Journal of Neuropathology and Experimental Neurology*, vol. 29, no. 2, pp. 217–224, 1970.

[38] B. B. Boycott and J. M. Hopkins, "Microglia in the retina of monkey and other mammals: its distinction from other types of glia and horizontal cells," *Neuroscience*, vol. 6, no. 4, pp. 679–688, 1981.

[39] P. L. Penfold, M. C. Madigan, and J. M. Provis, "Antibodies to human leucocyte antigens indicate subpopulations of microglia in human retina," *Visual neuroscience*, vol. 7, no. 4, pp. 383–388, 1991.

[40] P. Yang, P. K. Das, and A. Kijistra, "Localization and characterization of immunocompetent cells in the human retina," *Ocular Immunology and Inflammation*, vol. 8, no. 3, pp. 149–157, 2000.

[41] J. M. Provis, C. M. Diaz, and P. L. Penfold, "Microglia in human retina: a heterogeneous population with distinct ontogenies," *Perspectives on Developmental Neurobiology*, vol. 3, no. 3, pp. 213–222, 1996.

[42] D. A. Hume, V. H. Perry, and S. Gordon, "Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina: phagocytosis of dying neurons and differentiation in microglial cells to form a regular array in the plexiform layers," *Journal of Cell Biology*, vol. 97, no. 1, pp. 253–257, 1983.

[43] T. Huang, J. Cui, L. Li, P. F. Hitchcock, and Y. Li, "The role of microglia in the neurogenesis of zebrafish retina," *Biochemical and Biophysical Research Communications*, vol. 421, no. 2, pp. 214–220, 2012.

[44] D. Checchin, F. Sennlaub, E. Levavasseur, M. Leduc, and S. Chemtob, "Potential role of microglia in retinal blood vessel formation," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 8, pp. 3595–3602, 2006.

[45] A. M. Santos, R. Calvente, M. Tassi et al., "Embryonic and postnatal development of microglial cells in the mouse retina," *Journal of Comparative Neurology*, vol. 506, no. 2, pp. 224–239, 2008.

[46] C. M. Diaz-Araya, J. M. Provis, P. L. Penfold, and F. A. Billson, "Development of microglial topography in human retina," *Journal of Comparative Neurology*, vol. 363, no. 1, pp. 53–68, 1995.

[47] A. Ohta and M. Sitkovsky, "Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage," *Nature*, vol. 414, no. 6866, pp. 916–920, 2001.

[48] B. B. Fredholm, A. P. Ijzerman, K. A. Jacobson, K.-. Klotz, and J. Linden, "International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors," *Pharmacological Reviews*, vol. 53, no. 4, pp. 527–552, 2001.

[49] L. Luongo, F. Guida, R. Imperatore et al., "The A1 adenosine receptor as a new player in microglia physiology," *Glia*, vol. 62, no. 1, pp. 122–132, 2014.

[50] M. L. Haselkorn, D. K. Shellington, E. K. Jackson et al., "Adenosine A1 receptor activation as a brake on the microglial response after experimental traumatic brain injury in mice," *Journal of Neurotrauma*, vol. 27, no. 5, pp. 901–910, 2010.

[51] D. Choi, S. Pennathur, C. Perier et al., "Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice," *Journal of Neuroscience*, vol. 25, no. 28, pp. 6594–6600, 2005.

[52] J. Y. Lee, B. S. Jhun, Y. T. Oh et al., "Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF-α production through inhibition of PI 3-kinase/Akt and NF-κB activation in murine BV2 microglial cells," *Neuroscience Letters*, vol. 396, no. 1, pp. 1–6, 2006.

[53] R. A. Cunha, "Neuroprotection by adenosine in the brain: from A1 receptor activation to A2A receptor blockade," *Purinergic Signalling*, vol. 1, no. 2, pp. 111–134, 2005.

[54] C. V. Gomes, M. P. Kaster, A. R. Tomé, P. M. Agostinho, and R. A. Cunha, "Adenosine receptors and brain diseases: neuroprotection and neurodegeneration," *Biochimica et Biophysica Acta*, vol. 1808, no. 5, pp. 1380–1399, 2011.

[55] J. Chen, P. K. Sonsalla, F. Pedata et al., "Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation," *Progress in Neurobiology*, vol. 83, no. 5, pp. 310–331, 2007.

[56] C. Gomes, R. Ferreira, J. George et al., "Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *Journal of Neuroinflammation*, vol. 10, article 16, 2013.

[57] J. Saura, E. Angulo, A. Ejarque et al., "Adenosine A2A receptor stimulation potentiates nitric oxide release by activated microglia," *Journal of Neurochemistry*, vol. 95, no. 4, pp. 919–929, 2005.

[58] A. G. Orr, A. L. Orr, X. Li, R. E. Gross, and S. F. Traynelis, "Adenosine A2A receptor mediates microglial process retraction," *Nature Neuroscience*, vol. 12, no. 7, pp. 872–878, 2009.

[59] S. S. Dai, Y. G. Zhou, W. Li et al., "Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury," *The Journal of Neuroscience*, vol. 30, no. 16, pp. 5802–5810, 2010.

[60] J. L. Albasanz, S. Perez, M. Barrachina, I. Ferrer, and M. Martín, "Up-regulation of adenosine receptors in the frontal cortex in Alzheimer's disease," *Brain Pathology*, vol. 18, no. 2, pp. 211–219, 2008.

[61] D. M. Holtzman, J. C. Morris, and A. M. Goate, "Alzheimer's disease: the challenge of the second century," *Science Translational Medicine*, vol. 3, no. 77, Article ID 77sr1, 2011.

[62] L. Buée, T. Bussière, V. Buée-Scherrer, A. Delacourte, and P. R. Hof, "Tau protein isoforms, phosphorylation and role in neurodegenerative disorders," *Brain Research Reviews*, vol. 33, no. 1, pp. 95–130, 2000.

[63] T. F. Gendron and L. Petrucelli, "The role of tau in neurodegeneration," *Molecular Neurodegeneration*, vol. 4, no. 1, article 13, 2009.

[64] D. J. Selkoe and D. Schenk, "Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics," *Annual Review of Pharmacology and Toxicology*, vol. 43, pp. 545–584, 2003.

[65] P. L. McGeer and E. G. McGeer, "Glial reactions in Parkinson's disease," *Movement Disorders*, vol. 23, no. 4, pp. 474–483, 2008.

[66] E. C. Hirsch and S. Hunot, "Neuroinflammation in Parkinson's disease: a target for neuroprotection?" *The Lancet Neurology*, vol. 8, no. 4, pp. 382–397, 2009.

[67] T. Nagatsu and M. Sawada, "Inflammatory process in Parkinson's disease: role for cytokines," *Current Pharmaceutical Design*, vol. 11, no. 8, pp. 999–1016, 2005.

[68] E. Gómez-Tortosa, K. Newell, M. C. Irizarry, M. Albert, J. H. Growdon, and B. T. Hyman, "Clinical and quantitative pathologic correlates of dementia with Lewy bodies," *Neurology*, vol. 53, no. 6, pp. 1284–1291, 1999.

[69] E. Masliah, E. Rockenstein, I. Veinbergs et al., "Dopaminergic loss and inclusion body formation in α-synuclein mice: implications for neurodegenerative disorders," *Science*, vol. 287, no. 5456, pp. 1265–1269, 2000.

[70] B. A. Yankner and T. Lu, "Amyloid β-protein toxicity and the pathogenesis of Alzheimer disease," *The Journal of Biological Chemistry*, vol. 284, no. 8, pp. 4755–4759, 2009.

[71] P. L. McGeer, S. Itagaki, H. Tago, and E. G. McGeer, "Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR," *Neuroscience Letters*, vol. 79, no. 1-2, pp. 195–200, 1987.

[72] P. L. McGeer, S. Itagaki, B. E. Boyes, and E. G. McGeer, "Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains," *Neurology*, vol. 38, no. 8, pp. 1285–1291, 1988.

[73] S. V. More, H. Kumar, I. S. Kim, S. Y. Song, and D. K. Choi, "Cellular and molecular mediators of neuroinflammation in the pathogenesis of Parkinson's disease," *Mediators of Inflammation*, vol. 2013, Article ID 952375, 12 pages, 2013.

[74] X. Su, H. J. Federoff, and K. A. Maguire-Zeiss, "Mutant α-synuclein overexpression mediates early proinflammatory activity," *Neurotoxicity Research*, vol. 16, no. 3, pp. 238–254, 2009.

[75] Y. Ouchi, E. Yoshikawa, Y. Sekine et al., "Microglial activation and dopamine terminal loss in early Parkinson's disease," *Annals of Neurology*, vol. 57, no. 2, pp. 168–175, 2005.

[76] A. Gerhard, N. Pavese, G. Hotton et al., "In vivo imaging of microglial activation with [^{11}C](*R*)-PK11195 PET in idiopathic Parkinson's disease," *Neurobiology of Disease*, vol. 21, no. 2, pp. 404–412, 2006.

[77] A. Schuitemaker, M. A. Kropholler, R. Boellaard et al., "Microglial activation in Alzheimer's disease: an (*R*)-[^{11}C]PK11195 positron emission tomography study," *Neurobiology of Aging*, vol. 34, no. 1, pp. 128–136, 2013.

[78] A. Okello, P. Edison, H. A. Archer et al., "Microglial activation and amyloid deposition in mild cognitive impairment: a PET study," *Neurology*, vol. 72, no. 1, pp. 56–62, 2009.

[79] P. Edison, H. A. Archer, A. Gerhard et al., "Microglia, amyloid, and cognition in Alzheimer's disease: an [11C](R)PK11195-PET and [11C]PIB-PET study," *Neurobiology of Disease*, vol. 32, no. 3, pp. 412–419, 2008.

[80] W. Zhang, T. Wang, Z. Pei et al., "Aggregated α-synuclein activates microglia: a process leading to disease progression in Parkinson's disease," *The FASEB Journal*, vol. 19, no. 6, pp. 533–542, 2005.

[81] C. K. Combs, D. E. Johnson, J. C. Karlo, S. B. Cannady, and G. E. Landreth, "Inflammatory mechanisms in Alzheimer's disease: inhibition of β-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARγ agonists," *Journal of Neuroscience*, vol. 20, no. 2, pp. 558–567, 2000.

[82] L. Qin, Y. Liu, C. Cooper, B. Liu, B. Wilson, and J. Hong, "Microglia enhance β-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species," *Journal of Neurochemistry*, vol. 83, no. 4, pp. 973–983, 2002.

[83] M. Ii, M. Sunamoto, K. Ohnishi, and Y. Ichimori, "β-Amyloid protein-dependent nitric oxide production from microglial cells and neurotoxicity," *Brain Research*, vol. 720, no. 1-2, pp. 93–100, 1996.

[84] L. Meda, M. A. Cassatella, G. I. Szendrei et al., "Activation of microglial cells by β-amyloid protein and interferon-γ," *Nature*, vol. 374, no. 6523, pp. 647–650, 1995.

[85] M. F. McCarty, "Down-regulation of microglial activation may represent a practical strategy for combating neurodegenerative disorders," *Medical Hypotheses*, vol. 67, no. 2, pp. 251–269, 2006.

[86] D. Wu, P. Teismann, K. Tieu et al., "NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6145–6150, 2003.

[87] S. Hunot, F. Boissière, B. Faucheux et al., "Nitric oxide synthase and neuronal vulnerability in Parkinson's disease," *Neuroscience*, vol. 72, no. 2, pp. 355–363, 1996.

[88] C. Knott, G. Stern, and G. P. Wilkin, "Inflammatory regulators in Parkinson's disease: iNOS, lipocortin-1, and cyclooxygenases-1 and -2," *Molecular and Cellular Neuroscience*, vol. 16, no. 6, pp. 724–739, 2000.

[89] R. Lee Mosley, E. J. Benner, I. Kadiu et al., "Neuroinflammation, oxidative stress, and the pathogenesis of Parkinson's disease," *Clinical Neuroscience Research*, vol. 6, no. 5, pp. 261–281, 2006.

[90] A. L. de Lella Ezcurra, M. Chertoff, C. Ferrari, M. Graciarena, and F. Pitossi, "Chronic expression of low levels of tumor necrosis factor-α in the substantia nigra elicits progressive neurodegeneration, delayed motor symptoms and microglia/macrophage activation," *Neurobiology of Disease*, vol. 37, no. 3, pp. 630–640, 2010.

[91] T. Nagatsu, M. Mogi, H. Ichinose, and A. Togari, "Changes in cytokines and neurotrophins in Parkinson's disease," *Journal of Neural Transmission, Supplement*, no. 60, pp. 277–290, 2000.

