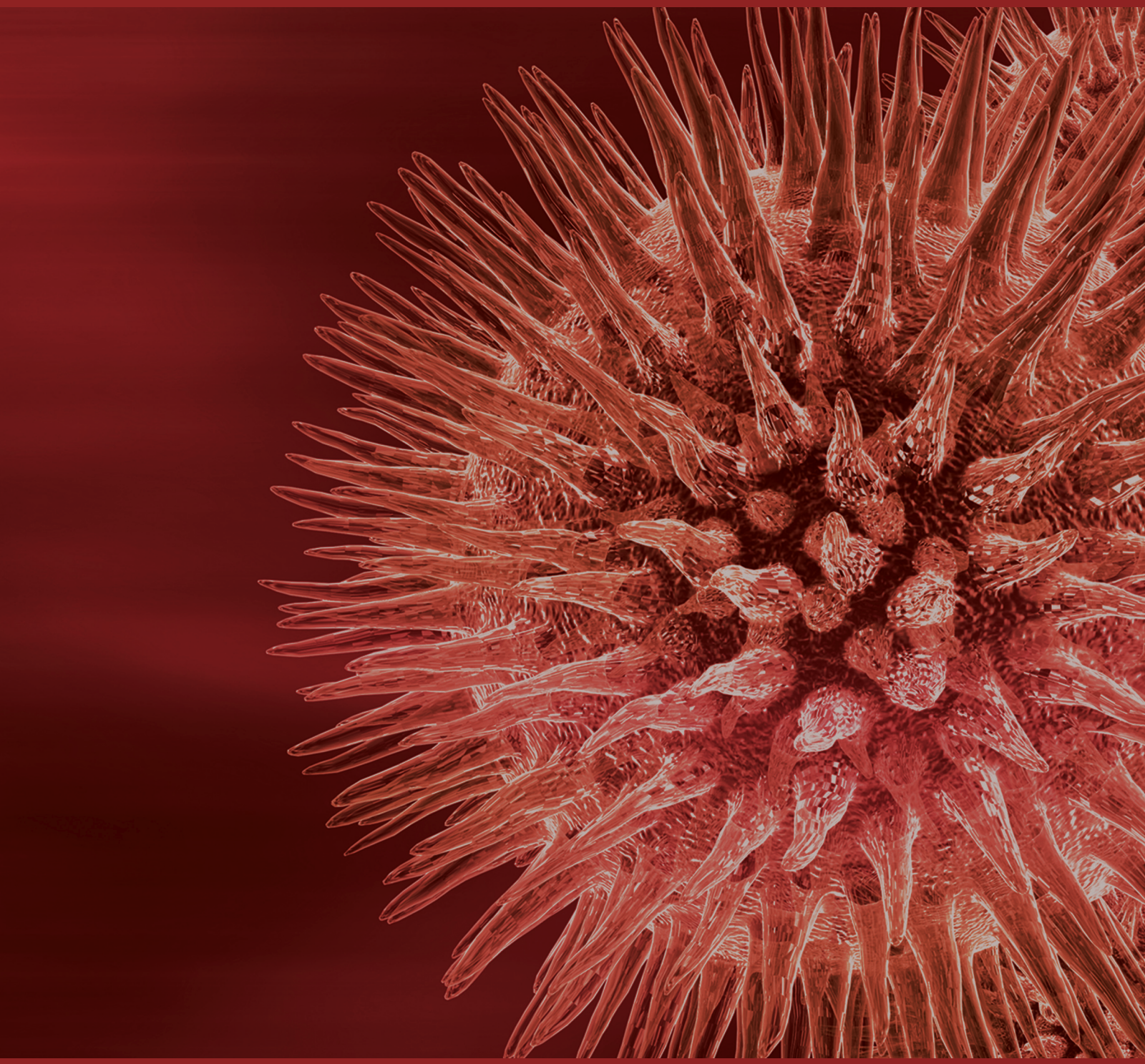
BioMed Research International

Modulation of Lung Immune Response

Guest Editors: Alexandre de Paula Rogerio, Carlo José Freire de Oliveira, Edinéia Lemos de Andrade, and Oliver Haworth



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Editorial

Modulation of Lung Immune Response

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The knowledge of the participation of the innate and adaptive immune response on the pathophysiological process of infectious and noninfectious airway injuries is rapidly expanding and the advances in the fields of biomedicine and biotechnology have been decisive to this expansion. The innate and adaptive immune system, as well as structural cells, modulates the quantity and quality of airway inflammatory response. Aberrant immune responses, including those induced for allergens, environmental pollutants, infectious agents, acids, and others promote excessive leukocyte recruitment, production of proinflammatory cytokines, chemokines, and other immunomodulatory mediators which are critical to initiate and maintain airway disorders. In recent decades, considerable progress has been made in the understanding of the genetic and immunological factors that contribute to the development and/or treatment of airway disorders. Thus, the use of molecular and cellular assays together with knockout animals has contributed significantly to this evolution. In despite of this, the current therapy for the treatment of airway disorders has not changed to the same degree and is still far from ideal. This special issue covers the most relevant research regarding the immunological aspects of airways disorders including asthma, airway inflammation, cancer and fungal and viral infections.

Allergic asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness, eosinophilic inflammation, and hypersecretion of mucus. The asthma physiopathology involves chemical mediators and many cells, mainly eosinophils which plays an important role in inflammation establishment through the release of specific mediators. The effect of corticosteroids on the asthma treatment as well as their adverse effects is widely known. Montelukast (Singulair) is a cysteinyl-leukotriene [$CysLT_1$]-receptor antagonist and demonstrates improvement of asthma; however the chronic use, also, causes adverse effects. It could be used in synergism with corticosteroids and in this case a lower dose of corticosteroids or both could be used and consequently less adverse effect could be evidenced. Using a classical airways allergic inflammation experimental model (induced by ovalbumin) N. B. Gobbato et al. demonstrated that either montelukast or dexamethasone was effective in reducing the recruitment of eosinophils to the lung and other parameters such as eotaxin, RANTES, and IGF-I concentrations possibly by interfering in the NF-κB signaling pathway. These data suggest that both treatments contribute to a better control of the inflammatory response in airways.

In the asthma, several cytokines, mainly Th2 cytokines, modulate and coordinate the airways immune response. L. Gallelli et al. reviewed the most current advances of anticytokine therapies in asthma, suggesting that antibodies anti-IL-4, anti-IL5, and anti-IL13 (all Th2 cytokines) may be beneficial in the treatment of atopic asthma and other allergic diseases but antibodies against cytokines such as

IL-9, GM-CSF, TNF-α, IL-23, IL-17, IL-25, and IL-33 must also be considered. Regarding the role of cytokines in the lung immune response, IL-33 has been highlighted in recent years. IL-33 is a novel member of the IL-1 family. M. M. Bunting et al. demonstrated in an airways allergic inflammation experimental model (induced by ovalbumin) that the expression of IL-33 in the airways is enhanced. In addition, they demonstrated that neutralization of IL-33 (antibody anti-IL-33) decreased both airway inflammation and the expression of proinflammatory cytokines by airways macrophages. These results demonstrated that IL-33 could be an important target to the treatment of airways allergic inflammation.

Chemoattractant cytokines, named chemokines, are also involved in asthma pathogenesis. CXCL12 is a homeostatic CXC chemokine widely expressed with a broad range of functions (from recruitment of mature B and T cells to migration of haematopoietic progenitor cells from the bone marrow). H. Rafatpanah et al. analyzed for CXCL12 polymorphisms in asthmatic individuals and demonstrated polymorphisms at position +801 of CXCL12. Interestingly, the authors suggest that the systemic concentration of CXCL12 could be used as one of the important markers in asthma diagnosis.

Carbon nanotubes (CNT) and carbon nanofibers (CNF) are particles present in an increasing number of consumer products and are also incidental components in indoor air. In an airways allergic inflammation experimental model (induced by ovalbumin), U. C. Nygaard et al. demonstrated that CNF and CNT (administrated by intranasal route) modulated the airway responses to allergens, resulting in airway inflammation and production of allergen-specific IgE. This study provided a basis for better understanding the mechanisms underlying to the effect of nanoparticles such as CNF and CNT promoting airway allergy.

Oral tolerization with proteins modulates the immune response to promote attenuation of hypersensitivity reactions. Xavier-Elsas et al. using direct and indirect experimental model of tolerization (induced by ovalbumin) demonstrated that oral tolerization suppresses eosinopoiesis in the bone-marrow.

In the lung, the vagal parasympathetic nervous system via muscarinic receptors represents the autonomic control of airways muscle tone. Cholinergic system is involved in bronchoconstriction as well as smooth regulation of inflammation. In this context, Castro et al. evaluated the airways responses to the cholinergic agonist methacholine (MCh) in two mouse lines selected to respond maximally (AIRmax) or minimally (AIRmin) to an innate inflammatory stimuli, focusing mainly on the role of M2 receptors. In basal condition AIRmin mice responded more vigorously to MCh than AIRmax. Gallamine treatment, a specific M2 antagonist, increased airway response of AIRmax but not of AIRmin mice. The expression of M2 receptors in the lung was significantly lower in AIRmin when compared to AIRmax animals. AIRmax mice developed a more intense allergic inflammation than AIRmin and both allergic mouse lines increased airway responses to MCh. These authors suggested that AIRmin and AIRmax mouse lines represent a model to study lung muscarinic receptor functions and its relation with the development of an allergic lung disease.

In chronic pulmonary disorders including cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) neutrophils play an important role in airway inflammation by damaging the airways tissues. Secretory leukoprotease inhibitor (SLPI) is an anti-inflammatory protein present in respiratory secretions. The SLPI associated to neutrophils is still poorly characterized. In an elegant study, F. P. Reeves et al. demonstrated that recombinant human SLPI significantly inhibited fMet-Leu-Phe (fMLP) and interleukin (IL)-8 induced neutrophil chemotaxis as well as decreased degranulation of matrix metalloprotease-9 (MMP-9), hCAP-18, and myeloperoxidase (MPO) from neutrophils, probably due to modulation of cytosolic IP_3 production and downstream Ca^{2+} influx. They suggest that SLPI could have potential therapeutic effects in neutrophilic airways diseases.

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS) are caused by several stimuli as direct (such as pneumonia, aspiration of gastric contents, and others) as indirect (such as sepsis, burns, pancreatitis, and others). No therapeutic agents have demonstrated a clear benefit in ALI treatment and corticosteroids have been used for the treatment of ALI for many years and present a range of side effects. D. C. Favarin et al. cover a wide range of studies, mainly in experimental model, in a review describing the natural compounds (extracts from medicinal plants and secondary metabolites isolated from them) with potential to be used as an alternative treatment for ALI. In another study demonstrated by S. F. Fernandez et al., airways macrophages exposures to a low pH (1.75) for 1 min resulted in reduction in all lipopolysaecharide (LPS) and lipoteichoic acid (LTA)-mediated cytokine release. In addition, they used a nonlethal experimental model of ALI from acid aspiration followed by pulmonary bacterial infection (*Escherichia coli* and *Streptococcus pneumonia*) and demonstrated reduction of bacterial clearance. They suggested a strong suppressive direct effect of low pH stress on the ability of alveolar macrophages to mount an adequate antibacterial proinflammatory response following TLR2 and TLR4 activation.

Methamidophos, an organophosphate insecticide, is a well-known toxicant, and its use is restricted during planting of vegetables. W. Watanabe et al. evaluated the developmental immunotoxicity of methamidophos using a respiratory syncytial virus (RSV) infection mouse model. Pregnant mice were exposed to methamidophos and offspring born to these dams were intranasally infected with RSV. They demonstrated that methamidophos suppressed the production of proinflammatory cytokines in the bronchoalveolar lavage fluids and the inflammation in the lung in response to RSV infection in the offspring generation of mice. So, they suggested that exposure of the mother to methamidophos during pregnancy could cause an irregular immune response in the lung tissues in the offspring mice.