[92] P. Teismann, K. Tieu, D. K. Choi et al., "Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 9, pp. 5473–5478, 2003.

[93] S. Hunot, M. Vila, P. Teismann et al., "JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 2, pp. 665–670, 2004.

[94] R. Sánchez-Pernaute, A. Ferree, O. Cooper, M. Yu, A. Brownell, and O. Isacson, "Selective COX-2 inhibition prevents progressive dopamine neuron degeneration in a rat model of Parkinson's disease," *Journal of Neuroinflammation*, vol. 1, article 6, 2004.

[95] G. T. Liberatore, V. Jackson-Lewis, S. Vukosavic et al., "Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease," *Nature Medicine*, vol. 5, no. 12, pp. 1403–1409, 1999.

[96] K. Imamura, N. Hishikawa, M. Sawada, T. Nagatsu, M. Yoshida, and Y. Hashizume, "Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains," *Acta Neuropathologica*, vol. 106, no. 6, pp. 518–526, 2003.

[97] E. Croisier, L. B. Moran, D. T. Dexter, R. K. B. Pearce, and M. B. Graeber, "Microglial inflammation in the parkinsonian substantia nigra: relationship to α-synuclein deposition," *Journal of Neuroinflammation*, vol. 2, article 14, 2005.

[98] D. C. Wu, V. Jackson-Lewis, M. Vila et al., "Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease," *Journal of Neuroscience*, vol. 22, no. 5, pp. 1763–1771, 2002.

[99] D. M. Paresce, H. Chung, and F. R. Maxfield, "Slow degradation of aggregates of the Alzheimer's disease amyloid β- protein by microglial cells," *The Journal of Biological Chemistry*, vol. 272, no. 46, pp. 29390–29397, 1997.

[100] D. G. Walker and L.-F. Lue, "Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases," *Journal of Neuroscience Research*, vol. 81, no. 3, pp. 412–425, 2005.

[101] A. Majumdar, D. Cruz, N. Asamoah et al., "Activation of microglia acidifies lysosomes and leads to degradation of Alzheimer amyloid fibrils," *Molecular Biology of the Cell*, vol. 18, no. 4, pp. 1490–1496, 2007.

[102] G. Krabbe, A. Halle, V. Matyash et al., "Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology," *PLoS ONE*, vol. 8, no. 4, Article ID e60921, 2013.

[103] J. El Khoury, M. Toft, S. E. Hickman et al., "*Ccr2* deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease," *Nature Medicine*, vol. 13, no. 4, pp. 432–438, 2007.

[104] P. M. Canas, L. O. Porciúncula, G. M. A. Cunha et al., "Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogen-activated protein kinase pathway," *Journal of Neuroscience*, vol. 29, no. 47, pp. 14741–14751, 2009.

[105] N. Rebola, A. P. Simões, P. M. Canas et al., "Adenosine A2A receptors control neuroinflammation and consequent hippocampal neuronal dysfunction," *Journal of Neurochemistry*, vol. 117, no. 1, pp. 100–111, 2011.

[106] L. Minghetti, A. Greco, R. L. Potenza et al., "Effects of the adenosine A2A receptor antagonist SCH 58621 on cyclooxygenase-2 expression, glial activation, and brain-derived neurotrophic factor availability in a rat model of striatal neurodegeneration," *Journal of Neuropathology and Experimental Neurology*, vol. 66, no. 5, pp. 363–371, 2007.

[107] F. Di Virgilio, S. Ceruti, P. Bramanti, and M. P. Abbracchio, "Purinergic signalling in inflammation of the central nervous system," *Trends in Neurosciences*, vol. 32, no. 2, pp. 79–87, 2009.

[108] L. Yu, H. Shen, J. E. Coelho et al., "Adenosine A2A receptor antagonists exert motor and neuroprotective effects by distinct cellular mechanisms," *Annals of Neurology*, vol. 63, no. 3, pp. 338–346, 2008.

[109] A. P. Simões, J. A. Duarte, F. Agasse et al., "Blockade of adenosine A2A receptors prevents interleukin-1β-induced exacerbation of neuronal toxicity through a p38 mitogen-activated protein kinase pathway," *Journal of Neuroinflammation*, vol. 9, article 204, 2012.

[110] T. W. Stone and W. M. H. Behan, "Interleukin-1β but not tumor necrosis factor-α potentiates neuronal damage by quinolinic acid: protection by an adenosine A2A receptor antagonist," *Journal of Neuroscience Research*, vol. 85, no. 5, pp. 1077–1085, 2007.

[111] B. B. Fredholm, J. F. Chen, R. A. Cunha, P. Svenningsson, and J. M. Vaugeois, "Adenosine and brain function," *International Review of Neurobiology*, vol. 63, pp. 191–270, 2005.

[112] G. W. Arendash, T. Mori, C. Cao et al., "Caffeine reverses cognitive impairment and decreases brain amyloid-β levels in aged Alzheimer's disease mice," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 661–680, 2009.

[113] C. Cao, J. R. Cirrito, X. Lin et al., "Caffeine suppresses amyloid-β levels in plasma and brain of Alzheimerls disease transgenic mice," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 681–697, 2009.

[114] Y. F. Chu, W. H. Chang, R. M. Black et al., "Crude caffeine reduces memory impairment and amyloid β(1–42) levels in an Alzheimer's mouse model," *Food Chemistry*, vol. 135, no. 3, pp. 2095–2102, 2012.

[115] O. P. Dall'Igna, P. Fett, M. W. Gomes, D. O. Souza, R. A. Cunha, and D. R. Lara, "Caffeine and adenosine A_{2a} receptor antagonists prevent β-amyloid (25–35)-induced cognitive deficits in mice," *Experimental Neurology*, vol. 203, no. 1, pp. 241–245, 2007.

[116] R. A. Cunha and P. M. Agostinho, "Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline," *Journal of Alzheimer's Disease*, vol. 20, supplement 1, pp. S95–S116, 2010.

[117] O. P. Dall'Igna, L. O. Porciuncula, D. O. Souza, R. A. Cunha, and D. R. Lara, "Neuroprotection by caffeine and adenosine A2A receptor blockade of beta-amyloid neurotoxicity," *British Journal of Pharmacology*, vol. 138, no. 7, pp. 1207–1209, 2003.

[118] H. M. Brothers, Y. Marchalant, and G. L. Wenk, "Caffeine attenuates lipopolysaccharide-induced neuroinflammation," *Neuroscience Letters*, vol. 480, no. 2, pp. 97–100, 2010.

[119] M. A. Lynch, "Long-term potentiation and memory," *Physiological Reviews*, vol. 84, no. 1, pp. 87–136, 2004.

[120] E. Angulo, V. Casadó, J. Mallol et al., "A1 adenosine receptors accumulate in neurodegenerative structures in Alzheimer disease and mediate both amyloid precursor protein processing and tau phosphorylation and translocation," *Brain Pathology*, vol. 13, no. 4, pp. 440–451, 2003.

[121] W. Chen, H. Wang, H. Wei, and S. Gu, "Istradefylline, an adenosine A_{2A} receptor antagonist, for patients with Parkinson's disease: a meta-analysis," *Journal of the Neurological Sciences*, vol. 324, no. 1, pp. 21–28, 2013.

[122] M. Morelli, A. R. Carta, and P. Jenner, "Adenosine A_{2A} receptors and Parkinson's disease," *Handbook of Experimental Pharmacology*, vol. 193, pp. 589–615, 2009.

[123] M. Pierri, E. Vaudano, T. Sager, and U. Englund, "KW-6002 protects from MPTP induced dopaminergic toxicity in the mouse," *Neuropharmacology*, vol. 48, no. 4, pp. 517–524, 2005.

[124] S. Yadav, S. P. Gupta, G. Srivastava, P. K. Srivastava, and M. P. Singh, "Role of secondary mediators in caffeine-mediated neuroprotection in maneb- and paraquat-induced Parkinson's disease phenotype in the mouse," *Neurochemical Research*, vol. 37, no. 4, pp. 875–884, 2012.

[125] S. Stayte and B. Vissel, "Advances in non-dopaminergic treatments for Parkinson's disease," *Frontiers in Neuroscience*, vol. 8, p. 113, 2014.

[126] J. F. Chen, K. Xu, J. P. Petzer et al., "Neuroprotection by caffeine and A_{2A} adenosine receptor inactivation in a model of Parkinson's disease.," *Journal of Neuroscience*, vol. 21, no. 10, p. RC143, 2001.

[127] M. A. Hernán, B. Takkouche, F. Caamaño-Isorna, and J. J. Gestal-Otero, "A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease," *Annals of Neurology*, vol. 52, no. 3, pp. 276–284, 2002.

[128] J. Costa, N. Lunet, C. Santos, J. Santos, and A. Vaz-Carneiro, "Caffeine exposure and the risk of Parkinson's disease: a systematic review and meta-analysis of observational studiess," *Journal of Alzheimer's Disease*, vol. 20, supplement 1, pp. S221–S238, 2010.

[129] R. Liu, X. Guo, Y. Park et al., "Caffeine intake, smoking, and risk of parkinson disease in men and women," *American Journal of Epidemiology*, vol. 175, no. 11, pp. 1200–1207, 2012.

[130] M. van der Mark, P. C. Nijssen, J. Vlaanderen et al., "A case-control study of the protective effect of alcohol, coffee, and cigarette consumption on Parkinson disease risk: time-since-cessation modifies the effect of tobacco smoking," *PLoS ONE*, vol. 9, no. 4, Article ID e95297, 2014.

[131] K. Sääksjärvi, P. Knekt, H. Rissanen, M. A. Laaksonen, A. Reunanen, and S. Männistö, "Prospective study of coffee consumption and risk of Parkinson's disease," *European Journal of Clinical Nutrition*, vol. 62, no. 7, pp. 908–915, 2008.

[132] G. W. Ross, R. D. Abbott, H. Petrovitch et al., "Association of coffee and caffeine intake with the risk of Parkinson disease," *Journal of the American Medical Association*, vol. 283, no. 20, pp. 2674–2679, 2000.

[133] R. A. Popat, S. K. Van Den Eeden, C. M. Tanner et al., "Coffee, *ADORA2A*, and *CYP1A2*: the caffeine connection in Parkinson's disease," *European Journal of Neurology*, vol. 18, no. 5, pp. 756–765, 2011.

[134] S. Gyoneva, L. Shapiro, C. Lazo et al., "Adenosine A_{2A} receptor antagonism reverses inflammation-induced impairment of microglial process extension in a model of Parkinson's disease," *Neurobiology of Disease*, vol. 67, pp. 191–202, 2014.

[135] *World Population Ageing 2013*, Department of Economic and Social Affairs, Population Division, New York, NY, USA, 2013.

[136] G. Dagnelie, "Age-related psychophysical changes and low vision," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 14, pp. ORSF88–ORSF93, 2013.

[137] S. Resnikoff, D. Pascolini, D. Etya'ale et al., "Global data on visual impairment in the year 2002," *Bulletin of the World Health Organization*, vol. 82, no. 11, pp. 844–851, 2004.

[138] W. Cheung, L. Guo, and M. F. Cordeiro, "Neuroprotection in glaucoma: drug-based approaches," *Optometry and Vision Science*, vol. 85, no. 6, pp. E406–E416, 2008.

[139] International Diabetes Federation, *IDF Diabetes Atlas*, International Diabetes Federation, Brussels, Belgium, 6th edition, 2013.

[140] S. J. McKinnon, "The cell and molecular biology of glaucoma: common neurodegenerative pathways and relevance to glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 5, pp. 2485–2487, 2012.

[141] J. M. Sivak, "The aging eye: common degenerative mechanisms between the Alzheimer's brain and retinal disease," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 1, pp. 871–880, 2013.

[142] G. I. Liou, J. A. Auchampach, C. J. Hillard et al., "Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A_{2A} adenosine receptor," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 12, pp. 5526–5531, 2008.

[143] R. J. Casson, G. Chidlow, J. P. M. Wood, J. G. Crowston, and I. Goldberg, "Definition of glaucoma: clinical and experimental concepts," *Clinical and Experimental Ophthalmology*, vol. 40, no. 4, pp. 341–349, 2012.

[144] T. Kersey, C. I. Clement, P. Bloom, and M. F. Cordeiro, "New trends in glaucoma risk, diagnosis & management," *Indian Journal of Medical Research*, vol. 137, no. 4, pp. 659–668, 2013.

[145] M. F. Cordeiro and L. A. Levin, "Clinical evidence for neuroprotection in glaucoma," *American Journal of Ophthalmology*, vol. 152, no. 5, pp. 715–716, 2011.

[146] G. Tezel, "The role of glia, mitochondria, and the immune system in glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 50, no. 3, pp. 1001–1012, 2009.

[147] A. Baltmr, J. Duggan, S. Nizari, T. E. Salt, and M. F. Cordeiro, "Neuroprotection in glaucoma: is there a future role?" *Experimental Eye Research*, vol. 91, no. 5, pp. 554–566, 2010.

[148] R. Naskar, M. Wissing, and S. Thanos, "Detection of early neuron degeneration and accompanying microglial responses in the retina of a rat model of glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 9, pp. 2962–2968, 2002.

[149] R. M. Sappington, M. Chan, and D. J. Calkins, "Interleukin-6 protects retinal ganglion cells from pressure-induced death," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 7, pp. 2932–2942, 2006.

[150] G. Tezel, "TNF-α signaling in glaucomatous neurodegeneration," *Progress in Brain Research*, vol. 173, pp. 409–421, 2008.

[151] X. Zhou, F. Li, L. Kong, H. Tomita, C. Li, and W. Cao, "Involvement of inflammation, degradation, and apoptosis in a mouse model of glaucoma," *The Journal of Biological Chemistry*, vol. 280, no. 35, pp. 31240–31248, 2005.

[152] A. Bosco, M. R. Steele, and M. L. Vetter, "Early microglia activation in a mouse model of chronic glaucoma," *Journal of Comparative Neurology*, vol. 519, no. 4, pp. 599–620, 2011.

[153] S. Taylor, C. J. Calder, J. Albon, J. T. Erichsen, M. E. Boulton, and J. E. Morgan, "Involvement of the CD200 receptor complex in microglia activation in experimental glaucoma," *Experimental Eye Research*, vol. 92, no. 5, pp. 338–343, 2011.

[154] R. de Hoz, B. I. Gallego, A. I. Ramirez et al., "Rod-like microglia are restricted to eyes with laser-induced ocular hypertension but absent from the microglial changes in the contralateral untreated eye," *PLoS ONE*, vol. 8, no. 12, Article ID e83733, 2013.

[155] T. M. Tikka and J. E. Koistinaho, "Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia," *Journal of Immunology*, vol. 166, no. 12, pp. 7527–7533, 2001.

[156] A. Bosco, D. M. Inman, M. R. Steele et al., "Reduced retina microglial activation and improved optic nerve integrity with minocycline treatment in the DBA/2J mouse model of glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 4, pp. 1437–1446, 2008.

[157] D. S. Fong, L. P. Aiello, F. L. Ferris III, and R. Klein, "Diabetic retinopathy," *Diabetes Care*, vol. 27, no. 10, pp. 2540–2553, 2004.

[158] A. R. Santiago, A. J. Cristóvão, P. F. Santos, C. M. Carvalho, and A. F. Ambrósio, "High glucose induces caspase-independent cell death in retinal neural cells," *Neurobiology of Disease*, vol. 25, no. 3, pp. 464–472, 2007.

[159] A. J. Barber, "A new view of diabetic retinopathy: a neurodegenerative disease of the eye," *Progress in NeuroPsychopharmacology and Biological Psychiatry*, vol. 27, no. 2, pp. 283–290, 2003.

[160] A. J. Barber, E. Lieth, S. A. Khin, D. A. Antonetti, A. G. Buchanan, and T. W. Gardner, "Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin," *Journal of Clinical Investigation*, vol. 102, no. 4, pp. 783–791, 1998.

[161] A. M. A. El Asrar, D. Maimone, P. H. Morse, S. Gregory, and A. T. Reder, "Cytokines in the vitreous of patients with proliferative diabetic retinopathy," *American Journal of Ophthalmology*, vol. 114, no. 6, pp. 731–736, 1992.

[162] T. Yuuki, T. Kanda, Y. Kimura et al., "Inflammatory cytokines in vitreous fluid and serum of patients with diabetic vitreoretinopathy," *Journal of Diabetes and Its Complications*, vol. 15, no. 5, pp. 257–259, 2001.

[163] J. I. Patel, G. M. Saleh, P. G. Hykin, Z. J. Gregor, and I. A. Cree, "Concentration of haemodynamic and inflammatory related cytokines in diabetic retinopathy," *Eye*, vol. 22, no. 2, pp. 223–228, 2008.

[164] M. T. Schram, N. Chaturvedi, C. G. Schalkwijk, J. H. Fuller, and C. D. A. Stehouwer, "Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes—the EURODIAB Prospective Complications Study," *Diabetologia*, vol. 48, no. 2, pp. 370–378, 2005.

[165] N. Demircan, B. G. Safran, M. Soylu, A. A. Ozcan, and S. Sizmaz, "Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy," *Eye*, vol. 20, no. 12, pp. 1366–1369, 2006.

[166] C. Gustavsson, E. Agardh, B. Bengtsson, and C. D. Agardh, "TNF-α is an independent serum marker for proliferative retinopathy in type 1 diabetic patients," *Journal of Diabetes and Its Complications*, vol. 22, no. 5, pp. 309–316, 2008.

[167] A. Carmo, J. G. Cunha-Vaz, A. P. Carvalho, and M. C. Lopes, "L-arginine transport in retinas from streptozotocin diabetic rats: Correlation with the level of IL-1β and NO synthase activity," *Vision Research*, vol. 39, no. 23, pp. 3817–3823, 1999.

[168] R. A. Kowluru and S. Odenbach, "Role of interleukin-1β in the pathogenesis of diabetic retinopathy," *British Journal of Ophthalmology*, vol. 88, no. 10, pp. 1343–1347, 2004.

[169] C. Gerhardinger, M. B. Costa, M. C. Coulombe, I. Toth, T. Hoehn, and P. Grosu, "Expression of acute-phase response proteins in retinal Müller cells in diabetes," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 1, pp. 349–357, 2005.

[170] J. K. Krady, A. Basu, C. M. Allen et al., "Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy," *Diabetes*, vol. 54, no. 5, pp. 1559–1565, 2005.

[171] A. M. Joussen, V. Poulaki, N. Mitsiades et al., "Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression," *The FASEB Journal*, vol. 16, no. 3, pp. 438–440, 2002.

[172] Y. Behl, P. Krothapalli, T. Desta, A. DiPiazza, S. Roy, and D. T. Graves, "Diabetes-enhanced tumor necrosis factor-α production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy," *The American Journal of Pathology*, vol. 172, no. 5, pp. 1411–1418, 2008.

[173] C. Gustavsson, E. Agardh, B. Bengtsson, and C. D. Agardh, "TNF-α is an independent serum marker for proliferative retinopathy in type 1 diabetic patients," *Journal of Diabetes and Its Complications*, vol. 44, no. 5, pp. 2184–2191, 2003.

[174] E. C. Leal, A. Manivannan, K. Hosoya et al., "Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 11, pp. 5257–5265, 2007.

[175] C. A. Aveleira, C. M. Lin, S. F. Abcouwer, A. F. Ambrósio, and D. A. Antonetti, "TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability," *Diabetes*, vol. 59, no. 11, pp. 2872–2882, 2010.

[176] G. N. Costa, J. Vindeirinho, C. Cavadas, A. F. Ambrósio, and P. F. Santos, "Contribution of TNF receptor 1 to retinal neural cell death induced by elevated glucose," *Molecular and Cellular Neuroscience*, vol. 50, no. 1, pp. 113–123, 2012.

[177] H. Zeng, W. R. Green, and M. O. M. Tso, "Microglial activation in human diabetic retinopathy," *Archives of Ophthalmology*, vol. 126, no. 2, pp. 227–232, 2008.

[178] A. J. Barber, D. A. Antonetti, T. S. Kern et al., "The Ins2Akita mouse as a model of early retinal complications in diabetes," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 6, pp. 2210–2218, 2005.

[179] A. S. Ibrahim, A. B. El-Remessy, S. Matragoon et al., "Retinal microglial activation and inflammation induced by amadori-glycated albumin in a rat model of diabetes," *Diabetes*, vol. 60, no. 4, pp. 1122–1133, 2011.

[180] X. X. Zeng, Y. K. Ng, and E. A. Ling, "Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats," *Visual Neuroscience*, vol. 17, no. 3, pp. 463–471, 2000.

[181] S. Omri, F. Behar-Cohen, Y. de Kozak et al., "Microglia/macrophages migrate through retinal epithelium barrier by a transcellular route in diabetic retinopathy: role of PKCzeta in the Goto Kakizaki rat model," *The American Journal of Pathology*, vol. 179, no. 2, pp. 942–953, 2011.

[182] N. N. Osborne, R. J. Casson, J. P. M. Wood, G. Chidlow, M. Graham, and J. Melena, "Retinal ischemia: mechanisms of damage and potential therapeutic strategies," *Progress in Retinal and Eye Research*, vol. 23, no. 1, pp. 91–147, 2004.

[183] T. T. Lam, A. S. Abler, and M. O. M. Tso, "Apoptosis and caspases after ischemia-reperfusion injury in rat retina," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 5, pp. 967–975, 1999.

[184] H. A. Quigley, "Neuronal death in glaucoma," *Progress in Retinal and Eye Research*, vol. 18, no. 1, pp. 39–57, 1999.

[185] N. N. Osborne, J. Melena, G. Chidlow, and J. P. M. Wood, "A hypothesis to explain ganglion cell death caused by vascular insults at the optic nerve head: possible implication for the treatment of glaucoma," *The British Journal of Ophthalmology*, vol. 85, no. 10, pp. 1252–1259, 2001.

[186] G. J. Ghiardi, J. M. Gidday, and S. Roth, "The purine nucleoside adenosine in retinal ischemia-reperfusion injury," *Vision Research*, vol. 39, no. 15, pp. 2519–2535, 1999.

[187] B. Li, P. S. Rosenbaum, N. M. Jennings, K. M. Maxwell, and S. Roth, "Differing roles of adenosine receptor subtypes in retinal ischemia- reperfusion injury in the rat," *Experimental Eye Research*, vol. 68, no. 1, pp. 9–17, 1999.

[188] T. Konno, A. Sato, T. Uchibori, A. Nagai, K. Kogi, and N. Nakahata, "Adenosine A_{2A} receptor mediated protective effect of 2-(6-cyano-1-hexyn-1-yl)adenosine on retinal ischaemia/reperfusion damage in rats," *British Journal of Ophthalmology*, vol. 90, no. 7, pp. 900–905, 2006.

[189] K. D. Steinsapir and R. A. Goldberg, "Traumatic optic neuropathy," *Survey of Ophthalmology*, vol. 38, no. 6, pp. 487–518, 1994.

[190] S. Ahmad, N. Fatteh, N. M. El-Sherbiny et al., "Potential role of A2A adenosine receptor in traumatic optic neuropathy," *Journal of Neuroimmunology*, vol. 264, no. 1-2, pp. 54–64, 2013.

[191] E. Garcia-Valenzuela and S. C. Sharma, "Laminar restriction of retinal macrophagic response to optic nerve axotomy in the rat," *Journal of Neurobiology*, vol. 40, no. 1, pp. 55–66, 1999.

[192] C. Zhang and M. O. M. Tso, "Characterization of activated retinal microglia following optic axotomy," *Journal of Neuroscience Research*, vol. 73, no. 6, pp. 840–845, 2003.

[193] S. G. Wohl, C. W. Schmeer, O. W. Witte, and S. Isenmann, "Proliferative response of microglia and macrophages in the adult mouse eye after optic nerve lesion," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 5, pp. 2686–2696, 2010.

[194] J. Vindeirinho, G. N. Costa, M. B. Correia, C. Cavadas, and P. F. Santos, "Effect of diabetes/hyperglycemia on the rat retinal adenosinergic system," *PLoS ONE*, vol. 8, no. 6, Article ID e67499, 2013.

[195] N. M. Elsherbiny, S. Ahmad, M. Naime et al., "ABT-702, an adenosine kinase inhibitor, attenuates inflammation in diabetic retinopathy," *Life Sciences*, vol. 93, no. 2-3, pp. 78–88, 2013.

[196] M. Madeira, F. Elvas, J. Martins, A. F. Ambrósio, and A. R. Santiago, "denosine A2AR blockade prevents retinal microglial activation," in *Proceedings of the 22nd IUBMB 37th FEBS Congress*, Blackwell, Seville, Spain, 2012.