Bronchiectasis is an abnormal and irreversible thick-walled dilatation of the bronchi caused by the inflammatory response of the bronchi as consequence of recurrent lower respiratory tract infections. In case-control cross-sectional

study, M. Luján et al. demonstrated that the prevalence of bronchiectasis was higher (20%) in the steroid-dependent asthma (SDA) group when compared to nonsteroid-dependent asthma (NSDA) group (4%). In addition, patients with asthma-associated bronchiectasis presented lower FEV_1 (forced expiratory volume in one second) values than patients without bronchiectasis, while the levels of IgG immunoglobulins showed no differences between the tested groups. Therefore, steroid-dependent asthma group seems to be associated with a higher risk of developing bronchiectasis than nonsteroid-dependent asthma.

Some individuals could be infected by *Aspergillus* moulds and can develop as airways as invasive diseases due to predisposing factors or defects in immune responses. In a review S. H. Chotirmall et al. covered the most important aspects involved in the airways infection caused by *Aspergillus* moulds such as clinical spectrum of disease, innate and inflammatory defenses against distinct *Aspergillus* species, and modulating the immune and inflammatory response for therapeutic purpose. In another article H. Sales-Campos et al. described the aspects immune response such as recognition, activation of innate and adaptive immune responses, pathogen virulence factors, and the development of novel therapeutic approaches for invasive lung aspergillosis.

Rheumatoid arthritis (RA) is a chronic, inflammatory condition that mainly affects joints and causes pain, erosion, and disability. In addition, interstitial lung disease (ILD) is a relevant extra-articular manifestation of RA that may occur either in early stages or as a complication of long-standing disease which was reviewed by L. Cavagna et al. The authors described the multifaceted aspects of interstitial lung disease in rheumatoid arthritis bringing important information in the field of epidemiology and prognosis, risk factors, histological and radiological patterns, and all the immunological involvement.

A. Antczak et al. demonstrated that the polymorphism of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), an immunoregulatory molecule that downregulates T-cell activation, is associated to nonsmall cell lung cancer (NSCLC). These authors showed that allelic and genotypic distribution of +49 A/G and −318 C/T CTLA-4 SNPs (single nucleotide polymorphisms) are present in a group of NSCLC patients and revealed that CTLA-4 genotype distribution shifts from one genotype in the blood to another genotype in the tumor.

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Research Article

Perinatal Exposure to Insecticide Methamidophos Suppressed Production of Proinflammatory Cytokines Responding to Virus Infection in Lung Tissues in Mice

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Methamidophos, a representative organophosphate insecticide, is regulated because of its severe neurotoxicity, but it is suspected of contaminating agricultural foods in many countries due to illicit use. To reveal unknown effects of methamidophos on human health, we evaluated the developmental immunotoxicity of methamidophos using a respiratory syncytial virus (RSV) infection mouse model. Pregnant mice were exposed to methamidophos (10 or 20 ppm) in their drinking water from gestation day 10 to weaning on postnatal day 21. Offsprings born to these dams were intranasally infected with RSV. The levels of interleukin-6 (IL-6) and interferon-gamma in the bronchoalveolar lavage fluids after infection were significantly decreased in offspring mice exposed to methamidophos. Treatment with methamidophos did not affect the pulmonary viral titers but suppressed moderately the inflammation of lung tissues of RSV-infected offspring, histopathologically. DNA microarray analysis revealed that gene expression of the cytokines in the lungs of offspring mice exposed to 20 ppm of methamidophos was apparently suppressed compared with the control. Methamidophos did not suppress IL-6 production in RSV-infected J774.1 cell cultures. Thus, exposure of the mother to methamidophos during pregnancy and nursing was suggested to cause an irregular immune response in the lung tissues in the offspring mice.

1. Introduction

Methamidophos, an organophosphate insecticide, is a well-known toxicant, and its usage is restricted during planting of vegetables [1]. Recently, it was reported that the levels of methamidophos in horticultural greenhouse workers continuously exposed to it were similar to those who ingested methamidophos-contaminated food [2]. A representative mechanism of action of methamidophos is inhibition of cholinesterase in the central nervous system, but delayed neuropathy is speculated to be due to no association with acetylcholinesterase [3]. Toxic effects of methamidophos on male reproductive organs were also reported [4]. However, except for those on the central nervous system, the cumulative effects of exposure to methamidophos, either directly or in utero, on living organism have not been elucidated clearly. To protect mothers and children from environmental contaminants including organophosphate

insecticides, the developmental immunotoxicity of methamidophos should be strictly assessed.

A novel assay system for evaluating the developmental immunotoxicity of environmental contaminants, brominated flame retardants (BFRs), using a mouse model infected with respiratory syncytial virus (RSV) has been established and reported previously [5]. RSV, a member of the family *Paramyxoviridae*, is the most prevalent agent of acute lower respiratory infections in infants and young children [6]. Because it was reported that clinically severe RSV infection is seen primarily in young children with naïve immune systems and/or genetic predispositions [7], patients with suppressed T-cell immunity [6], and the elderly [8], we applied the virus to an assay system evaluating the immunotoxicity of BFRs. Perinatal exposure to a representative BFR, decabrominated diphenyl ether (DBDE), was previously shown to increase viral titers in the lungs of RSV-infected offspring mice on day 5 after infection, resulting in exacerbation of pneumonia [9]. Moreover, perinatal exposure to DBDE was suggested to cause a functional disorder of the primary immunity responding to RSV infection [10].

In this study, we measured several parameters associated with immune response and/or inflammation in lung tissues of RSV-infected offspring to evaluate effects of maternal and postnatal exposure to methamidophos on the immune system in a murine model. Perinatal exposure to methamidophos was shown to suppress production of the specific cytokines responding to RSV infection in the lung tissues in the offspring mice.

2. Materials and Methods

2.1. Animals. Female (6 weeks old) and male (8 weeks old) BALB/c mice was purchased from Kyudo Animal Laboratory (Kumamoto, Japan) and housed at 25 ± 2°C. The mice were allowed free access to a conventional solid diet, CRF-1 (Oriental Yeast Co., Chiba, Japan), and water and used in this experiment after 7 d acclimation. The animal experimentation guideline of the Kyushu University of Health and Welfare were followed in the animal studies.

2.2. Cells and Virus. The mouse macrophage cell line J774.1 (JCRB0018) was purchased from JCRB Cell Bank (Ibaraki, Japan) and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with heat-inactivated 10% fetal calf serum. The A2 strain of RSV was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in HEp-2 cell (human epidermoid carcinoma, ATCC CCL-23) cultures.

2.3. Chemical Compound. Methamidophos was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Methamidophos was completely dissolved in tap water.

2.4. Perinatal Exposure to Methamidophos. Seven-week-old female mice and 9-week-old male mice were paired for 3 d. The day of conception was determined by observation of a vaginal plug in the female. At 3 d after conception, the females were randomly divided into three groups for methamidophos exposure at 0, 10, or 20 ppm. Each group was composed of six mice. These mice continuously ingested methamidophos in drinking water from 10 days after conception to weaning on postnatal day 21. After weaning, offspring mice were given the CRF-1 diet and tap water. On postnatal day 28, offspring mice in each group were used for an RSV infection test. Throughout these experiments, both chows and drinking water were given ad libitum, and the consumption was checked weekly.

2.5. RSV Infection Test. An RSV infection test was performed according to our previous report [5]. Briefly, four-week-old offspring mice were infected intranasally with 5×10^6 PFU of the A2 strain of RSV under anesthesia. In a mock-infected group, offspring mice were given phosphate-buffered saline (PBS) intranasally. On day 1 or 5 after infection, bronchoalveolar lavage fluid (BALF) was obtained from the mice under anesthesia by instilling 1.0 mL of cold PBS into the lungs and aspirating it from the trachea using a tracheal cannula. For a DNA microarray test of the lungs, mock- or RSV-infected mice were sacrificed by cervical dislocation on day 1 after infection, and the lungs were removed and placed in RNAlater reagent (Qiagen, Germany) and stored at 4°C until use.

For histological examination of the infected lungs, RSV-infected mice were sacrificed by cervical dislocation on day 5 after infection, and the lungs were removed and placed in buffered formalin for a minimum of 24 h. The tissue was then embedded in low-melting point paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin.

2.6. Enzyme-Linked Immunosorbent Assays. Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α levels in BALF were measured using specific ELISA kits (Ready-set-go, eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions. IL-12 levels in BALF were also measured using a specific kit (Ready-set-go, eBioscience Inc.) for IL-12 p70, without interference by the p40 monomer or the related protein IL-23, according to the manufacturer's instructions. Levels of colony stimulating factor 3 (G-CSF) in BALF were measured using specific ELISA kit (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The lower limits of detection of the kits are 8 (pg/mL) for IL-1β, 4 (pg/mL) for IL-4, 4 (pg/mL) for IL-6, 8 (pg/mL) for IL-10, 15 (pg/mL) for IL-12 p70, 4 (pg/mL) for IFN-γ, 8 (pg/mL) for TNF-α, and 14 (pg/mL) for G-CSF. The intra- and interassay coefficients of variation for the ELISA results were less than 10%.

2.7. Real-Time RT-PCR. Viral titers in lung tissues were measured by real-time RT-PCR using specific RSV primers. Briefly, RNA was isolated from lung tissues stabilized in RNAlater reagent using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Theisolated RNA was

transcribed into cDNA by ReverTra Ace α (Toyobo Co. Ltd., Osaka, Japan) using the primer (5′-ATGGCTC TTAGCAAAGTCAAGTTG-3′) for the RSV N gene region according to the manufacturer's instructions. The RSV cDNA was amplified and analyzed on a Roche LightCycler P2000 real-time PCR machine using a Roche LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Indianapolis, IN, USA) with a pair of RSV-specific primers (forward primer: 5′-AGATCAACTTCTGTCATCC AGCAAATACACCAT-3′, reverse primer: 5′-TGTTTCTGC ACATCATAATTAGGAGTATCAATA-3′). The amounts of RSV cDNA were determined by comparing the crossing point value of the cDNA sample to those of pWRSN-1, a TA vector harboring a part of the RSV N gene (nt. 1096–1347).

2.8. DNA Microarray Test. DNA microarray analysis of the lungs of mock- or RSV-infected mice was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Briefly, RNA was isolated from lung tissues using an RNeasy kit according to the manufacturer's instructions. After a quality check of the isolated RNA, cDNA was synthesized and amplified from the RNA sample using a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Exhaustive analysis of the gene expression of the sample was performed using a SurePrint G3 Mouse GE 8×60 K 1 color system (Agilent Technologies). Significant changes in the gene expression were discovered and analyzed using the software program GeneSpring (Agilent Technologies).

2.9. Assay of IL-6 Production from J774.1 Cells. Subcultured J774.1 cells were suspended in RPMI-1640 medium supplemented with 2% heat-inactivated FCS, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin (maintenance medium). One hundred microliters of J774.1 cell suspension (1×10^5 cells/mL) was seeded in each well in a 96-well microtiter plate and incubated at 37°C for 24 h in humidified air with 5% CO_2. After incubation, the culture medium was removed by aspiration and replaced by maintenance medium with or without methamidophos at 10, 30, or 100 μM. Following 24 h incubation, the cells were inoculated with RSV at three multiplicities of infection and then incubated at 37°C for 1 h. Following virus adsorption, each well was washed and maintenance medium with or without methamidophos was added. The plates were further incubated at 37°C for 3 d in humidified air with 5% CO_2. After incubation, the culture supernatant was harvested from each well, and the amount of IL-6 was measured by ELISA.

2.10. Statistical Analysis. Comparisons of body weight, food consumption, and the levels of cytokines of the controls with experimental groups were carried out using Student's t-test. Student's t-test was also used for the result for the levels of IL-6 in the culture supernatant of J774.1 cells. A P value of 0.05 or less was considered to be significant.