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Review Article

Purinergic Receptors in Ocular Inflammation

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Inflammation is a complex process that implies the interaction between cells and molecular mediators, which, when not properly "tuned," can lead to disease. When inflammation affects the eye, it can produce severe disorders affecting the superficial and internal parts of the visual organ. The nucleoside adenosine and nucleotides including adenine mononucleotides like ADP and ATP and dinucleotides such as P^1,P^4-diadenosine tetraphosphate (Ap_4A), and P^1,P^5-diadenosine pentaphosphate (Ap_5A) are present in different ocular locations and therefore they may contribute/modulate inflammatory processes. Adenosine receptors, in particular A_{2A} adenosine receptors, present anti-inflammatory action in acute and chronic retinal inflammation. Regarding the A_3 receptor, selective agonists like N^6-(3-iodobenzyl)-5′-N-methylcarboxamidoadenosine (CF101) have been used for the treatment of inflammatory ophthalmic diseases such as dry eye and uveoretinitis. Sideways, diverse stimuli (sensory stimulation, large intraocular pressure increases) can produce a release of ATP from ocular sensory innervation or after injury to ocular tissues. Then, ATP will activate purinergic P2 receptors present in sensory nerve endings, the iris, the ciliary body, or other tissues surrounding the anterior chamber of the eye to produce uveitis/endophthalmitis. In summary, adenosine and nucleotides can activate receptors in ocular structures susceptible to suffer from inflammatory processes. This involvement suggests the possible use of purinergic agonists and antagonists as therapeutic targets for ocular inflammation.

1. Introduction

Inflammation is the biological process triggered when a vascular tissue needs to be repaired from an injury or faces a microbial challenge. This process is normally self-limited; however, in the absence of a proper return to homeostasis it can turn into a damaging condition for tissues [1, 2]. It works throughout complex and specific interactions between cells and mediator molecules in the damaged tissue that require a fine and well-tuned molecular regulation. In the absence of a prompt resolution of the acute inflammatory response, affected tissue may progress to a chronic inflammatory status that leads to disease [3].

As many organs, human eye suffers important and sometimes devastating inflammatory diseases. In such cases, inflammation can be either cause or consequence of disorders affecting different structures in the anterior part of the eye, the intraocular compartment, or both. Examples of such conditions include lacrimal keratoconjunctivitis, severe allergic diseases, severe cicatrizing conjunctivitis, uveitis (intraocular inflammation of the uvea, retina, vitreous body, and/or optic nerve head), age-related macular degeneration, diabetic retinopathy, and its major complication, and proliferative vitreoretinopathy. Also, it is important to note that ocular infections, traumas, and surgery also involve inflammatory processes that can lead to a vision-threatening situation if not properly controlled. Many efforts have been invested in the development of therapeutic strategies to confront such diseases (for recent reviews see [4–6]), even using the aid of

novel technology-related strategies, such as drug delivery systems or gene therapy (for recent reviews see [7, 8]). However, a much deeper knowledge of molecular pathophysiology mechanisms and mediators underlying ocular inflammation-related disorders is necessary.

The eye has its own mechanism to protect itself from inflammation, which is the so called immune privilege [9]. The biological significance of such "privilege" is the active tolerance to foreign antigens exerted by the ocular immune system. In addition, the tightly sealed blood-ocular barriers prevent the passage of inflammatory cells and molecules from the blood into the eye. Altogether, the collateral inflammation that is associated with the normal immune response is avoided. However, the immune privilege can fail and inflammation can eventually develop with the involvement of different mediators.

There are several families of molecules that have been described as active participants in ocular inflammatory diseases. These include immune mediators such as cytokines and chemokines and enzymes such as matrix metalloproteinases, lipids, growth factors and their receptors, and neurotransmitters and their receptors. These last ones are especially interesting because they are involved in maintaining the immune privilege [10], among other physiological activities. Different neuropeptides and their receptors have been identified in ocular structures, including the cornea, sclera, iris, ciliary body, ciliary process, and the retina [11]. In addition, there are examples of the involvement of not only neuropeptides, but also biogenic amines, amino acids, acetylcholine, or purines, in physiological processes of the eye that can be affected by inflammation. Thus, the knowledge of the clinical impact that neuropeptides and their receptors may have is growing in parallel with their envisioned therapeutical applications in ocular inflammatory diseases.

The role of parasympathetic and sympathetic nerves in conjunctival goblet cell functioning has been reported (for review see [12]). The parasympathetic nerves contain the neurotransmitters acetylcholine and vasoactive intestinal peptide (VIP), and the sympathetic nerves contain norepinephrine and neuropeptide Y. Also, purinergic receptor $P2Y_2$ agonists, such as UTP and ATP, are capable of stimulating both goblet cell mucin secretion and stratified squamous cell fluid (water and electrolytes) secretion to tear film [12]. Sensory nerves of cornea and conjunctiva contain neurotransmitters such as substance P, calcitonin gene-related peptide, and galanin, which can activate a neural reflex to stimulate conjunctival goblet cell secretion [13]. The reduction in corneal sensation that takes place in chronic inflammatory disease of the ocular surface can impair the secretion of mucin and water components of the tear film.

Histamine produced by conjunctival sensitized mast cells is a well-known mediator of the allergic pathology affecting the eye [14]. It is secreted to conjunctival tissues and the tear film, along with other mast cell-derived irritant mediators, after the allergen challenge and triggers different allergic inflammation-related processes [15]. It not only increases vasodilation and vascular permeability to immune cells, but also directly acts upon specific receptors present in conjunctival epithelial cells to stimulate goblet cell mucin secretion [16]. Besides, histamine exerts a chemotactic effect on various immune cell types through a complex cytokine network thus amplifying its biological activities.

The eye has a small piece of brain in it, that is, the retina. It is long known the importance of neurotransmitters and their receptors for the processing of visual information within the retinal tissue [17]. Catecholamine neurotransmitters, mainly dopamine, are key for retinal neuron functioning in the vertebrate retina. Also, the role exerted by excitatory and inhibitory amino acids as well as acetylcholine in the visual process is well established [18]. Glutamate, aspartate, gamma-aminobutyric acid, taurine, and glycine are normal neurotransmitter or neuromodulatory agents for photoreceptors and other retinal neurons, such as horizontal and amacrine cells. Müller cells, the main glial cell of the retina, also secrete neurotransmitters and express a wide variety of neurotransmitter receptors, reflecting their participation in the physiological signaling between neurons and glial cells [19]. However, glutamate-mediated loss of retinal ganglion cells occurs in glaucoma and retinal vessels occlusion (central and branch retinal artery and retinal vein) [20]. Cooperation between inflammatory cytokines and glutamate receptors has been proposed as one of the mechanisms responsible for a toxic damage on retinal cells related to glutathione depletion [21]. Müller cells protect neurons from glutamate toxicity. It is now widely accepted that almost any retinal degenerative disease is associated with Müller cell gliosis, which is a complex series of functional changes that occurs as a consequence of retinal inflammation accompanying the degenerative process. Gliosis impedes Müller cell protective role against glutamate toxicity and impairs their neurotransmitter recycling activity in the glioneuronal interactions [22].

Among the plethora of transmitters present in the ocular structures, nucleosides and nucleotides emerge as remarkable molecules with the ability to regulate many biochemical and physiopathological processes. Their actions are mediated by membrane receptors termed purinergic receptors that can be divided into adenosine P1 or A receptors and nucleotide receptors named as P2. Adenosine receptors can be divided into A_1, A_{2A}, A_{2B}, and A_3 and these receptors are only sensitive to the nucleoside adenosine. On the contrary, P2 receptors are divided into two main groups, ionotropic P2X and metabotropic P2Y receptors, and are sensitive to adenine, to guanine nucleotides, and also to dinucleotides such as dinucleoside polyphosphates.

The diversity of receptors in the eye structures reflects the importance of these molecules in processes such as tear secretion, intraocular pressure homeostasis, lens accommodation, or retinal functioning. Moreover, use of nucleosides and nucleotides, naturally occurring and synthetic, has been suggested to rescue the eye from some pathological conditions. Indeed there is a chance for the development of patents based on nucleotides as a therapeutic approach since it has been possible to relate nucleoside/nucleotide levels with pathological conditions, such as dry eye or glaucoma, for example. The review of the patent literature did not bring any document related to ocular inflammatory processes. In this sense it might be of interest the development of new

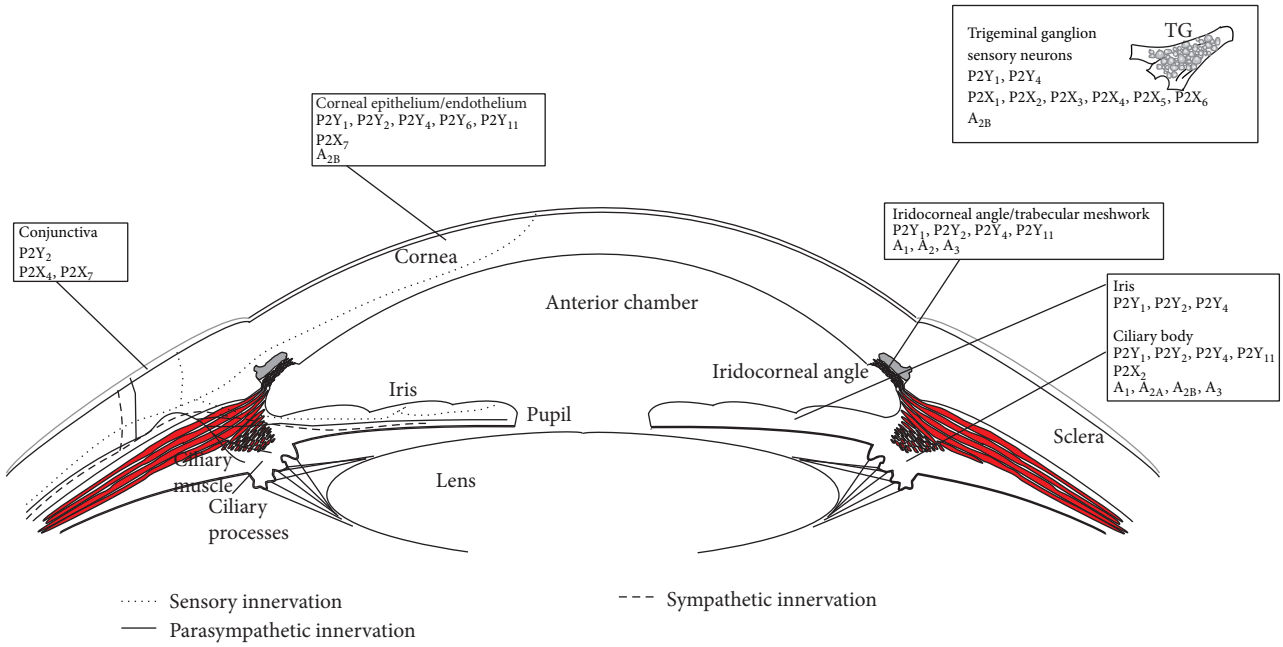

FIGURE 1: Purinergic receptors identified in the ocular anterior segment. Purinergic receptors localized in the different ocular parts/structures of the ocular anterior segment are shown.

inventions for the treatment of ocular inflammation based on purinergic agonists and antagonists.

The scientific literature that studies the relation of purines and the eye have provided a disperse number of papers describing the involvement of these molecules in ocular inflammatory processes. In this sense, the present work reviews and groups the existing works in the field by structuring them in two main groups, on the one hand, actions mediated by means of adenosine receptors and on the other hand those occurring by nucleotide receptors.

2. Adenosine Receptors

Adenosine is elevated at sites of tissue damage resulting from inflammation or hypoxia [23, 24]. Adenosine can be formed intracellularly and diffuse into the extracellular space via equilibrative nucleoside transporter, or extracellularly from released ATP by ectonucleotidases, CD39 and CD73. Under stress and ischemic conditions, the local tissue concentration of extracellular adenosine is increased due to its synthesis from the released ATP. Adenosine has been proposed to modulate a variety of physiological responses including inflammation and immunity by stimulating specific adenosine receptors (AR) [25, 26]. To date, four adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3 have been identified that belong to the family of seven transmembrane G protein-coupled receptors [27]. The A_1 and A_3 adenosine receptors preferentially couple to G_i protein to inhibit adenylate cyclase and consequently the production of cyclic AMP (cAMP), and the A_{2A} and A_{2B} subtypes stimulate the production of cAMP by coupling to G_s. Expression of adenosine receptors has been described in different eye locations (Figure 1). The presence of A_{2B} adenosine receptors [28] has been detected on bovine corneal endothelium. In the ciliary epithelium, A_1, A_{2A}, and A_{2B} adenosine receptor mRNAs were found in the ciliary processes of rat using *in situ* hybridization [29]. Later, A_3 adenosine receptor mRNA expression was also detected in cultured human ciliary epithelial cells and rabbit ciliary processes by RT-PCR [30]. In the retina, A_{2A} adenosine receptor mRNA expression was mainly found in the inner nuclear layer and ganglion cell layer and to a lesser extent in the outer nuclear layer. Likewise, A_1 and A_3 adenosine receptor mRNAs were identified in the ganglion cell layer of the retina [29, 31]. In addition, A_{2A} and A_{2B} adenosine receptors are also present in retinal pigment epithelial cells [29, 32] as well as in Müller cells [33].

2.1. A_1 Adenosine Receptors. Conflicting conclusions about the effect of A_1 adenosine receptors on inflammation have been reported. Thus, A_1 adenosine receptor has been implicated as a potent anti-inflammatory mediator in various inflammatory models of several organ systems, including the kidney [34], heart [35], liver [36], and brain [37]. On the contrary, in the lung, pharmacologic blocking of A_1 adenosine receptors attenuated lipopolysaccharide (LPS)-induced lung injury in cats [38]. Likewise, in an allergic mouse model of asthma, A_1 adenosine receptors have been shown responsible for altered vascular reactivity, increased airway hyperresponsiveness, and systemic inflammation [39].

In the eye, there is no data about the role of this receptor in ocular inflammation. To date, it has been only showed that A_1 adenosine receptor mediates IL-6 trophic effect on retinal ganglion cells [40]. IL-6 is a pleiotropic cytokine classically denominated proinflammatory, but, additionally,

it has been demonstrated that this cytokine is able to increase the survival of retinal ganglion cells [41]. It remains unknown whether A_1 adenosine receptor could also take part in some proinflammatory actions induced by this cytokine in the retina apart from the IL-6 trophic effect on retinal ganglion cells.

2.2. A_{2A} Adenosine Receptors. Substantial lines of evidence have suggested that the anti-inflammatory effects of extracellular adenosine are mainly mediated by A_{2A} adenosine receptors [25, 42]. The anti-inflammatory action of A_{2A} adenosine receptors in acute and chronic retinal inflammation has been demonstrated [43, 44]. Using cultured retinal microglia cells activated by LPS as an *in vitro* model of acute neuroinflammation, Liou et al. [44] showed that A_{2A} adenosine receptor activation in the stressed retinal microglial cells efficiently inhibited LPS-induced TNF-α release. The protective role of A_{2A} adenosine receptor in chronic retinal inflammation associated to diabetic retinopathy has also been examined [43, 45]. Diabetic retinopathy has been categorized as a vascular-neuroinflammatory disease. Among the early signs of diabetic retinopathy are retinal inflammatory reactions, breakdown of the blood-retinal barrier function, and loss of retinal neurons [46–48]. As the disease progresses, the retina may be damaged by oxidative stress induced by hyperglycemia, or advanced glycation end products [49, 50]. This stress damages vascular and neuronal tissues of the retina and activates microglial cells [51]. Activated microglia further exacerbate the damage by releasing cytotoxic molecules (glutamate, reactive oxygen species) and proinflammatory mediators, such as TNF-α [52, 53]. Thus, local inflammation has a relevant contribution in the pathogenesis of diabetic retinopathy. To elucidate the role of A_{2A} adenosine receptor in diabetic retinopathy, the effect of A_{2A} adenosine receptor ablation on diabetic mice was analyzed [43]. Knockout A_{2A} adenosine receptor mice had significantly more retinal terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells, TNF-α release, and intercellular adhesion molecule 1 (ICAM-1) expression compared with diabetic wild type [43]. Interestingly, together with these changes, an altered microglia phenotype was observed in the knockout A_{2A} adenosine receptor mice. In this sense, in a diabetic milieu, microglia transformed from their ramified resting state into an amoeboid shape, the activated and cytokine-releasing state, and this phenotypic configuration was more evident in the knockout A_{2A} adenosine receptor diabetic mice than in diabetic wild-type [43]. Moreover, treatment of diabetic mice with the A_{2A} adenosine receptor agonist CGS21680 (3-[4-[2-[[6-amino-9-[(2R, 3R, 4S, 5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid) attenuated the morphological transformation of ramified microglia into an activated ameboid microglia and resulted in marked decreases in diabetes-induced retinal cell death and TNF-α release [43]. Inhibition of reactive microglial phenotype acquisition is not the only mechanism by which A_{2A} adenosine receptor regulates inflammation in diabetic retinopathy. Additional studies using microglial retinal cells treated with amadori-glycated albumin (AGA) (a risk factor in diabetic disorders) showed that activation of A_{2A} adenosine receptor attenuated AGA-induced TNF-α release by repressing the inflammatory cascade C-Raf/extracellular signal-regulated kinase (ERK) in activated microglia (Figure 2) [43, 45].

Considering these findings about the protective role of A_{2A} adenosine receptor activation in diabetes-induced retinal inflammation, abnormality in adenosine metabolism could have influence on retinal complications in diabetic retinopathy. In this context, an increased expression and activity of catabolic enzyme adenosine deaminase-2 (ADA2), which represent a critical checkpoint in the regulation of extracellular adenosine levels and, consequently, in the control of receptor stimulation and function, have been identified in human and porcine retinas with diabetes as well as in AGA-treated porcine and human microglia cells [54]. Moreover, TNF-α release was induced in AGA-treated microglia cells and that TNF-α release was blocked by ADA2-neutralizing antibody or ADA2 siRNA [54]. These results confirm that abnormality in adenosine metabolism can contribute to retinal inflammation in diabetic retinopathy and suggest that the anti-inflammatory activity of A_{2A} adenosine receptor signaling can be impaired in diabetic retinopathy due to increased ADA2 activity.

Taking into advance the ability of A_{2A} adenosine receptor to offer protection against retinal inflammation in diabetic retinopathy, the use of the A_{2A} adenosine receptor agonist CGS21680 in other ocular retinal pathologies in which proinflammatory mediators are released has also been examined [55]. The A_{2A} adenosine receptor agonist administration significantly attenuated the expression of inflammatory (TNF-α, IL-6, and ICAM-1) and cell death markers in a mouse model of traumatic optic neuropathy (a disease characterized by retinal ganglion cell death, which is closely related to the local production of reactive oxygen species and inflammatory mediators from activated microglial cells) [55]. A_{2A} adenosine receptor agonist anti-inflammatory action was mediated by blocking ERK activation and subsequent cytokine release in traumatic optic neuropathy activated microglia cells (Figure 2).

On the other hand, it has been described the contribution of adenosinergic pathway through A_{2A} adenosine receptor on protective regulatory immunity in a mouse model of human autoimmune uveitis [56]. Thus, A_{2A} adenosine receptor activation on T cells was associated with antigen-presenting cells (APC) induction and activation of Tregs (regulatory T cells), which mediate a postexperimental autoimmune uveoretinitis regulatory immune response to ocular autoantigens protecting from recurrence of uveitis [56].

2.3. A_{2B} Adenosine Receptors. Discrepancy between anti-inflammatory and proinflammatory effects has been observed in several tissues for A_{2B} adenosine receptors [57]. This apparent contradiction might be related to differences between the acute and chronic models of inflammation studied, playing the receptor different roles at different points during the progression of inflammation. Furthermore, A_{2B} adenosine receptors may play different roles even in similar types of inflammation but occurring in different tissues [57–59].

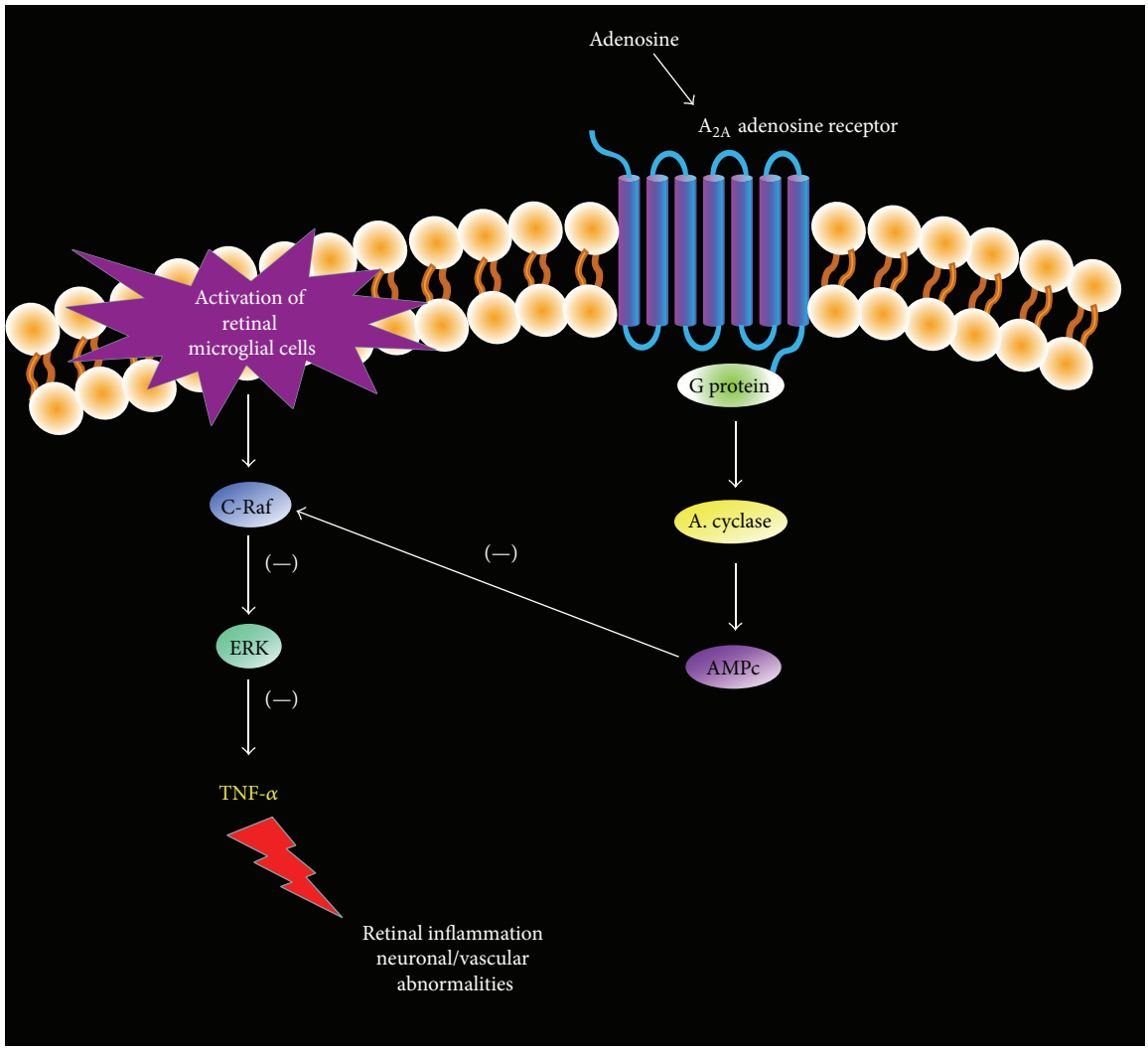

FIGURE 2: Regulation of retinal inflammation by A_{2A} adenosine receptor. Pathways proposed to be involved in anti-inflammatory effect of A_{2A} adenosine receptor in the retinal microglial cells during pathologies such as diabetes or traumatic optic neuropathy. A_{2A} adenosine receptor activation reduces TNF-α release by repressing the inflammatory cascade C-Raf/ERK in activated retinal microglia.

Little is known about the role of A_{2B} adenosine receptor in the eye. A gradual increase in A_{2B} adenosine receptor has been reported after alkali burn-induced corneal inflammation and neovascularization. As A_{2B} adenosine receptor was not expressed by normal cornea, it suggests that the A_{2B} adenosine receptor detected after alkali burns was produced in the cornea by infiltrated inflammatory cells [60]. In agreement with this finding, it has been detected that A_{2B} adenosine receptor seems to be mainly expressed in inflammatory cells [61].

2.4. A_3 Adenosine Receptors. The A_3 adenosine receptor is highly expressed in inflammatory cells whereas low or almost no expression is found in normal cells [62], rendering the A_3 adenosine receptor as a potential therapeutic target. A_3 adenosine receptor upregulation can be attributed to several factors, including elevated adenosine and cytokines, which are characteristic of the microenvironment of inflammatory cells [63]. Under these conditions, the binding of adenosine to their cell surface receptors might induce, through an autocrine pathway, the expression of its own receptors [64, 65]. Additionally, it has been proposed that the proinflammatory cytokine TNF-α can induce an increase of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB)/Akt expression levels, resulting in upregulation of cAMP response element-binding (CREB) and nuclear factor-kappaB (NF-κB) which translocate to the nucleus to act as A_3 adenosine receptor transcription factors [62].