3. Results

3.1. Toxicological Effects of Perinatal Exposure to Methamidophos. Outline of the assay system was represented in Figure 1. In this study, general toxicological signs such as suppression of body weight gain and food consumption in dams and of body weight of offspring were monitored. Body weight of dams exposed to methamidophos was suppressed approximately 20% compared to the control (Table 1). Reduced food consumption was also observed in dams exposed to methamidophos at 20 ppm. However, no loss of body weight was detected in the offspring exposed perinatally to methamidophos (Table 1). From 5 to 8 offsprings were born to each dam in control and methamidophos-exposed groups. After weaning, no significant difference in the size of litters, survival rates, or food consumption of pups after weaning was detected between control and methamidophos-exposed groups (data not shown). No particular toxicological sign or abnormal behavior was observed in offspring mice. Then, offspring mice were infected intranasally with the A2 strain of RSV, and the following analyses were performed.

3.2. Effects of Perinatal Exposure to Methamidophos on Cytokine Production in RSV-Infected Offspring. To investigate the effects of perinatal exposure to methamidophos on the immune system of RSV-infected offspring, the levels of various cytokines in BALF were measured by ELISA on days 1 and 5 after infection (Table 2). In mock-infected offspring mice treated with or without methamidophos, the levels of cytokines in BALF were under the limit of detection (data not shown). On day 1 after infection, the levels of IL-6 in offspring mice exposed to methamidophos at 10 and 20 ppm were significantly decreased to approximately 66% ($P < 0.05$) and 50% ($P < 0.01$), respectively, of the control. But, the levels of TNF-α and G-CSF were suppressed in a dose-dependent manner, but not significant due to perinatal exposure to methamidophos. The levels of IL-1β and IL-12 were under the limit of detection. On day 5 after infection, the levels of IFN-γ, a representative marker of pneumonia due to RSV infection, in BALF from offsprings exposed to methamidophos were significantly ($P < 0.05$) decreased to approximately 54% of the control. Regarding Th2 cytokines, the levels of IL-10 were not significantly reduced due to perinatal exposure to methamidophos compared with the control, and those of IL-4 were under the limit of detection. Thus, perinatal exposure to methamidophos suppressed production of the proinflammatory cytokines IL-6 and IFN-γ in RSV-infected offspring mice.

3.3. Effect of Perinatal Exposure to Methamidophos on Severity of RSV Infection in RSV-Infected Offspring. The effect of methamidophos on the severity of RSV infection in mice was investigated. Viral titers of lung tissues in RSV-infected offspring on day 5 after infection were measured by real-time RT-PCR using the specific primer for RSV (Table 2). The viral titers in methamidophos-exposed offsprings were almost equivalent to those in the control. The effects of

Table 1: Body weights and food consumption of mice perinatally exposed to methamidophos.

Group/methamidophos (ppm)	Body weight (g)	Food consumption (g/week)
Dam		
0	28.7 ± 2.0	60.6 ± 11.5
10	22.1 ± 0.8**	59.4 ± 2.6
20	23.0 ± 2.0**	37.8 ± 12.4*
Offspring		
0	17.2 ± 1.1	0
10	16.3 ± 0.7	0
20	16.5 ± 1.7	0

The data represent mean ± standard deviation values of at least six mice. Body weight was assessed on postnatal day 21. Food consumption of each mouse was checked weekly and is expressed as a mean value ± standard deviation during methamidophos exposure.
*Significantly different from control at $P < 0.05$ (Student's t-test).
**Significantly different from control at $P < 0.01$ (Student's t-test).

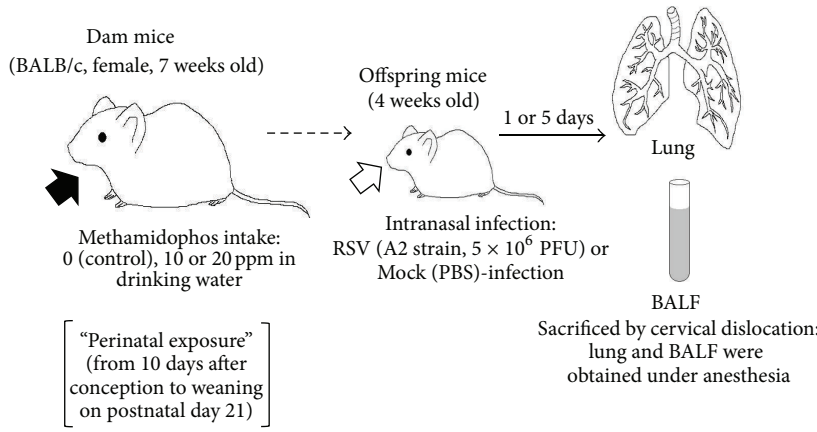


Figure 1: Schematic diagram of assay system.

perinatal exposure to methamidophos on lung tissues of RSV-infected offspring mice were analyzed histopathologically (Figures 2(a) and 2(b)). While moderate peri bronchiolar inflammation was observed on day 5 after infection in the control mouse (Figure 2(a)), slight peri bronchiolar inflammation was found in the infected mouse treated with methamidophos (Figure 2(b)). Suppression of infiltration of mononuclear cells was typically observed (Figure 2(b)). No histopathological change was found in lung tissues of mock-infected offspring mice treated with or without methamidophos (data not shown). These results indicated that perinatal exposure to methamidophos did not affect the growth of RSV but suppressed moderately the expansion of the virus-induced pneumonia in offsprings.

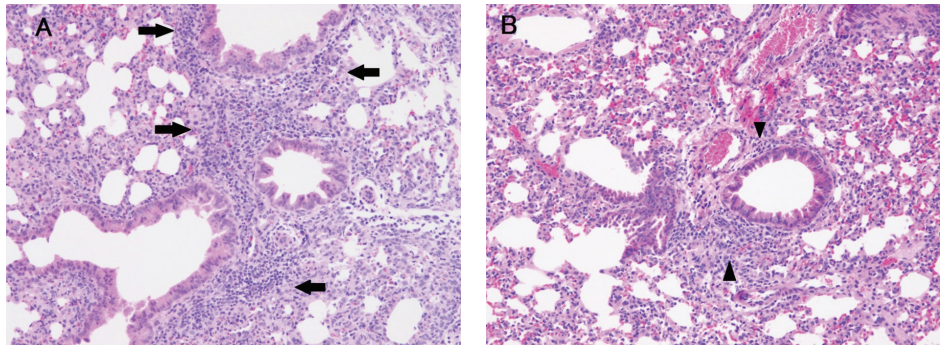
3.4. Gene Expression in Lung Tissues of Offspring. Because effects of methamidophos on the immune system in RSV-infected offspring mice were observed, a change of the gene expression in lung tissues of offspring was sought by exhaustive analysis using a DNA microarray. We integrated the data of gene expressions that changed during RSV infection compared with mock infection and then changed due to methamidophos-exposure compared with the control. Data with a more than 2-fold change of gene expression were selected and, finally, 573 genes were determined. Ten genes related to the immune and/or inflammatory system were found among them (Table 3). Due to perinatal exposure to methamidophos, expressions of seven genes were suppressed. Particularly, the expression of IL-6 and IFN-γ genes was strongly suppressed to 24% and 34%, respectively. These results accorded almost with those of cytokine levels in BALF from RSV-infected offspring (Table 2). The gene expression of G-CSF was also suppressed to 29%. Then, the level of G-CSF in BALF was suppressed in a dose-dependent manner, but not statistically significant (Table 2). Changes of gene expression of both TNF-α and IL-10 were within 2-fold and not determined in this analysis, although the levels of those in BALF were quantitative by ELISA (Table 2). Changes of gene expression of IL-1, IL-4, and IL-12 in which their levels were under the limit of detection of ELISA in BALF (Table 2), were also within 2-fold and not determined in this analysis (Table 3). Thus, perinatal exposure to methamidophos affected the expression of specific cytokine genes in lung tissues of RSV-infected offspring mice.

3.5. Effects of Methamidophos on IL-6 Production by J774.1 Cells. We evaluated the suppressive effect of methamidophos on cytokine production using the macrophage-like cell line

Table 2: Effects of methamidophos on cytokine levels in BALF and pulmonary viral titers in RSV-infected offspring mice.

Methamidophos (ppm)	Day 1 after infection					Day 5 after infection			
	Cytokine level in BALF (ng/mL)					Cytokine level in BALF (ng/mL)			Pulmonary viral titer (copy/mL)
	TNF-α	IL-6	IL-1β	IL-12	G-CSF	IFN-γ	IL-4	IL-10	
0	0.83 (0.24)	0.50 (0.10)	<0.04	<0.08	0.08 (0.01)	1.50 (0.47)	<0.04	0.26 (0.13)	3.4×10^5 (1.3×10^5)
10	0.68 (0.28)	0.33* (0.02)	<0.04	<0.08	0.07 (0.01)	1.33 (0.78)	<0.04	0.25 (0.13)	—
20	0.53 (0.18)	0.25** (0.07)	<0.04	<0.08	0.06 (0.01)	0.81* (0.38)	<0.04	0.14 (0.02)	2.5×10^5 (4.0×10^4)

Data represent mean of values of 6 mice. Numbers in parentheses indicate standard deviation of values.
*Significantly different from control at $P < 0.05$ (Student's t-test).
**Significantly different from control at $P < 0.01$ (Student's t-test).



(a) (b)

Figure 2: Lungs of mice 5 d after RSV infection. In this experiment, 6 mice per group were used, and representative data are shown. A, Control mouse with RSV infection. B, Methamidophos-treated (20 ppm) mouse with RSV infection. Marked changes in histological findings are indicated as follows: arrow, moderate infiltration of mononuclear cells; arrowhead, slight infiltration of mononuclear cells. Hematoxylin and eosin stain (×100).

J774.1 (Figure 3). The cell line was established from BALB/c mouse and has been used to produce the proinflammatory cytokines due to some stimulation such as lipopolysaccharide stimulation and virus infection [11]. The levels of IL-6 in the culture supernatant of mock-infected J774.1 cells treated with or without methamidophos were under the limit of detection of ELISA (data not shown). Treatment with up to 100 μM methamidophos did not suppress directly the production of IL-6 from RSV-infected J774.1 cells.

4. Discussion

Use of methamidophos, an organophosphate insecticide, is strictly regulated due to its severe neurotoxicity, but illegal use is suspected to cause contamination of agricultural foods in many countries. In addition, acephate, which belongs to the same category of insecticide, is used widely and converted to methamidophos by hydrolysis in animals [12]. The immunotoxicity of organophosphates is not known widely, but modulation of the immune system by organophosphates has been reported [13, 14]. We therefore evaluated the effects of exposure to methamidophos during development on the immune system response to a virus infection (Figure 1).

Before starting RSV infection, general toxicological effects of methamidophos were examined (Table 1). Suppression of body weights and food consumption was observed in dams, but no toxicological sign was seen in offspring mice. Rats were similarly exposed to methamidophos during gestation, and no particular toxicological signs including behavioral abnormality were observed in pups [15]. Prenatal exposure to methamidophos resulted in a transient decrease in cholinergic receptors, but they had returned to normal by 3-4 weeks of age in rats [16]. In our study, the contribution of methamidophos to toxicity in the nervous system might be slight in offspring mice.

In a previous study, we evaluated the effects of environmental contaminant brominated flame retardants (BFRs) on developmental immunotoxicity using an RSV infection mouse model and found that decabrominated diphenyl ether (DBDE) exacerbated pneumonia due to an increase in pulmonary viral titers and IFN-γ production in lung tissues [9]. Disorders of primary immunity, such as reduced productions of both TNF-α and IL-6, and increased those of IL-1β, were also obtained in RSV-infected offspring mice [10]. Although the level of IL-6 in BALF of RSV-infected offspring mice was significantly suppressed by methamidophos treatment, the level of IFN-γ and pulmonary viral titers were suppressed and not increased, respectively, (Table 2). In a histopathological analysis, pneumonia was moderately lessened due to perinatal exposure to methamidophos (Figure 2). Particularly, the inflammatory was marked by suppression of infiltration of mononuclear cells, such as lymphocyte and monocyte/macrophage (Figure 2(b)). This might be consisted in the low levels of the proinflammatory cytokines in BALF

Table 3: Effects of perinatal exposure to methamidophos on gene expression in lung tissues of RSV-infected offspring mice by microarray analysis.

Fold change	Gene name	GenBank accession no.
4.50	Membrane-associated ring finger (C3HC4) 1	NM_175188
4.42	C-type lectin domain family 4, member a2	NM_001170333
3.26	Lipoic acid synthetase	AK013575
0.34	Interferon gamma	NM_008337
0.29	Colony stimulating factor 3 (granulocyte)	NM_009971
0.27	CD1d2 antigen	NM_007640
0.27	Interleukin 18 receptor accessory protein	NM_010553
0.24	Interleukin 6	NM_031168
0.22	Chemokine (C motif) ligand 1	NM_008510
0.14	Regenerating islet-derived 3 gamma	NM_011260

Data with more than 2-fold change in gene expression compared with RSV-infected control are included.
Ten genes related to the immune and/or inflammatory system were selected using the software GeneSpring.

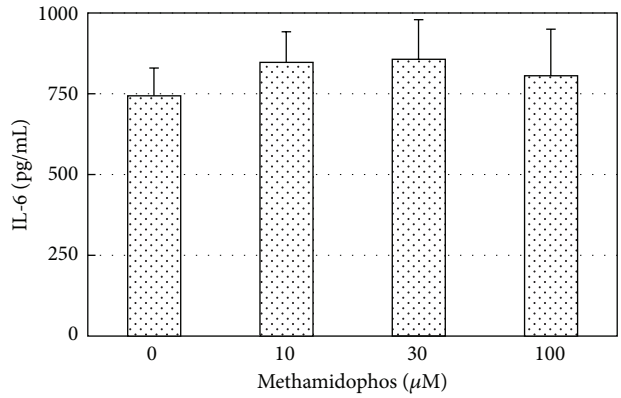


Figure 3: Effects of treatment of methamidophos on IL-6 production from RSV-infected J774.1 cells. J774.1 cells were incubated at 37°C for 24 h with or without methamidophos at 10, 30, or 100 μM. After incubation, the cells were infected with RSV at three multiplicities of infection and further incubated with or without methamidophos. Following 3 d incubation, the culture supernatant was harvested and the amount of IL-6 was measured by ELISA. Data represent mean ± standard deviation (vertical bars).

(Table 2), because IL-6 and IFN-γ activate T lymphocyte and macrophage, respectively.

To clarify the mechanism of action of methamidophos on the immune system in RSV-infected offspring mice, a DNA microarray test was performed on day 1 after infection (Table 3). It was verified that gene expressions of both IL-6 and IFN-γ were strongly suppressed in the lung tissues of RSV-infected offspring treated with methamidophos. Although gene expression of G-CSF was also suppressed, the results for the level of protein were not statistically significant (Table 2). This discrepancy may consist in the low levels of the cytokine in BALF. It is possible that methamidophos affected production of the cytokine in endothelial cells and monocytes/macrophages remaining in lung tissues of offsprings. Moreover, G-CSF may work in the initial phase of RSV infection, such as within 8 hours after infection. In a DNA microarray test, TNF-α and IL-10 were not selected (Table 3), although the levels of them in BALF were clearly detected in RSV-infected offspring (Table 2). Methamidophos might affect the process of RSV antigen and induced subsequently the suppression of production of TNF-α in macrophages [10]. As for IL-10, change of gene expression of the cytokine occurred probably in late phase responding to RSV infection. Suppression of gene expression of IL-18 receptor accessory protein might contribute to reduction of pneumonia in RSV-infected offspring mice treated with methamidophos (Table 3), because IL-18 is an inducer of IFN-γ. IFN-γ is a common marker of the progression of RSV-induced pneumonia in humans and mice [9, 10]. Although it is controversial whether the cytokine exacerbates the pneumonia [17], repression of progression of pneumonia due to methamidophos exposure might be due to suppression of IFN-γ production.

The *in vitro* experiment using J774.1 cell culture showed that methamidophos did not suppress RSV-induced production of IL-6, suggesting that the compound worked on the immune system in an indirect manner *in vivo* (Figure 3). Thus, perinatal exposure to methamidophos suppressed indirectly productions of proinflammatory cytokines due to reduction of their gene expressions, resulting in moderate repression of pneumonia without affecting the RSV growth in the lung tissues of RSV-infected offspring mice.

In contrast to environmental contaminant BFRs [9, 10], developmental exposure to methamidophos did not degenerate pneumonia and suppressed the production of the proinflammatory cytokines in response to RSV infection in the lung tissues in the offspring generation of mice. However, it is doubtful that methamidophos only affects the proinflammatory cytokines. Therefore, further studies on the other immune responses, including humoral immunity, are needed. Results for food contaminant methamidophos on immune system will be useful in the health sciences to manage risk in both mothers and children. Recently, an outbreak of food poisoning due to consumption of Chinese dumplings that had been intentionally contaminated by methamidophos was reported [18]. Thus, to protect the health of mothers and children from threatening environmental contaminants such as organophosphates, the mechanism of action of methamidophos on suppression of the proinflammatory cytokines presented in this study should be elucidated as soon as possible.

5. Conclusion

Perinatal exposure to methamidophos, a representative organophosphate insecticide, suppressed the production of proinflammatory cytokines in response to RSV infection in the lung tissues in the offspring generation of mice.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Clinical Study

Prevalence of Bronchiectasis in Asthma according to Oral Steroid Requirement: Influence of Immunoglobulin Levels

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Purpose. To establish the prevalence of bronchiectasis in asthma in relation to patients' oral corticosteroid requirements and to explore whether the increased risk is due to blood immunoglobulin (Ig) concentration. *Methods*. Case-control cross-sectional study, including 100 sex- and age-matched patients, 50 with non-steroid-dependent asthma (NSDA) and 50 with steroid-dependent asthma (SDA). Study protocol: (a) measurement of Ig and gG subclass concentration; (b) forced spirometry; and (c) high-resolution thoracic computed tomography. When bronchiectasis was detected, a specific etiological protocol was applied to establish its etiology. *Results*. The overall prevalence of bronchiectasis was 12/50 in the SDA group and 6/50 in the NSDA group (p = ns). The etiology was documented in six patients (four NSDA and two SDA). After excluding these patients, the prevalence of bronchiectasis was 20% (10/50) in the SDA group and 2/50 (4%) in the NSDA group ($P < 0.05$). Patients with asthma-associated bronchiectasis presented lower FEV_1 values than patients without bronchiectasis, but the levels of Ig and subclasses of IgG did not present differences. *Conclusions*. Steroid-dependent asthma seems to be associated with a greater risk of developing bronchiectasis than non-steroid-dependent asthma. This is probably due to the disease itself rather than to other influencing factors such as immunoglobulin levels.

1. Introduction

Bronchiectasis is defined as abnormal, irreversible thick-walled dilatation of the bronchi and represents the end stage of a variety of pathological processes. Caused by the inflammatory reaction of the bronchi and their frequent chronic bacterial colonization, bronchiectasis usually presents with recurrent lower respiratory tract infections and chronic mucopurulent sputum production.

Bronchiectasis often goes unrecognized, even when characteristic features are present and appropriate diagnostic techniques are readily available. In a recent study the etiology of the condition was identified in only 57% of patients [1]. The etiological spectrum of the disease has changed notably since it was first described. In the preantibiotic era, bronchiectasis was almost exclusively due to untreated or suboptimally treated lung infections, including pneumonia or tuberculosis [2]. Today, although the majority of identified causes of

bronchiectasis are infections (24% [2, 3] and 29% [4]), associations have been reported with new entities like adulthood cystic fibrosis [5, 6], humoral immunodeficiency including common variable immunodeficiency [7, 8], and systemic diseases [9]. On the other hand, despite the improvements in the control of pulmonary infectious diseases, the incidence of bronchiectasis does not seem to be declining—perhaps because the widespread use of high-resolution chest tomography (HRCT) has allowed easy diagnosis of its clinically silent minor forms. The use of CT to diagnose bronchiectasis has been validated in various studies [10]. Overall, today, the etiology is identified in fewer than 50% of patients with the condition [4, 11].

Several studies have attempted to establish an association between asthma and bronchiectasis [12–14]. Recently, in a study of 1680 patients with asthma, around 3% had radiographic bronchiectasis, and around 50% of patients with bronchiectasis had severe asthma. Unfortunately, the authors did not rule out other causes of bronchiectasis [15]. In this connection, a role for immunoglobulin (Ig) and IgG subclass deficiency, classically associated with the development of bronchiectasis [7, 8], has been suggested in certain cases of bronchial asthma [16, 17].

The aim of the present study was to compare the prevalence of bronchiectasis in a cohort of patients with severe steroid-dependent asthma (SDA) and in another with non-steroid-dependent asthma (NSDA), in order to establish whether the SDA group has an increased associated risk of developing bronchiectasis. We also determined the Ig and IgG subclass blood levels in an attempt to correlate them with the development of bronchiectasis in either group.

2. Material and Methods

The study was performed between 2004 and 2011 at the Corporació Sanitària Universitària Parc Taulí, a 760-bed university hospital with a reference population of 400,000 inhabitants. The study design was a single-center, prospective case-control study. Each patient in the SDA group was matched for age and sex status with one in the NSDA group. Patients were consecutively recruited from our institution's asthma out-patient clinic [18] and complete patient evaluation was performed prospectively and lasted less than three months per patient.

2.1. Patients

2.1.1. Inclusion Criteria. All patients were nonsmokers and were treated in accordance with the severity of their disease following international guidelines [19].

2.1.2. NSDA (GINA Steps I to IV). The maximum treatment received by NSDA patients was salmeterol 50 μg bid, fluticasone 500 μg bid, salbutamol as rescue medication, and oral antileukotrienes. None had received oral steroids during the year prior to inclusion in the study.

2.1.3. SDA (GINA Step V). In addition to the treatment just described, SDA patients were also given oral steroids at least 7.5 mg/day of oral prednisone (or equivalent). No patient was receiving immunomodulatory treatment [20–22] since in accordance with our policy these treatments are not prescribed to our patients until corticodependence is verified [23].

A stabilization period of three months was required before admission, during which the FEV_1 did not change more than 5% and patients did not report any deterioration in their clinical symptoms. Failure in full-tapering of corticosteroids for at least three months was also required.

2.2. Study Protocol. A general protocol was applied to the entire study population (general instrumentation) to establish the prevalence of bronchiectasis and, in a second step, a specific etiologic protocol to determinate the cause(s) of bronchiectasis in patients in which this entity was documented (specific instrumentation).

2.2.1. General Instrumentation. The following tests were performed in all patients. (a) Blood analysis was performed including red and white blood cell count, platelet count, renal, hepatic biochemical parameters, rheumatoid factor, autoantibody screening test and determination of IgG, IgM, and IgA by nephelometry, and IgE. IgG subclass levels were determined by radiated immunodiffusion. (b) Forced spirometry with bronchodilator test was performed in accordance with local guidelines [24] (MedGraphics system 1070 Series 2E/1085). The bronchodilator test was considered positive when the FEV1 increased by 12% and 200 mL. Reference values for the Mediterranean population were used [25], and normal spirometry was defined as FEV1 $\geq$ 80% of predicted value and FEV1/FVC $\geq$ 0.7. (c) Multidetector CT scanner (Sensation 16; Siemens, Erlangen, Germany) that acquires thin-slice images of the whole chest, with 0.5–1 mm thick slices, during a single breathhold was performed. These scanners produce true isotropic voxels, allowing image reconstructions in which the Z dimension (slice thickness) is the same dimension as the X and Y (in plane) resolution. A radiologist skilled in HRCT and blind to the study interpreted the scans, classifying patients as having or not bronchiectasis and assessing the severity of the bronchiectasis in accordance with Reiff et al.'s classification [26]. Following this score, each lung was evaluated according to individual lobes, with the lingula being considered a separate lobe. Each lobe was scored for extent of involvement: 0 = none, 1 = involvement of one or a partial segment, 2 = involvement of two or more segments, and (d) Skin prick test was performed.