Selective A_3 adenosine receptor agonists are being developed for the treatment of inflammatory diseases such as rheumatoid arthritis, osteoarthritis, psoriasis, and inflammatory bowel diseases [66]. One of these agonists is the compound CF101 (N^6-(3-iodobenzyl)-5′-N-methylcarboxamidoadenosine) which exerts a robust anti-inflammatory effect in experimental animal models of inflammatory diseases [67–70]. The mechanism of action

mediating the anti-inflammatory effect of CF101 includes downregulation of NF-κB signaling pathway, leading to inhibition of proinflammatory cytokines (TNF-α, IL-6, and IL-12), macrophage inflammatory proteins (MIPs-1a, MIP-2), and receptor activator of NF-κB ligand (RANKL), resulting in apoptosis of inflammatory cells [68, 71]. In addition, a direct antiproliferative effect of CF101 towards autoreactive T cells has been observed [72].

The anti-inflammatory effects of CF101 via A_3 adenosine receptor has prompted to explore its use for the treatment of inflammatory ophthalmic diseases such as dry eye and uveoretinitis. Dry eye syndrome is an inflammatory condition of the eye characterized by a massive production of proinflammatory cytokines [73–75]. Desiccating stress induces tear hyperosmolarity, activating intracellular signaling pathways that initiate the production of proinflammatory cytokines. These inflammatory mediators promote the activation (maturation) of immature APCs and induce their migration to draining lymphoid tissues. The APCs are responsible for priming naive T cells in the lymphoid compartment, leading to the expansion of autoreactive $CD4^+$ helper T cell (TH) subtype 1 and T_H17 cell subsets. T cells subsequently infiltrate the ocular surface, where they secrete additional proinflammatory cytokines [76].

A phase II clinical study (randomized, multicenter, double-masked, placebo-controlled, and parallel group) exploring the effect of CF101 on patients with moderate to severe dry eye syndrome has been performed. CF101 administrated orally (1 mg/day for 12 weeks), induced a statistically significant improvement in the corneal staining and an improvement in the tear break-up time and tear meniscus height in patients with dry eye syndrome [77]. In good agreement with previous trials [78], the drug was very well tolerated and no severe adverse effects were detected. It has been suggested that the improvement in the corneal staining and tear break-up time in the study group might be due to reduced inflammation on the ocular surface following direct interaction between CF101 and its receptors on inflammatory cells [79]. However, additional proofs of reduction of inflammation are required to fully confirm this notion.

An experimental mice model of uveitis has been used to test the anti-inflammatory action of CF101. Oral treatment with CF101 (10 μg/kg, twice daily), initiated upon disease onset, improved uveitis clinical score measured by fundoscopy and ameliorated the pathological manifestations of the disease [72]. A decrease in PI3K and STAT (signal transducer and activator of transcription) protein levels in the lymph nodes of experimental autoimmune uveitis mice was detected upon CF101 treatment. Both proteins are known to be involved in the production of proinflammatory cytokines [80, 81] and, indeed, inhibition of interleukin-2, TNF-α, and interferon-γ (IFN-γ) production was also found in CF101-treated animals [72]. Furthermore, CF101 suppressed the antigen-specific proliferation of autoreactive T cells. Overall these results indicate the marked anti-inflammatory effect of CF101 and support further investigation of this drug for uveitis treatment.

3. Ocular Sensory Innervation and Purinergic Receptors P2 Involved in Ocular Inflammation

The trigeminal ganglion through the ophthalmic nerve provides nonvisual sensory innervation of the eye. Sensory neurons innervating the eye detect noxious or potentially noxious stimuli in order to protect the eyeball, elaborate responses to minimize damage, and promote tissue repair. These sensory neurons transduce mechanical, thermal, and chemical stimuli in the noxious range or close to it. Most of the sensory nerve endings innervate the front of the eye, in particular, the cornea and conjunctiva, but important innervation is present in the uvea, where it has a critical role on ocular inflammation [82].

Autonomic parasympathetic innervation of the eye is supplied by the Edinger-Westphal nucleus in the brainstem through the oculomotor nerve [83, 84]. Parasympathetic nerve fibers synapse in the ciliary ganglion and enter the ocular globe through the short ciliary nerves to innervate the iris, the ciliary body and ciliary muscle, and parts of the iridocorneal angle (uveal trabecular meshwork and scleral spur). Some parasympathetic fibers come from the pons through the geniculate ganglion (Petrosal). Later they synapse in the pterygopalatine ganglion before entering the eye [85]. In parallel, sympathetic nerve fibers arise from the superior cervical ganglion and enter the eyeball though the long and short ciliary nerves. They innervate the ciliary body (central stroma and stroma of the ciliary processes), the iris, and parts of the iridocorneal angle. Nonsignificant autonomic innervation is present in the cornea, which is innervated exclusively by sensory fibers.

Different studies have provided evidence for the presence of purinergic receptors in sensory neurons from the trigeminal ganglion (Figure 1). $P2X_3$ receptor mRNA and protein are found in the cell bodies of both small and large sensory neurons, which has the highest level of expression among these neurons and, in particular, in peptidergic neurons [86]. In contrast, only a small percentage of IB4-binding neurons express this receptor in trigeminal ganglia. Lower levels are found for $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ [86–88]. mRNA and protein for $P2Y_1$ and $P2Y_4$ receptors are also present and in many neurons, colocalized with $P2X_3$ receptors [89]. Despite the studies in trigeminal ganglion neurons, there is a lack of specific studies on purinergic receptors in the sensory nerve endings innervating the anterior part of the eye (cornea, sclera, and conjunctiva) or the uvea (iris and ciliary body). Although no information is available for ocular nerves, purinergic receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are present in the corneal epithelium and endothelium cells [90] (Figure 1). In fact, injury to corneal epithelial cells results in nucleotide release and mobilization of a calcium wave from the epithelium to the neurons [91]. It has been hypothesized that ATP is initially released from epithelial cells and then followed by a release of ATP and glutamate from neuronal processes that activate purinergic and N-methyl-D-aspartate (NMDA) receptors, contributing to the wound response [91]. In humans, $P2X_7$ receptor mRNA is also found in the cornea

and upregulated in diabetic patients. Evidence indicates that corneal epithelial cells express full-length and truncated forms of $P2X_7$, allowing $P2X_7$ to function as a multifaceted receptor that can mediate cell proliferation and migration or cell death [92].

In parallel, the conjunctiva, the wet mucosal membrane of the eye, is highly exposed to the environment and at the same time very sensitive to the damaging effects of inflammation. The ocular surface therefore requires a carefully balanced mechanism to initiate inflammation only when absolutely necessary. Here, hybridization to $P2Y_2$ receptor mRNA has been observed in the palpebral and bulbar conjunctival epithelium, including goblet cells, the corneal epithelium, and in meibomian gland sebaceous and ductal cells [93]. In addition, recent studies [94] have reported that the purinergic receptors $P2X_4$ and $P2X_7$ and the bacterial Toll-like receptor 2 (TLR2) are present and functional in conjunctival goblet cells and are involved in the priming and activation of the NLRP3 inflammasome, initiated by danger associated molecular patterns (DAMPs) such as ATP. The $P2X_7$ receptor-NLRP3 inflammasome complex modulates the release of the inflammatory cytokines IL-1b and IL-18 and it seems to be involved in the primary Sjögren's syndrome pathology in the salivary glands and likely in Sjögren's derived ocular dryness (xerophthalmia) [95].

In the anterior uvea, purinergic receptors $P2Y_1$, $P2Y_2$, and $P2Y_4$ have been found in the iris [90]. The same receptors and $P2Y_{11}$ have also been observed in both layers of the ciliary body epithelium (pigmented and nonpigmented) in the rabbit and monkey eye (Figure 1), in addition to a variety of structures within the choroid [90, 93]. Functional evidence of $P2Y_2$ receptor activity has also been reported in these tissues [96, 97]. In turn, ocular ciliary epithelial cells are known to store and release ATP, an endogenous $P2Y_2$ receptor agonist, providing a potential source of extracellular nucleotides for autocrine regulation of intraocular pressure [98]. In this sense, ATP it is known to be released from antidromically stimulated trigeminal sensory nerve endings in the ciliary body and, as a consequence, a significant increase of ATP is found in the aqueous humor [99]. This provides evidence that ATP released by ocular sensory innervation or after injury of ocular tissues can activate both sensory nerve endings and purinergic receptors present in the iris, ciliary body, or other tissues surrounding the anterior chamber of the eye to produce uveitis/endophthalmitis. In addition to the cornea and sclera, abundant sensory nerve terminals are present in the iris and anterior uvea, which detect mechanical, thermal, and chemical stimuli, contributing to neurogenic inflammation (inflammation of neural origin) by releasing proinflammatory neuropeptides like substance P and CGRP [82, 100]. As stated before, released ATP might stimulate these sensory nerve endings to enhance neurogenic inflammation and to maintain an inflamed state in the eye after a noxious insult.

Circulating ATP, nucleotides, and dinucleotides released into the aqueous humor can also stimulate purinergic receptors present in the trabecular meshwork, a tissue located at the iridocorneal angle of the anterior chamber of the eye and involved in the regulation of aqueous humor outflow. mRNA, protein, and functional evidence have been found for purinergic receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ in the bovine trabecular meshwork (Figure 1) [101, 102] and in the human HTM-3 cell line [103]. Depending on the purinergic receptor activated, an increase or decrease in aqueous humor outflow is found. In this sense, selective agonists of $P2Y_1$ receptor increase the facility of aqueous humor outflow and have been proposed as possible drugs for ocular hypertension [102]. On the other hand, ocular inflammation/uveitis produces the opposite effect on outflow facility (decrease) and it has been proposed that ATP and other inflammatory mediators might be involved in this effect [101, 104–106].

4. Conclusions

The eye has evolved to curb intraocular inflammation protecting the delicate visual elements from damage that would be detrimental to visual acuity. This ability of the eye to limit and control immune responses is known as ocular immune privilege. However, the immune privilege can fail and inflammatory processes can occur. The nucleoside adenosine and nucleotides such as ATP are emerging as novel molecules related to ocular inflammatory diseases. To date, the anti-inflammatory effects of adenosine and their agonists CGS21680 and CF101 acting via A_{2A} and A_3 adenosine receptors, respectively, have encouraged exploring their use for the treatment of inflammatory ophthalmic conditions such as ocular retinal pathologies and dry eye and clinical trials are being developed. In contrast to adenosine, the nucleotide ATP exhibits proinflammatory actions mediated by purinergic P2 receptors present in sensory nerve endings or in other eye locations. Altogether the effects of nucleotides and dinucleotides suggest the development of some of these compounds as therapeutic agents mainly based on the use of P2 receptor antagonists. Also, indirectly, the use of $P2Y_2$ agonists on the ocular surface to treat dry eye could reduce ocular surface inflammation, but it is necessary to be aware that the anti-inflammatory effect is a consequence of the restorage of aqueous and mucin production. Under these new normal conditions, friction of the lids with the ocular surface is diminished and therefore inflammation is reduced. In any case, to our knowledge, apart from the commented effects on dry eye, there is a lack of patents claiming the use of agonists or antagonists for the treatment of ocular inflammation, although, in the recent years, our knowledge about the relation of these molecules with ocular inflammatory processes is increasing. However a better understanding of their exact contribution in the different ocular inflammatory diseases (dry eye, severe cicatrizing conjunctivitis, uveitis, and so forth) is an important step to reveal additional pathologic mechanisms and designing new therapies based on the use of purinergic agonists and antagonists.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] V. Chesnokova and S. Melmed, "Minireview: neuro-immuno-endocrine modulation of the hypothalamic-pituitary-adrenal (HPA) axis by gp130 signaling molecules," *Endocrinology*, vol. 143, no. 5, pp. 1571–1574, 2002.

[2] C. Nathan, "Points of control in inflammation," *Nature*, vol. 420, no. 6917, pp. 846–852, 2002.

[3] C. N. Serhan, "Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways," *Annual Review of Immunology*, vol. 25, pp. 101–137, 2007.

[4] C. Evereklioglu, "Ocular Behçet disease: current therapeutic approaches," *Current Opinion in Ophthalmology*, vol. 22, no. 6, pp. 508–516, 2011.