2.2.2. Specific Instrumentation. This part of the protocol was only performed in patients who had shown radiological evidence of bronchiectasis ruling out other causes of the disease: (a) clinical assessment to detect chronic sputum production and remote infections in childhood or prior to the onset of asthma symptoms; (b) blood analysis: alpha-1-antitrypsin deficiency, precipitins for aspergillus, and genetic

Table 1: Spirometric and Ig/IgG subclass plasmatic values in SDA and NSDA patients.

		SDA	NSDA	P
Forced spirometry	FEV1 L (%)	1.52 ± 0.53 (52.1 ± 18.6)	2.02 ± 0.81 (78.13 ± 21.09)	0.01 0.01
	FVC L (%)	2.70 ± 0.67 (82.62 ± 15.96)	2.92 ± 1.06 (84 ± 15.97)	Ns
	FEV1/FVC %	56.27 ± 12.47	69.38 ± 13.16	<0.01
Ig (mg/dL)	IgA (N = 70–400)	207 ± 114	255 ± 105	0.03
	IgM (40–230)	152 ± 137	113 ± 56	ns
	IgG (700–1600)	784 ± 226	1032 ± 318	<0.001
IgG subclass (mg/dL)	IgG1 (N = 550–1270)	485 ± 177	586 ± 241	0.01
	IgG2 (N = 130–700)	267 ± 108	334 ± 144	0.01
	IgG3 (N = 21–180)	58 ± 28	87 ± 78	0.01
	IgG4 (N = 20–150)	23 ± 16	29 ± 33	ns

profile of cystic fibrosis; (c) sweat test; (d) nasal potential difference; (e) saccharin test; and (f) seminogram in males.

2.2.3. Classification of Patients according to the Specific Protocol. In patients with a diagnosis other than asthma, the bronchiectasis was attributed to this alternative diagnosis. These patients were classified as having non-asthma-related bronchiectasis and were excluded from the study. Bronchiectasis in patients without alternative etiological diagnosis was considered asthma to be related to. The alternative diagnostics were defined as follows: (a) alpha-1-antitrypsin deficiency was diagnosed if serum levels were below 80 m/dL; (b) common variable immunodeficiency: presence of IgG or subclass deficiency (below 700 mg/dL for total IgG, and 550, 130, 21 and 20 mg/dL for IgG1 to IgG4 subclasses, resp.) with IgA or IgM levels below normal lower limit (below 70 and 40 mg/dL, resp.); (c) adulthood cystic fibrosis: presence of a mutation associated in the literature with cystic fibrosis with altered sweat test; (d) bronchopulmonary allergic aspergillosis: central bronchiectasis plus positive precipitins for aspergillus; (e) postinfectious bronchiectasis: medical history of documented pulmonary infection (tuberculosis or not) in the site of bronchiectasis.

2.3. Statistical Methods

2.3.1. Sample Size Calculation. Since the only reliable data on the prevalence of bronchiectasis in steroid-dependent asthmatic patients were reported in our earlier pilot study [27], we calculated the sample size from our preliminary results. We also assumed that the prevalence in the non-steroid-dependent group would be very low (probably similar to the general population) and calculated the sample size assuming a prevalence of 20% in the SDA group and 4% in the NSDA group, an alpha risk of 0.05 and a beta risk of 0.20. In a unilateral contrast, we needed 48 patients in each branch. We estimated a loss rate <0.05 and therefore included 100 patients divided into two groups of 50 according to their oral steroid requirements. An interim analysis was not planned. For the sample size calculation we used the Gramno program (IMIM-Barcelona).

2.3.2. Data Entry and Analysis. Statistical Package for the Social Sciences (SPSS version 19.0) was used for data entry and analysis. The results are given as mean (SD) values. Statistical comparisons between groups were performed using the two-tailed unpaired Student's t test for continuous variables. The Chi-squared test was used to compare proportions between the two groups. To detect the risk of developing the disease a stratified analysis (Mantel and Hanszel method) was used and a stepwise regression logistic analysis was performed later to detect independent factors for developing bronchiectasis; the model included all variables with $P < 0.1$ in the univariate analysis.

The protocol was approved by the Ethics Committee of our Institution, and informed consent was obtained from each participant.

3. Results

Fifty patients were included in the SDA group and 50 age- and sex-matched controls in the NSDA. None of them had a history of tobacco exposure. No differences in age (56.9 ± 13.1 for controls and 57.8 ± 10.8 for cases, p = ns) or sex (M/F 15/35 for each group) were found. Time from clinical diagnosis to inclusion in the study was 23.7 ± 14.5 years in the SDA group and 16.02 ± 11.4 years in the NSDA group ($P < 0.05$). The mean daily steroid dose in SDA group was 14.9 ± 8 mg of methylprednisolone/day or equivalent.

3.1. Spirometry and Immunology. As stated in Table 1, the SDA group presented more severe obstruction than the NSDA group. Moreover, 38% of patients (19/50) presented normal spirometric values in the NSDA group and only 4/50 (8%) in the SDA group ($P < 0.01$).

3.1.1. Immunology. Mean values of IgA, IgG, and IgG subclasses 1, 2, and 3 were lower in the SDA group. We did not detect combined deficiency of IgG (or subclasses) with low IgA or IgM values corresponding to a common variable immunodeficiency (Table 1).

Table 2: Comparison of demographic conditions, spirometric values, and plasmatic values of Ig and IgG subclass in patients with and without asthma-associated bronchiectasis.

		Asthma-associated bronchiectasis ($n = 12$)	Non-asthma-associated bronchiectasis ($n = 88$)	P value
Baseline conditions	Age	64.5 ± 8	56.4 ± 12.4	<0.05
	Time from diagnosis (yr)	30.14 ± 7.2	16.98 ± 12.4	<0.05
Spirometric values	FEV1 (L)	1.31 ± 0.51	1.82 ± 0.73	<0.05
	FEV1 %	61.9 ± 29.9	70.9 ± 19.8	ns
	FVC (L)	2.51 ± 0.65	2.85 ± 0.91	ns
	FVC %	83.7 ± 20.9	83.2 ± 15.2	ns
	FEV1/FVC	52.7 ± 15.9	63.9 ± 13.6	0.01
Ig (mg/dL)	IgA	182 ± 74	237 ± 114	ns
	IgM	309 ± 124	110 ± 53	<0.01
	IgG	812 ± 198	920 ± 310	ns
IgG subclass (mg/dL)	IgG1	472 ± 105	544 ± 226	ns
	IgG2	290 ± 128	302 ± 132	ns
	IgG3	63 ± 30	74 ± 63	ns
	IgG4	19 ± 17	26 ± 26	ns

3.1.2. Diagnosis of Bronchiectasis. Twelve out of 50 patients in the SDA group were diagnosed with bronchiectasis by the HRCT scan. Severity of the condition according to the classification of Reiff et al. [26] was 4.17 ± 3.29 points. In the NSDA group, six patients had bronchiectasis (p = ns in comparison with SDA group), with a score of 3.17 ± 1.16 on the classification of Reiff (p = ns).

Applying the etiology study protocol, an alternative diagnostic etiology was found in six cases: two of them in the SDA group (both adult cystic fibrosis) and four in the NSDA group (one tuberculosis, one bronchiectasis postpneumonia, and two cases of adult cystic fibrosis). After ruling out those cases with a definitive etiology of bronchiectasis, the prevalence of asthma-associated bronchiectasis was 10/50 (20%) in the SDA group and 2/50 (4%) in the NSDA group ($P < 0.05$). 11/12 (91%) of patients with asthma-associated bronchiectasis were documented in patients with abnormal spirometry (10 in the SDA group and 1 in the NSDA group, $P < 0.05$).

Table 2 compares the patients with asthma-related bronchiectasis ($n = 12$) and those patients without ($n = 88$). Patients with asthma-associated bronchiectasis were older, with longer-term asthma history, and had lower FEV_1 and FEV_1/FVC values. Conversely, the IgG, IgA, and IgG subclasses mean values did not differ between patients with and without asthma-associated bronchiectasis. Only IgM mean values were higher in the patients with bronchiectasis. Using normality cut-off points, 7 patients in the nonbronchiectasis group and 2 in the bronchiectasis group presented isolated low values of IgG (p = ns), and only 5 patients with isolated IgA deficiency were found (4 in nonbronchiectasis and 1 in bronchiectasis, p = ns). Moreover, comparing patients in the SDA group with asthma-related bronchiectasis ($n = 10$) with the remaining forty patients without, neither the daily steroid dose nor the levels of immunoglobulins and IgG subclasses (with the exception of IgM values) were associated with the presence of bronchiectasis (data not shown).

Finally, after adjusting for immunoglobulin levels, a stepwise regression logistic analysis showed that only age (aOR 1.08, 95% CI: 1.01–1.16) and steroid dependence (aOR 6.9, 95% CI: 1.35–35.09) were associated with the presence of asthma-related bronchiectasis.

4. Discussion

To establish the prevalence of a pathology, a preliminary appraisal using a cross-sectional study is required. Patient selection and sample size estimation must be performed with care. Given the lack of reliable data in the literature, we based our calculations of the sample size on our earlier pilot study [27]. To control the confounding variables and to increase the efficiency of the study, each patient with steroid-dependent asthma was matched with a patient from the non-steroid-dependent group of the same race, sex, and age range to ensure the comparability of the groups. There were no differences in terms of age or sex. Obviously, the degree of bronchial obstruction differed: SDA patients presented moderate/severe obstruction while in NSDA patients FEV_1 values were almost normal.

From the point of view of the methodology used to diagnose bronchiectasis and its causes, our study presents two innovative features. First, we established the diagnosis using a HRCT scan in stable patients. The original CT scans designed to assess airway structure involved thin-slice images (typically 1-2 mm axial), which were acquired using a "stop and shoot" protocol and were reconstructed using an edge-enhancing algorithm. Conversely, the high-resolution CT (HRCT) protocol allows the measurement of the airways

in true cross section at any location, using retrospective reconstruction of the images to achieve a cross-sectional image of the airway [28].

Second, after confirming the diagnosis, we performed an etiological diagnosis of bronchiectasis applying our pneumology service's protocol which includes innovative techniques for screening infrequent illnesses [29, 30] such as the determination of nasal potential difference [31], an examination now accepted worldwide as a diagnostic criterion for cystic fibrosis. So the comparison of proportions was carried out among patients with bronchiectasis correctly diagnosed by HRCT scan in whom other etiological causes of bronchiectasis had been ruled out.

To evaluate bronchiectasis severity, we used the easily reproducible CT grading system described by Reiff and coworkers [26]. The finding of at least two lobes and a minimum of three segments affected in bronchiectasis patients supports the idea that asthma causes a generalized inflammation of the airways. As can be seen, though SDA patients had higher scores, no statistical differences were found between groups.

The literature review takes us back to the 1960s, when Dunnill's autopsy findings recorded the presence of bronchiectasis in 4/20 (25%) asthmatic subjects [12]. For many years Dunnill's results were regarded as unreliable since they were obtained from necropsies of patients who died in status asthmaticus, a situation that increases the presence of mucous plugs which disrupt and dilate the airway wall. Years later, authors studying lung parenchyma of asthmatic patients using HRCT found that asthmatics present more radiological abnormalities than normal subjects, such as bronchial dilatation and even bronchiectasis, probably related to permanent airway remodeling.

Though some of these studies used HRCT scan, the method and/or the moment used for accepting the diagnosis of bronchiectasis were often inadequate, or no etiological differential diagnosis of bronchiectasis was performed. Consequently a wide range of prevalence results have been found. Paganin et al. [13] reported a prevalence of cylindric bronchiectasis of up to 80% in severe nonallergic asthmatics. Similarly, Lynch et al. [14] found bronchial dilatation in 77% of compensated asthmatic subjects. Since these findings have not been considered reliable, recent investigations [11] of the causative factors of bronchiectasis have excluded bronchial asthma from the disease's etiological spectrum.

In a recent study, Anwar et al. [1] found asthma to be the aetiology of bronchiectasis in 3% of the cases. Oguzulgen et al. [15] found that asthmatics with bronchiectasis mostly had severe persistent asthma (49.0%), while pure asthmatics mostly had mild persistent and intermittent asthma (69.4%). More recently, in a retrospective data based study, Bisaccioni et al. [32] found that among the 245 asthma patients evaluated in whom CT scans of the chest were taken for 105 patients, 26 (24.8%) showed bronchiectasis. Only 2.5% of their cohort was taking oral steroids, but 98.7% had an abnormal lung function and 94.6% were considered uncontrolled. Thus, many of these patients (probably the majority) were probably SDA patients who at that moment were not receiving oral steroids. Thus, our study is the first to establish a reliable prevalence of bronchiectasis in both stabilized SDA (18%) and NSDA (4%) obtained after ruling out causes other than asthma. The prevalence of bronchiectasis in SDA we found is thus probably in concordance with Bisaccioni's results [32]. Furthermore, using a comparison of proportions, we were able to establish the increased risk of developing bronchiectasis in the SDA group compared with the NSDA group.

Regarding the PFT results, we observed that the degree of airway obstruction was more severe in SDA than in NSDA. Asthma and bronchiectasis coexist in many patients, and it has been shown that bronchiectasis can contribute to severe and difficult-to-control asthma with pulmonary complications [33]. In this population, PFTs are frequently abnormal [32]. Therefore, our results are as expected.

In patients with asthma, especially in those with severe disease, several studies have reported a high incidence of Ig and IgG subclass deficiency and suggest that this may play a role in the pathogenesis of the disease [16, 17]. We observed that the level of immunoglobulins was lower in the SDA group, lower even than the normal values found in our laboratory. This may be because the steroids themselves lower the IgG_1 values (the most abundant fraction of the IgG subclasses) and thus the total IgG value as well. However, comparing the immunoglobulin values in patients with asthma-related bronchiectasis with those without bronchiectasis there were no significant differences; the only relevant finding was the higher IgM value in patients with bronchiectasis, possibly because they had presented infection more recently. Therefore, Ig values do not appear to play an important role in the pathogenesis of bronchiectasis in asthmatic patients.

The prevalence of bronchiectasis is statistically higher in SDA than in NSDA. Steroid dependency is associated with a higher risk of developing bronchiectasis, and the development of the condition is not due to the circulating levels of immunoglobulins. So it seems logical to attribute this increased risk to the evolution of steroid-dependent asthma, with higher levels of inflammation and bronchial remodeling, higher levels of formation of thick, difficult-to-clear mucous plugs, and progressive obstruction of the airway with a low degree of reversibility.

The study has several limitations. The first is the fact that we do not know the prevalence of bronchiectasis in the general nonsmoking population and in NSDA. For this reason we assumed a high prevalence (4%), to be sure that the calculation of the sample size was sufficient. The second is the selection bias in patient recruitment. The SDA patients were recruited from the study of difficult-to-control asthma carried out at our hospital [18] and the NSDA patients from the pneumology service at our institution. In the SDA group there is no chance of a selection bias, but in the NSDA it may be that the patients assessed, though not steroid dependent, presented higher severity than other apparently equally severe cases treated in primary care. If so, the prevalence of bronchiectasis obtained in this group would be greater than the real figure. However, this would not affect the significance of our results, and the value obtained for the increased risk of developing bronchiectasis in the SDA

group would still be valid. In addition, asthma symptoms had lasted longer in the SDA group than in NSDA. But although the duration of the disease differs in the two groups, we stress that in the NSDA group the mean duration period was also very long (16 years), which may have given these patients the opportunity to develop bronchiectasis. Finally, gastroesophageal reflux (GER) may be present in patients with asthma [32]. Although rare, GER can be the causative factor of bronchial aspirations. No special testing to rule out GER was performed, but, when present, bronchial aspirations are not usually the causative agent of bronchiectasis. Thus we do not believe that this entity played an important role in our cohort of patients.

Therefore, after having ruled out other potential causative factors, we believe that our results show a clear association between SDA and bronchiectasis that is related not to the immunological situation of the patient but probably to the disease itself. Future studies should explore whether the risk of infection and colonization by germs that are more frequent in patients with bronchiectasis than in patients with asthma is higher in SDA patients.

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Research Article

Low pH Environmental Stress Inhibits LPS and LTA-Stimulated Proinflammatory Cytokine Production in Rat Alveolar Macrophages

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Gastric aspiration increases the risks for developing secondary bacterial pneumonia. Cytokine elaboration through pathogen recognition receptors (PRRs) is an important mechanism in initiating innate immune host response. Effects of low pH stress, a critical component of aspiration pathogenesis, on the PRR pathways were examined, specifically toll-like receptor-2 (TLR2) and TLR4, using isolated rat alveolar macrophages (aMØs). We assessed the ability of aMØs after brief exposure to acidified saline to elaborate proinflammatory cytokines in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) stimulation, known ligands of TLR4 and TLR2, respectively. Low pH stress reduced LPS- and LTA-mediated cytokine release (CINC-1, MIP-2, TNF-α, MCP-1, and IFN-β). LPS and LTA increased intracellular Ca^{2+} concentrations while Ca^{2+} chelation by BAPTA decreased LPS- and LTA-mediated cytokine responses. BAPTA blocked the effects of low pH stress on most of LPS-stimulated cytokines but not of LTA-stimulated responses. *In vivo* mouse model demonstrates suppressed *E. coli* and *S. pneumoniae* clearance following acid aspiration. In conclusion, low pH stress inhibits antibacterial cytokine response of aMØs due to impaired TLR2 (MyD88 pathway) and TLR4 signaling (MyD88 and TRIF pathways). The role of Ca^{2+} in low pH stress-induced signaling is complex but appears to be distinct between LPS- and LTA-mediated responses.

1. Introduction

Gastric aspiration pneumonitis is a major cause of death and disability. An important complication of this acute lung injury is the development of a secondary pulmonary bacterial pneumonia from inhalation of oropharyngeal or gastric bacterial flora. Patients that are specifically susceptible to gastric aspiration pneumonitis are those with impaired airway defense reflexes such as anesthetized patients or patients with altered mental status. Consequences of low pH gastric aspiration can range from benign aseptic pneumonitis to acute respiratory distress syndrome (ARDS) with or without a fulminant secondary bacterial infection. It is estimated that complications from gastric aspirations can be seen in 1 of every 2,000–4,000 anesthetic cases every year and possibly 10–15% of all community acquired pneumonia [1, 2].

Common bacterial organisms implicated in aspiration pneumonia include *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae*, and Enterobacteriaceae [1]. Although gastric components are highly variable with varying proportions of gastric particles, acidity, and bacterial load, it appears that the extent of pulmonary injury is directly related to the aspirate volume, acidity, and composition [3, 4]. In rat models of gastric aspiration injury, intratracheal administration of acidified solution (pH of 1.25) results in an early direct corrosive irritation to the airway epithelium

followed by a delayed inflammatory response (4–6 hours after exposure) characterized by neutrophil activation and extravasation into the air spaces and elaboration of proinflammatory mediators and chemotactic cytokines [3, 5, 6]. Gastric aspiration is an important risk factor for bacterial pneumonia [7]. Using an *in vivo* rat model, our laboratory has demonstrated that a preceding gastric aspiration injury results in reduced clearance of *Escherichia coli* instilled into the lungs [7]. The mechanism by which transient pulmonary low pH stress promotes bacterial infection is the focus of this investigation.

Our major hypothesis is that a transient low pH environment resulting from gastric aspiration adversely affects alveolar macrophage (aMØ) antimicrobial function. Using isolated rat aMØs, we investigated the effects of a transient low pH exposure in impairing the ability of these cells to express cytokines in response to ligation of pathogen recognition receptors (PRRs). Macrophages were stimulated, *in vitro*, with the pathogen associated molecular pattern (PAMP) ligands of *E. coli* lipopolysaccharide (LPS) or pneumococcal lipoteichoic acid (LTA), known ligands of toll-like receptor (TLR) 4 and TLR2, respectively. TLRs are PRRs utilized by immune sentinel cells to recognize microbial PAMPs to illicit antibacterial proinflammatory responses [8]. The aMØs' response to receptor ligation was quantified based on protein levels of cytokine-induced neutrophil chemoattractant-1 (CINC-1), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-α (TNF-α), macrophage chemoattractant protein-1 (MCP-1), and interferon-β (IFN-β) in the culture supernatant, as assessed by ELISA. The role of cytosolic Ca^{2+} in mediating the effects of low pH stress was also investigated [9, 10] using the Ca^{2+}-sensitive dye, Fura-2, in single cell imaging studies or the Ca^{2+} chelator, BAPTA, in cell culture stimulation studies. These findings will help identify the mechanisms by which gastric aspiration can alter MØ function and modify innate immune responses that may predispose patients to secondary pulmonary bacterial infections.

2. Materials and Methods

2.1. Rat Alveolar Macrophage Isolation. Bronchoalveolar lavage (BAL) was performed on naïve Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250–390 g to isolate alveolar macrophages for *in vitro* experiments. Briefly, rats were anesthetized with 2-3% halothane in 100% O_2 to full anesthetic effect and then exsanguinated by transecting the inferior vena cava. After thoracotomy, the thoracic and pulmonary vasculatures were flushed using 20 mL of Hank's balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS) injected directly into the right ventricle. A 14-gauge catheter was inserted and secured in the trachea and BAL performed with 50 mL of normal saline instilled in 10 mL aliquots. The instilled saline solution was recovered and kept in ice until processing.

2.2. Cell Plating. The recovered BAL fluid was centrifuged at 1,500 ×g for 3 min at 4°C and the cell-free supernatant discarded. The cell pellet was resuspended in 4 mL of culture media (RPMI-1640, Life Technologies, Carlsbad, CA), containing 10% heat-inactivated fetal calf serum and penicillin and streptomycin. A cytoslide was prepared with 5×10^4 cells plated onto a slide and centrifuged at 28 ×g (Cytospin 3 cytocentrifuge, Shandon, Pittsburgh, PA) for 5 min, air dried, and stained with Diff-Quik (Dade Behring, Newark, DE). Microscopic assessment demonstrated that plated cells were ≥98% macrophages. Alveolar macrophages were >95% viable prior to plating as determined by Trypan Blue exclusion. Cells were resuspended in warm culture media and seeded in poly-L-lysine-coated 24-well polystyrene tissue culture plates at a density of 2×10^5 cells/well, 500 μL/well for ELISA studies, or 200 μL/well in 96-well plates for WST-1 cell viability (Roche Diagnostics, Indianapolis, IN) studies. Cells were incubated at 37°C, 95% RH, and 5% CO_2 (all incubations were performed under these conditions unless otherwise noted) for 20–22 hrs to allow cells to attach and quiesce.

2.3. Treatment Conditions. Plated cells were washed with an unbuffered balanced salt solution (UBSS, 144 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$); then 100 μL/well of appropriate injury solution, UBSS (uninjured control) or UBSS + 1 N HCl, pH = 1.75 (acidic injury), was added to each well. After 1 min of injury exposure, 400 μL of culture media was added (neutralizing the injury solution) and then removed and replaced with 500 μL/well fresh culture media containing 1.5 μg/mL LPS, 30 μg/mL LTA, or media alone (nonstimulated control). The cultures were incubated, as above, for 24 hrs and the supernatant collected and stored at −80°C for subsequent analysis. A pH = 1.75 was specifically chosen to represent the most acidic injury that did not cause significant reduction in macrophage viability. This was based on a study examining a 1 min exposure to a range of pH from 1.25 to 3.00 using a WST-1 cell viability assay (Roche Diagnostics, Indianapolis, IN), a colorimetric measure of cellular metabolic activity. Incubation with pH 1.75 for 1 min was established as the most appropriate pH to maximize low pH injury without producing cell death. For intracellular calcium sequestration experiments, macrophages were incubated with 5 μM BAPTA-AM (an intracellular Ca^{2+} chelator) in culture media (Life Technologies, Grand Island, NY) for 30 min prior to initiating the acidic injury and LPS or LTA stimulation procedures.