[5] S. C. Maloney, K. D. Godeiro, A. N. Odashiro, and M. N. Burnier Jr., "Current and emerging concepts in the management of neovascular age-related macular degeneration," *Cardiovascular and Hematological Agents in Medicinal Chemistry*, vol. 5, no. 2, pp. 147–154, 2007.

[6] I. Offiah and V. L. Calder, "Immune mechanisms in allergic eye diseases: what is new?" *Current Opinion in Allergy and Clinical Immunology*, vol. 9, no. 5, pp. 477–481, 2009.

[7] C. J. Chu, S. E. Barker, A. D. Dick, and R. R. Ali, "Gene therapy for noninfectious uveitis," *Ocular Immunology and Inflammation*, vol. 20, no. 6, pp. 394–405, 2012.

[8] Y. Diebold, L. Contreras-Ruiz, I. Arranz-Valsero, and L. García-Posadas, "Drug delivery systems for ophthalmic administration of antiinflammatory agents," *Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry*, vol. 10, no. 3, pp. 203–214, 2011.

[9] J. Y. Niederkorn and J. Stein-Streilein, "History and physiology of immune privilege," *Ocular Immunology and Inflammation*, vol. 18, no. 1, pp. 19–23, 2010.

[10] A. W. Taylor, "Neuroimmunomodulation and immune privilege: the role of neuropeptides in ocular immunosuppression," *NeuroImmunoModulation*, vol. 10, no. 4, pp. 189–198, 2002.

[11] T. Tervo, K. Tervo, and L. Eranko, "Ocular neuropeptides," *Medical Biology*, vol. 60, no. 2, pp. 53–60, 1982.

[12] D. A. Dartt, "Regulation of mucin and fluid secretion by conjunctival epithelial cells," *Progress in Retinal and Eye Research*, vol. 21, no. 6, pp. 555–576, 2002.

[13] T. L. Kessler, H. J. Mercer, J. D. Zieske, D. M. McCarthy, and D. A. Dartt, "Stimulation of goblet cell mucous secretion by activation of nerves in rat conjunctiva," *Current Eye Research*, vol. 14, no. 11, pp. 985–992, 1995.

[14] A. S. Bacon, P. Ahluwalia, A. Irani et al., "Tear and conjunctival changes during the allergen-induced early- and late-phase responses," *Journal of Allergy and Clinical Immunology*, vol. 106, no. 5, pp. 948–954, 2000.

[15] M. Ohbayashi, B. Manzouri, K. Morohoshi, K. Fukuda, and S. J. Ono, "The role of histamine in ocular allergy," *Advances in Experimental Medicine and Biology*, vol. 709, pp. 43–52, 2010.

[16] D. Hayashi, D. Li, C. Hayashi, M. Shatos, R. R. Hodges, and D. A. Dartt, "Role of histamine and its receptor subtypes in stimulation of conjunctival goblet cell secretion.," *Investigative ophthalmology & visual science*, vol. 53, no. 6, pp. 2993–3003, 2012.

[17] R. G. Pourcho, "Neurotransmitters in the retina," *Current Eye Research*, vol. 15, no. 7, pp. 797–803, 1996.

[18] C. J. Pycock, "Retinal neurotransmission," *Survey of Ophthalmology*, vol. 29, no. 5, pp. 355–365, 1985.

[19] R. A. de Melo Reis, A. L. M. Ventura, C. S. Schitine, M. C. F. de Mello, and F. G. de Mello, "Müller glia as an active compartment modulating nervous activity in the vertebrate retina: neurotransmitters and trophic factors," *Neurochemical Research*, vol. 33, no. 8, pp. 1466–1474, 2008.

[20] N. J. Sucher, S. A. Lipton, and E. B. Dreyer, "Molecular basis of glutamate toxicity in retinal ganglion cells," *Vision Research*, vol. 37, no. 24, pp. 3483–3493, 1997.

[21] T. Harada, C. Harada, K. Nakamura et al., "The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma," *Journal of Clinical Investigation*, vol. 117, no. 7, pp. 1763–1770, 2007.

[22] A. Bringmann, T. Pannicke, J. Grosche et al., "Müller cells in the healthy and diseased retina," *Progress in Retinal and Eye Research*, vol. 25, no. 4, pp. 397–424, 2006.

[23] C. Martin, M. Leone, X. Viviand, M. Ayem, and R. Guieu, "High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock," *Critical Care Medicine*, vol. 28, no. 9, pp. 3198–3202, 2000.

[24] B. Sperlágh, M. Dóda, M. Baranyi, and G. Haskó, "Ischemic-like condition releases norepinephrine and purines from different sources in superfused rat spleen strips," *Journal of Neuroimmunology*, vol. 111, no. 1-2, pp. 45–54, 2000.

[25] S. Gessi, S. Merighi, D. Fazzi, A. Stefanelli, K. Varani, and P. A. Borea, "Adenosine receptor targeting in health and disease," *Expert Opinion on Investigational Drugs*, vol. 20, no. 12, pp. 1591–1609, 2011.

[26] G. Haskó, J. Linden, B. Cronstein, and P. Pacher, "Adenosine receptors: therapeutic aspects for inflammatory and immune diseases," *Nature Reviews Drug Discovery*, vol. 7, no. 9, pp. 759–770, 2008.

[27] B. B. Fredholm, A. P. Ijzerman, K. A. Jacobson, K. Klotz, and J. Linden, "International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors," *Pharmacological Reviews*, vol. 53, no. 4, pp. 527–552, 2001.

[28] R. J. Walkenbach and W.-T. Chao, "Adenosine regulation of cyclic AMP in corneal endothelium," *Journal of Ocular Pharmacology*, vol. 1, no. 4, pp. 337–342, 1985.

[29] A. Kvanta, S. Seregard, S. Sejersen, B. Kull, and B. B. Fredholm, "Localization of adenosine receptor messenger RNAs in the rat eye," *Experimental Eye Research*, vol. 65, no. 5, pp. 595–602, 1997.

[30] C. H. Mitchell, K. Peterson-Yantorno, D. A. Carré et al., "A3 adenosine receptors regulate Cl- channels of nonpigmented ciliary epithelial cells," *The American Journal of Physiology—Cell Physiology*, vol. 276, no. 3, part 1, pp. C659–C666, 1999.

[31] M. Zhang, M. T. Budak, W. Lu et al., "Identification of the A3 adenosine receptor in rat retinal ganglion cells," *Molecular Vision*, vol. 12, pp. 937–948, 2006.

[32] C. Blazynski, "Characterization of adenosine A2 receptors in bovine retinal pigment epithelial membranes," *Experimental Eye Research*, vol. 56, no. 5, pp. 595–599, 1993.

[33] E. A. Newman, "Calcium increases in retinal glial cells evoked by light-induced neuronal activity," *Journal of Neuroscience*, vol. 25, no. 23, pp. 5502–5510, 2005.

[34] H. T. Lee, G. Gallos, S. H. Nasr, and C. W. Emala, "A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice," *Journal of the American Society of Nephrology*, vol. 15, no. 1, pp. 102–111, 2004.

[35] Y. Liao, S. Takashima, Y. Asano et al., "Activation of adenosine A1 receptor attenuates cardiac hypertrophy and prevents heart failure in murine left ventricular pressure-overload model," *Circulation Research*, vol. 93, no. 8, pp. 759–766, 2003.

[36] J. Kim, M. Kim, J. H. Song, and H. T. Lee, "Endogenous A_1 adenosine receptors protect against hepatic ischemia reperfusion injury in mice," *Liver Transplantation*, vol. 14, no. 6, pp. 845–854, 2008.

[37] S. Tsutsui, J. Schnermann, F. Noorbakhsh et al., "A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis," *Journal of Neuroscience*, vol. 24, no. 6, pp. 1521–1529, 2004.

[38] C. F. Neely, J. Jin, and I. M. Keith, "A1-adenosine receptor antagonists block endotoxin-induced lung injury," *The American Journal of Physiology*, vol. 272, no. 2, pp. L353–L361, 1997.

[39] D. S. Ponnoth, A. Nadeem, S. Tilley, and S. J. Mustafa, "Involvement of A1 adenosine receptors in altered vascular responses and inflammation in an allergic mouse model of asthma," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 299, no. 1, pp. H81–H87, 2010.

[40] R. Perígolo-Vicente, K. Ritt, M. R. Pereira, P. M. M. Torres, R. Paes-de-Carvalho, and E. Giestal-de-Araujo, "IL-6 treatment increases the survival of retinal ganglion cells in vitro: The role of adenosine A1 receptor," *Biochemical and Biophysical Research Communications*, vol. 430, no. 2, pp. 512–518, 2013.

[41] P. M. M. Torres and E. G. De Araujo, "Interleukin-6 increases the survival of retinal ganglion cells in vitro," *Journal of Neuroimmunology*, vol. 117, no. 1-2, pp. 43–50, 2001.

[42] G. Haskó and P. Pacher, "A2A receptors in inflammation and injury: lessons learned from transgenic animals," *Journal of Leukocyte Biology*, vol. 83, no. 3, pp. 447–455, 2008.

[43] A. S. Ibrahim, M. M. El-shishtawy, W. Zhang, R. B. Caldwell, and G. I. Liou, "A2A adenosine receptor (A2AAR) as a therapeutic target in diabetic retinopathy," *The American Journal of Pathology*, vol. 178, no. 5, pp. 2136–2145, 2011.

[44] G. I. Liou, J. A. Auchampach, C. J. Hillard et al., "Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A2A adenosine receptor," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 12, pp. 5526–5531, 2008.

[45] G. I. Liou, S. Ahmad, M. Naime, N. Fatteh, and A. S. Ibrahim, "Role of adenosine in diabetic retinopathy," *Journal of Ocular Biology, Diseases, and Informatics*, vol. 4, no. 1-2, pp. 19–24, 2011.

[46] A. J. Barber, E. Lieth, S. A. Khin, D. A. Antonetti, A. G. Buchanan, and T. W. Gardner, "Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin," *Journal of Clinical Investigation*, vol. 102, no. 4, pp. 783–791, 1998.

[47] A. B. El-Remessy, M. Al-Shabrawey, Y. Khalifa, N. Tsai, R. B. Caldwell, and G. I. Liou, "Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes," *American Journal of Pathology*, vol. 168, no. 1, pp. 235–244, 2006.

[48] A. M. Joussen, V. Poulaki, M. L. Le et al., "A central role for inflammation in the pathogenesis of diabetic retinopathy," *The FASEB Journal*, vol. 18, no. 12, pp. 1450–1452, 2004.

[49] M. Al-Shabrawey, M. Rojas, T. Sanders et al., "Role of NADPH oxidase in retinal vascular inflammation," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 7, pp. 3239–3244, 2008.

[50] A. W. Stitt, T. Bhaduri, C. B. T. McMullen, T. A. Gardiner, and D. B. Archer, "Advanced glycation end products induce blood-retinal barrier dysfunction in normoglycemic rats," *Molecular Cell Biology Research Communications*, vol. 3, no. 6, pp. 380–388, 2000.

[51] E. Rungger-Brändle, A. A. Dosso, and P. M. Leuenberger, "Glial reactivity, an early feature of diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 7, pp. 1971–1980, 2000.

[52] G. W. Kreutzberg, "Microglia: a sensor for pathological events in the CNS," *Trends in Neurosciences*, vol. 19, no. 8, pp. 312–318, 1996.

[53] M. Sayyah, M. Javad-Pour, and M. Ghazi-Khansari, "The bacterial endotoxin lipopolysaccharide enhances seizure susceptibility in mice: involvement of proinflammatory factors: nitric oxide and prostaglandins," *Neuroscience*, vol. 122, no. 4, pp. 1073–1080, 2003.

[54] N. M. Elsherbiny, M. Naime, S. Ahmad et al., "Potential roles of adenosine deaminase-2 in diabetic retinopathy," *Biochemical and Biophysical Research Communications*, vol. 436, no. 3, pp. 355–361, 2013.

[55] S. Ahmad, N. Fatteh, N. M. El-Sherbiny et al., "Potential role of A2A adenosine receptor in traumatic optic neuropathy," *Journal of Neuroimmunology*, vol. 264, no. 1-2, pp. 54–64, 2013.

[56] D. J. Lee and A. W. Taylor, "Both MC5r and A2Ar are required for protective regulatory immunity in the spleen of post-experimental autoimmune uveitis in mice," *The Journal of Immunology*, vol. 191, no. 8, pp. 4103–4111, 2013.

[57] I. Feoktistov and I. Biaggioni, "Role of adenosine A_{2B} receptors in inflammation," *Advances in Pharmacology*, vol. 61, pp. 115–144, 2011.

[58] V. L. Kolachala, M. Vijay-Kumar, G. Dalmasso et al., "A2B adenosine receptor gene deletion attenuates murine colitis," *Gastroenterology*, vol. 135, no. 3, pp. 861–870, 2008.

[59] Y. Zhou, A. Mohsenin, E. Morschl et al., "Enhanced airway inflammation and remodeling in adenosine deaminase-deficient mice lacking the A_{2B} adenosine receptor," *Journal of Immunology*, vol. 182, no. 12, pp. 8037–8046, 2009.

[60] Y. Han, Y. Shao, Z. Lin et al., "Netrin-1 simultaneously suppresses corneal inflammation and neovascularization," *Investigative Ophthalmology & Visual Science*, vol. 53, no. 3, pp. 1285–1295, 2012.

[61] P. Rosenberger, J. M. Schwab, V. Mirakaj et al., "Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia," *Nature Immunology*, vol. 10, no. 2, pp. 195–202, 2009.

[62] A. Ochaion, S. Bar-Yehuda, S. Cohen et al., "The anti-inflammatory target A3 adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn's disease," *Cellular Immunology*, vol. 258, no. 2, pp. 115–122, 2009.

[63] L. Madi, S. Cohen, A. Ochayin, S. Bar-Yehuda, F. Barer, and P. Fishman, "Overexpression of A3 adenosine receptor in peripheral blood mononuclear cells in rheumatoid arthritis:

involvement of nuclear factor-κB in mediating receptor level," *Journal of Rheumatology*, vol. 34, no. 1, pp. 20–26, 2007.

[64] A. Ochaion, S. Bar-Yehuda, S. Cohn et al., "Methotrexate enhances the anti-inflammatory effect of CF101 via up-regulation of the A3 adenosine receptor expression," *Arthritis Research and Therapy*, vol. 8, no. 6, article R169, 2006.

[65] U. Schlotzer-Schrehardt, M. Zenkel, U. Decking et al., "Selective upregulation of the A3 adenosine receptor in eyes with pseudoexfoliation syndrome and glaucoma," *Investigative Ophthalmology & Visual Science*, vol. 46, no. 6, pp. 2023–2034, 2005.

[66] P. Fishman, S. Bar-Yehuda, B. T. Liang, and K. A. Jacobson, "Pharmacological and therapeutic effects of A3 adenosine receptor agonists," *Drug Discovery Today*, vol. 17, no. 7-8, pp. 359–366, 2012.

[67] S. Bar-Yehuda, L. Rath-Wolfson, L. Del Valle et al., "Induction of an antiinflammatory effect and prevention of cartilage damage in rat knee osteoarthritis by CF101 treatment," *Arthritis and Rheumatism*, vol. 60, no. 10, pp. 3061–3071, 2009.

[68] P. Fishman, S. Bar-Yehuda, L. Madi et al., "The PI3K-NF-κB signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis," *Arthritis Research and Therapy*, vol. 8, no. 1, article R33, 2006.

[69] J. Mabley, F. Soriano, P. Pacher et al., "The adenosine A3 receptor agonist, N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide, is protective in two murine models of colitis," *European Journal of Pharmacology*, vol. 466, no. 3, pp. 323–329, 2003.

[70] L. Rath-Wolfson, S. Bar-Yehuda, L. Madi et al., "IB-MECA, an A3 adenosine receptor agonist prevents bone resorption in rats with adjuvant induced arthritis," *Clinical and Experimental Rheumatology*, vol. 24, no. 4, pp. 400–406, 2006.

[71] C. Szabó, G. S. Scott, L. Virág et al., "Suppression of macrophage inflammatory protein (MIP)-1α production and collagen-induced arthritis by adenosine receptor agonists," *British Journal of Pharmacology*, vol. 125, no. 2, pp. 379–387, 1998.

[72] S. Bar-Yehuda, D. Luger, A. Ochaion et al., "Inhibition of experimental auto-immune uveitis by the A3 adenosine receptor agonist CF101," *International Journal of Molecular Medicine*, vol. 28, no. 5, pp. 727–731, 2011.

[73] A. Acera, G. Rocha, E. Vecino, I. Lema, and J. A. Durán, "Inflammatory markers in the tears of patients with ocular surface disease," *Ophthalmic Research*, vol. 40, no. 6, pp. 315–321, 2008.

[74] N. Boehm, A. I. Riechardt, M. Wiegand, N. Pfeiffer, and F. H. Grus, "Proinflammatory cytokine profiling of tears from dry eye patients by means of antibody microarrays," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 10, pp. 7725–7730, 2011.

[75] H. Lam, L. Bleiden, C. S. de Paiva, W. Farley, M. E. Stern, and S. C. Pflugfelder, "Tear cytokine profiles in dysfunctional tear syndrome," *American Journal of Ophthalmology*, vol. 147, no. 2, pp. 198–205, 2009.

[76] W. Stevenson, S. K. Chauhan, and R. Dana, "Dry eye disease: an immune-mediated ocular surface disorder," *Archives of Ophthalmology*, vol. 130, no. 1, pp. 90–100, 2012.

[77] I. Avni, H. J. Garzozi, I. S. Barequet et al., "Treatment of dry eye syndrome with orally administered CF101: data from a phase 2 clinical trial," *Ophthalmology*, vol. 117, no. 7, pp. 1287–1293, 2010.

[78] A.-R. van Troostenburg, E. V. Clark, W. D. H. Carey et al., "Tolerability, pharmacokinetics and concentration-dependent hemodynamic effects of oral CF101, an A3 adenosine receptor agonist, in healthy young men," *International Journal of Clinical Pharmacology and Therapeutics*, vol. 42, no. 10, pp. 534–542, 2004.

[79] J. N. Ashar, A. Mathur, and V. Sangwan, "CF101 for dry eye," *Ophthalmology*, vol. 118, no. 5, pp. 1011–1012, 2011.

[80] B. Renga, M. Migliorati, A. Mencarelli, and S. Fiorucci, "Reciprocal regulation of the bile acid-activated receptor FXR and the interferon-γ-STAT-1 pathway in macrophages," *Biochimica et Biophysica Acta*, vol. 1792, no. 6, pp. 564–573, 2009.

[81] S. G. Ward and P. Finan, "Isoform-specific phosphoinositide 3-kinase inhibitors as therapeutic agents," *Current Opinion in Pharmacology*, vol. 3, no. 4, pp. 426–434, 2003.

[82] C. Belmonte, J. Garcia-Hirschfeld, and J. Gallar, "Neurobiology of ocular pain," *Progress in Retinal and Eye Research*, vol. 16, no. 1, pp. 117–156, 1997.

[83] A. Reiner, H. J. Karten, P. D. R. Gamlin, and J. T. Erichsen, "Parasympathetic ocular control. Functional subdivisions and circuity of the avian nucleus of Edinger-Westphal," *Trends in Neurosciences*, vol. 6, no. 4, pp. 140–145, 1983.

[84] M. P. M. Ten Tusscher, H. J. M. Beckers, G. F. J. M. Vrensen, and J. Klooster, "Peripheral neural circuits regulating IOP? A review of its anatomical backbone," *Documenta Ophthalmologica*, vol. 87, no. 4, pp. 291–313, 1994.

[85] G. L. Ruskell, "The orbital branches of the pterygopalatine ganglion and their relationship with internal carotid nerve branches in primates," *Journal of Anatomy*, vol. 106, no. 2, pp. 323–339, 1970.

[86] V. Staikopoulos, B. J. Sessle, J. B. Furness, and E. A. Jennings, "Localization of P2X2 and P2X3 receptors in rat trigeminal ganglion neurons," *Neuroscience*, vol. 144, no. 1, pp. 208–216, 2007.

[87] P. M. Dunn, Y. Zhong, and G. Burnstock, "P2X receptors in peripheral neurons," *Progress in Neurobiology*, vol. 65, no. 2, pp. 107–134, 2001.

[88] H. Kuroda, Y. Shibukawa, M. Soya et al., "Expression of P2X1 and P2X4 receptors in rat trigeminal ganglion neurons," *NeuroReport*, vol. 23, no. 13, pp. 752–756, 2012.

[89] H. Z. Ruan and G. Burnstock, "Localisation of P2Y1 and P2Y4 receptors in dorsal root, nodose and trigeminal ganglia of the rat," *Histochemistry and Cell Biology*, vol. 120, no. 5, pp. 415–426, 2003.

[90] J. Pintor, J. Sánchez-Nogueiro, M. Irazu, A. Mediero, T. Peláez, and A. Peral, "Immunolocalisation of P2Y receptors in the rat eye," *Purinergic Signalling*, vol. 1, no. 1, pp. 83–90, 2004.

[91] D. J. Oswald, A. Lee, M. Trinidad et al., "Communication between corneal epithelial cells and trigeminal neurons is facilitated by purinergic (P2) and glutamatergic receptors," *PLoS ONE*, vol. 7, no. 9, Article ID e44574, 2012.

[92] C. Mankus, C. Rich, M. Minns, and V. Trinkaus-Randall, "Corneal epithelium expresses a variant of P2X 7 receptor in health and disease," *PLoS ONE*, vol. 6, no. 12, Article ID e28541, 2011.

[93] M. S. Cowlen, V. Z. Zhang, L. Warnock, C. F. Moyer, W. M. Peterson, and B. R. Yerxa, "Localization of ocular P2Y2 receptor gene expression by in situ hybridization," *Experimental Eye Research*, vol. 77, no. 1, pp. 77–84, 2003.

[94] V. E. McGilligan, M. S. Gregory-Ksander, D. Li et al., "*Staphylococcus aureus* activates the NLRP3 inflammasome in human and rat conjunctival goblet cells," *PLoS ONE*, vol. 8, no. 9, Article ID e74010, 2013.

[95] C. Baldini, C. Rossi, F. Ferro et al., "The P2X7 receptor-inflammasome complex has a role in modulating the inflammatory response in primary Sjögren's syndrome," *Journal of Internal Medicine*, vol. 274, no. 5, pp. 480–489, 2013.

[96] N. A. Farahbakhsh and M. C. Cilluffo, "P2 purinergic receptor-coupled signaling in the rabbit ciliary body epithelium," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 7, pp. 2317–2325, 2002.

[97] M. Shahidullah and W. S. Wilson, "Mobilisation of intracellular calcium by P2Y2 receptors in cultured, non-transformed bovine ciliary epithelial cells," *Current Eye Research*, vol. 16, no. 10, pp. 1006–1016, 1997.

[98] C. H. Mitchell, D. A. Carré, A. M. Mcglinn, R. A. Stone, and M. M. Civan, "A release mechanism for stored ATP in ocular ciliary epithelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 12, pp. 7174–7178, 1998.

[99] E. Maul and M. Sears, "ATP is released into the rabbit eye by antidromic stimulation of the trigeminal nerve," *Investigative Ophthalmology and Visual Science*, vol. 18, no. 3, pp. 256–262, 1979.

[100] G. M. Mintenig, M. V. Sanchez-Vives, C. Martin, A. Gual, and C. Belmonte, "Sensory receptors in the anterior uvea of the cat's eye: an in vitro study," *Investigative Ophthalmology & Visual Science*, vol. 36, no. 8, pp. 1615–1624, 1995.

[101] D. Soto, N. Comes, E. Ferrer et al., "Modulation of aqueous humor outflow by ionic mechanisms involved in trabecular meshwork cell volume regulation," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 10, pp. 3650–3661, 2004.

[102] D. Soto, J. Pintor, A. Peral, A. Gual, and X. Gasull, "Effects of dinucleoside polyphosphates on trabecular meshwork cells and aqueous humor outflow facility," *Journal of Pharmacology and Experimental Therapeutics*, vol. 314, no. 3, pp. 1042–1051, 2005.

[103] C. E. Crosson, P. W. Yates, A. N. Bhat, Y. V. Mukhin, and S. Husain, "Evidence for multiple P2Y receptors in trabecular meshwork cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 309, no. 2, pp. 484–489, 2004.

[104] P. Conquet, B. Plazonnet, and J. C. le Douarec, "Arachidonic acid induced elevation of intraocular pressure and anti inflammatory agents," *Investigative Ophthalmology*, vol. 14, no. 10, pp. 772–775, 1975.

[105] J. G. Ladas, F. Yu, R. Loo et al., "Relationship between aqueous humor protein level and outflow facility in patients with uveitis," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 11, pp. 2584–2588, 2001.

[106] R. S. Moorthy, A. Mermoud, G. Baerveldt, D. S. Minckler, P. P. Lee, and N. A. Rao, "Glaucoma associated with uveitis," *Survey of Ophthalmology*, vol. 41, no. 5, pp. 361–394, 1997.