2.4. Cytokine Analysis. Cell-free culture media were analyzed by ELISA to determine concentrations of CINC-1, MIP-2, and MCP-1 using their respective antibody pairs and recombinant protein standards from R&D Systems (Minneapolis, MN) and IFN-β from USCN Life Sciences (Houston, TX), as previously described [11]. TNF-α levels were assessed by cytotoxicity bioassay using WEHI 164, subclone 13 cells (a generous gift from Dr. Steven L. Kunkel, Department of Michigan, Ann Arbor, MI). This cell line is a TNF-α-sensitive line derived from a mouse fibrosarcoma which allows assessment of bioactive TNF-α as previously described [11].

2.5. Fluorescence Calcium Imaging. Freshly isolated alveolar macrophages (2×10^5 cells in 200 μL culture media) were dispensed into the 10 mm diameter microwell in the middle of a 35 mm diameter glass bottom plastic culture dish with a poly-L-lysine-coated no. 1.5 glass coverslip defining the well bottom (MatTek Corp., Ashland, MA). The cells were incubated for 2 hrs to allow cell attachment. An additional 2 mL of culture medium was added, and the cells were incubated for 20–22 hrs. The *in vitro* injury procedure described above (UBSS + HCl, pH = 1.75 for 1 min) was performed, the cells washed, and culture media containing 1.5 μg/mL LPS or 30 μg/mL LTA added. Following the appropriate duration of incubation, the cells were washed 2x with Buffer A (124 mM NaCl, 5.8 mM KCl, 1.4 mM KH_2PO_4, 18.7 mM HEPES, 14.1 mM dextrose, 0.68 mM mannitol, and 1.8 mM $CaCl_2$, pH = 7.4) and then incubated with 6 μg/mL Fura-2 AM (Life Technologies, Grand Island, NY) for 30 min. The cells were then washed twice with Buffer A and incubated for an additional 15 min in Buffer A to allow the Fura-2 AM to deesterfy. The culture dish was placed on an inverted microscope stage of a dual-excitation microscopic fluorescence spectrophotometer (Photon Technology International, Birmingham, NJ) that was used to excite the dye at 340 and 380 nm, alternating at a 2 Hz sampling rate. Fluorescence emissions at 510 nm were recorded as an excitation fluorescence ratio ($R_{340/380}$, which is directly proportional to the intracellular calcium concentration, $[Ca^{2+}]_i$) by a digital PMT detector that was masked to allow emission measurements from a single alveolar macrophage. Measurements were taken for one minute on 10 different cells for each time point. For the immediate and 30 min time points, the cells were loaded with Fura-2 AM prior to low pH and LPS or LTA exposure.

2.6. Mouse Acid Aspiration Injury Model. Eight-week-old CD-1 male mice (Charles River Laboratories, Wilmington, MA) were used for *in vivo* experiments to determine the effects of low pH injury on bacterial clearance. After induction of halothane anesthesia, a tracheotomy was performed by midline incision and blunt dissection. A 22 ga needle was inserted into the trachea under direct visualization, and 3.6 mL/kg normal saline, pH = 5.3 (NS), or NS + HCl, pH = 1.25, (Acid) was injected followed by a 0.2 mL bolus of air while the mouse was suspended by its incisors in a 60° supine position. The bacterial challenge inoculum, 50 μL, was instilled through the same needle 1 min following the NS or Acid instillation. Two separate bacterial inocula were prepared, *Escherichia coli* (*E. coli*, CP9, an extraintestinal pathogenic *E. coli* strain) and *Streptococcus pneumoniae* (*S. pneumoniae*, strain: EF3030). *E. coli* was prepared by culturing overnight at 37°C in Luria-Bertani medium and centrifuged at 8,000 ×g for 4 min at 4°C and then resuspending the pellet in sterile NS to 5×10^6 colony-forming units (cfu)/50 μL. *S. pneumonia* cultures were prepared from frozen stocks of known titer that were centrifuged as above and the pellet resuspended in sterile NS to 5×10^5 cfu/50 μL. Actual inocula titers were determined by serial titration just prior to each *in vivo* injury experiment. Mice were sacrificed at 24 hrs after intratracheal challenge by exsanguination during halothane anesthesia. Lungs were removed and homogenized by adding sterile lung homogenate buffer, pH = 7.4 (150 mM NaCl, 15 mM Tris base, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$ containing 500 μM AEBSF HCl, 150 nM aprotinin, 1 μM E-64, 0.5 mM disodium EDTA, and 1 μM leupeptin hemisulfate) (protease inhibitor cocktail set I, Calbiochem/EMD Chemicals, Gibbstown, NJ), such that the total lung plus buffer weight was 3 g (i.e., 3 mL) and homogenizing on ice with a Polytron PT-2000 tissue homogenizer (Brinkman Instruments, Westbury, NY). Bacterial titer (cfu/mL) of the homogenized tissue was determined by serial titration and the bacterial load determined by multiplying the lung homogenate titer by the total volume (i.e., 3 mL). Bacterial clearance was expressed as $\log_{10}$(starting inoculum/lung homogenate bacterial load at time of harvest). All procedures performed on animals were approved by the Institutional Animal Care and Use Committee at the University at Buffalo and complied with all state, federal, and National Institutes of Health regulations.

2.7. Statistical Analysis. All data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA for multiple group analysis and two-tailed Student's *t*-test for direct group comparisons with $P < 0.05$ considered as significant.

3. Results

3.1. Effects of Low pH Stress on LPS-Induced Cytokine Production in Alveolar Macrophages. Bacterial LPS is an important structural lipoprotein found in Gram-negative bacteria that acts as a major activator of the antibacterial innate immune response. LPS ligates TLR4 to initiate intracellular signaling that results in production of antibacterial proinflammatory cytokines [12, 13]. Isolated rat aMØs were stimulated with LPS (1.5 μg/mL for 24 hrs), and production of CINC-1, MIP-2, TNF-α, and MCP-1 was quantified (Figures 1(a)–1(d)). LPS significantly increased the production of CINC-1 from control levels of 153 ± 43 to 2944 ± 224 pg/mL ($P < 0.001$), MIP-2 from 250 ± 66 to 4347 ± 319 pg/mL ($P < 0.001$), TNF-α from 23 ± 10 to 462 ± 136 pg/mL ($P = 0.01$), and MCP-1 from 160 ± 28 to 2009 ± 172 pg/mL ($P < 0.001$).

To investigate the effects of low pH stress on LPS-stimulated cytokine profile, LPS-treated aMØs were first exposed to low pH stress (acidified saline at pH 1.75 for 1 min). This regimen of transient low pH exposure was specifically determined to allow maximal acid exposure without decreasing cell viability (see Section 2). This exposure protocol also mimics the transient gastric acid exposure that is rapidly neutralized *in vivo*. Following low pH stress, LPS-induced cytokine release was significantly blunted compared with LPS alone (Figure 1); CINC-1 was reduced by 58% ($P < 0.001$), MIP-2 by 50% ($P < 0.005$), TNF-α by 53% ($P < 0.05$), and MCP-1 by 83% ($P < 0.001$).

LPS ligation of TLR4 signals two distinct cytosolic pathways, the MyD88 pathway and the TRIF (MyD88 independent) pathway. MyD88 signaling increases production of CINC-1, MIP-2, TNF-α, and MCP-1 through activation of

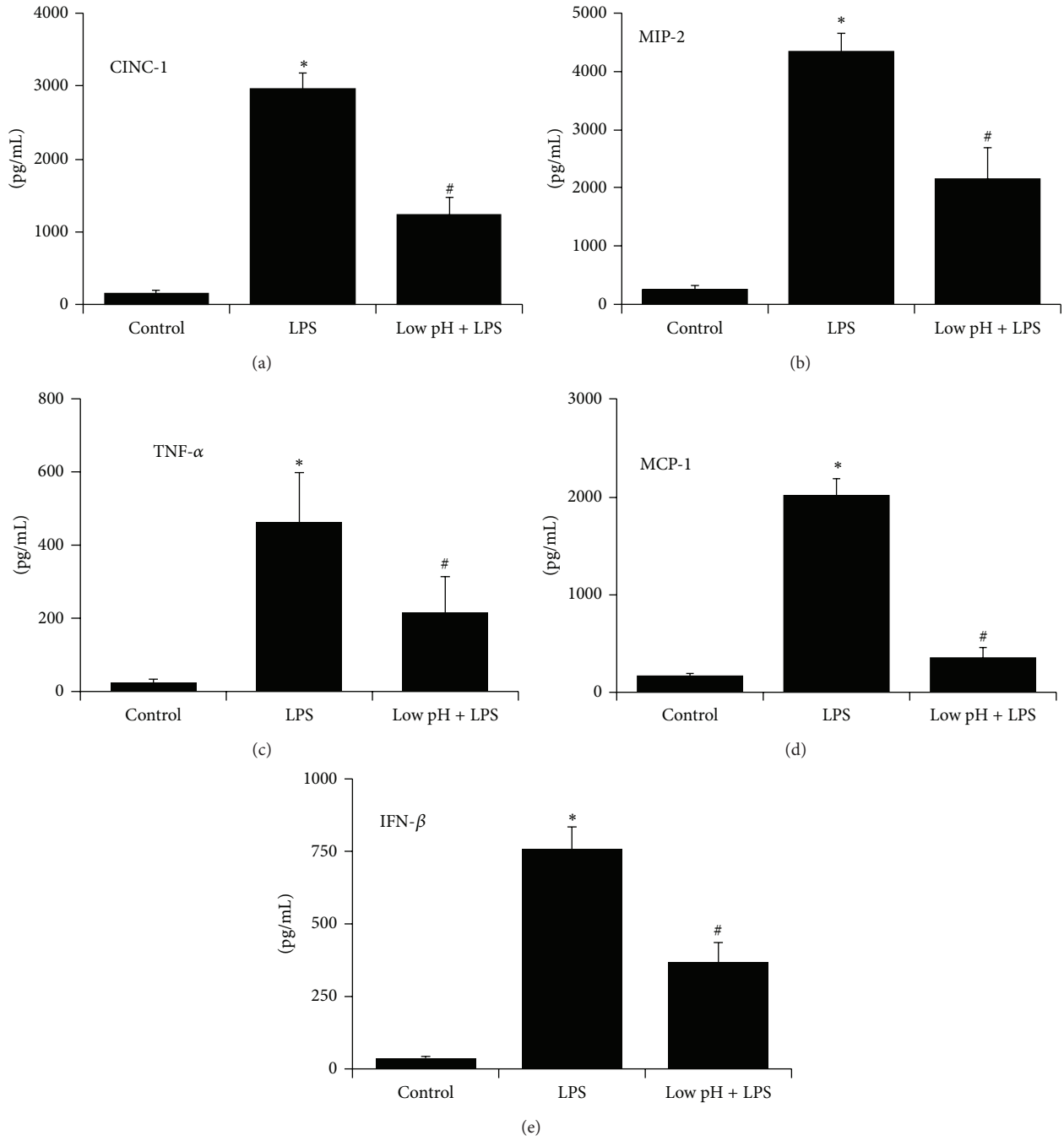

Figure 1: Effects of low pH stress on LPS-stimulated cytokine production in rat alveolar macrophages (aMØs). Isolated aMØs were stimulated *in vitro* with either saline (control), LPS alone (1.5 μg/mL for 24 hrs), or LPS in combination with a preceding transient low pH stress injury (pH 1.75 for 1 min, Low pH + LPS). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, MCP-1, and INF-β) or cytotoxicity bioassay (TNF-α). $^{*}P < 0.05$ LPS versus control, $^{\#}P < 0.05$ LPS versus Low pH + LPS.

the transcription factor, NF-κB, while TRIF signaling increases IFN-β production through activation of interferon regulatory transcription factor 3 (IRF3). To evaluate the involvement of the TRIF pathway, aMØs were stimulated with LPS for 24 hrs, and the release of IFN-β was quantified using ELISA (see Section 2). LPS stimulation resulted in a significant increase in IFN-β production from control levels of 37.8 ± 5.8 pg/mL to 758.7 ± 75.4 pg/mL ($P < 0.001$, Figure 1(e)). Exposure to low pH stress reduced LPS-induced IFN-β production by 52% ($P < 0.01$). These findings suggest that low pH stress inhibits LPS-induced cytokine release through inhibition of both MyD88 and TRIF pathways.

3.2. Effects of Low pH Stress on LTA-Induced Cytokine Production in Alveolar Macrophages. The PAMP, LTA, is a major component of the outer cell wall of Gram-positive bacteria,

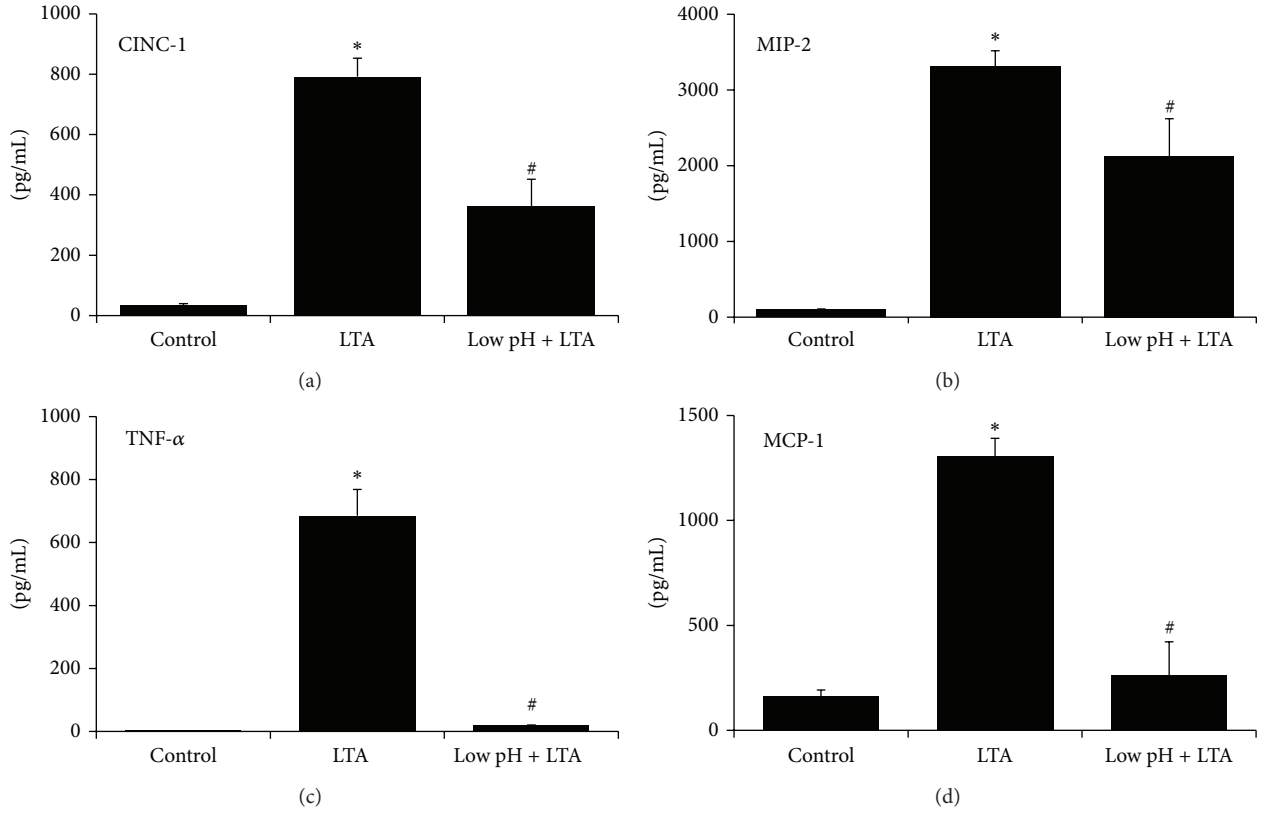

Figure 2: Effects of low pH stress on LTA-stimulated cytokine production in rat aMØs. Isolated aMØs were stimulated *in vitro* with either saline (control), LTA alone (30 mg/mL for 24 hrs), or LTA in combination with a preceding transient low pH stress injury (pH 1.75 for 1 min, Low pH + LTA). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, and MCP-1) or cytotoxicity bioassay (TNF-α). $^{*}P < 0.05$ LTA versus control, $^{\#}P < 0.05$ LTA versus Low pH + LTA.

and it shares many similarities with LPS in its proinflammatory effects. Cellular recognition of LTA is mediated by TLR2 ligation, which initiates cytosolic signaling that results in production of antibacterial cytokines [14, 15]. Isolated aMØs were stimulated *in vitro* with LTA (30 mg/mL for 24 hrs), and production of CINC-1, MIP-2, TNF-α, and MCP-1 was quantified (Figure 2). LTA increased the production of CINC-1 from control levels of 33 ± 5 to 792 ± 61 pg/mL ($P < 0.001$), MIP-2 from 99 ± 9 to 3316 ± 200 pg/mL ($P < 0.001$), TNF-α from 0.3 ± 0.04 to 685 ± 83 pg/mL ($P < 0.001$), and MCP-1 from 162 ± 29 to 1307 ± 84 pg/mL ($P < 0.001$).

To examine low pH stress on LTA-stimulated cytokines, aMØs were exposed to low pH stress (acidified saline at pH 1.75 for 1 min) prior to stimulation with LTA. Following low pH stress, LTA-induced cytokine production was significantly decreased compared to LTA alone (Figure 2); CINC-1 was reduced by 54% ($P < 0.05$), MIP-2 by 35% ($P = 0.02$), TNF-α by 97% ($P = 0.02$), and MCP-1 by 80% ($P < 0.001$).

3.3. Role of Intracellular Ca^{2+} in Mediating Effects of Low pH Stress on LPS-Stimulated Responses. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is an important regulator of macrophage function [16–18]. To assess the role of $[Ca^{2+}]_i$ in mediating the effects of low pH stress on LPS-mediated responses, changes in $[Ca^{2+}]_i$ were determined using the Ca^{2+}-sensitive fluorescent dye, Fura-2 AM (see Section 2). AMØs were exposed transiently to low pH stress and subsequently stimulated with LPS for 24 hrs. Fluorescence intensities ($R_{340/380}$) were measured at different time intervals within this 24 hr period to identify variations in the temporal Ca^{2+} levels: immediate postexposure to LPS, after 30 min, 1 hr, 4 hrs, and 24 hrs of LPS exposure.

First, changes in $[Ca^{2+}]_i$ due to low pH stress alone were assessed. Low pH stress increased $R_{340/380}$, correlating with increased $[Ca^{2+}]_i$, in a biphasic pattern (Figure 3(a)), increasing from a baseline value of 0.42 ± 0.01 to 1.04 ± 0.06, $P < 0.001$. This increase in $[Ca^{2+}]_i$ persisted to the 30 min time point. At 1 hr after low-pH exposure, the ratio dropped to baseline levels ($R_{340/380} = 0.42 \pm 0.01$, P = ns). This was followed by another rise in $R_{340/380}$ at 4 hrs and 24 hrs after exposure ($R_{340/380}$ of 1.09 ± 0.03, $P < 0.001$ and 0.96 ± 0.05, $P < 0.001$, resp.).

We then examined the effects of LPS alone on $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were assessed at increasing duration of LPS incubation as described earlier. $R_{340/380}$ increased immediately following LPS stimulation from control levels of 0.77 ± 0.01 to 1.04 ± 0.06 ($P = 0.02$) (Figure 3(b)). The elevation was short-lived with return to baseline levels at both 30 min and 1 hr. This was followed by low levels of $[Ca^{2+}]_i$ at 4 hrs and 24 hrs.

The effect of low pH stress on the LPS-mediated $[Ca^{2+}]_i$ profile was then investigated. AMØs exposed to a transient

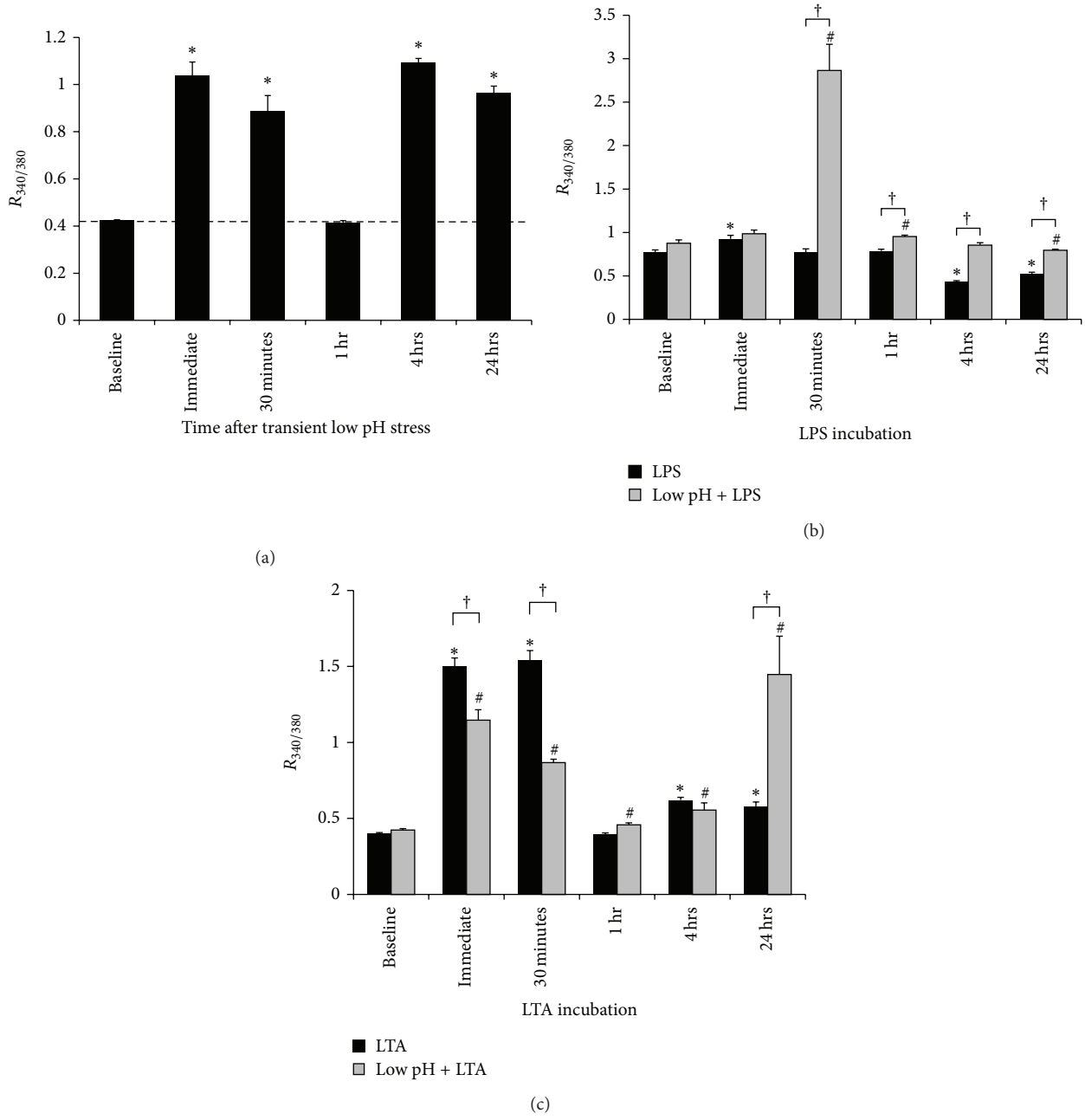

FIGURE 3: Intracellular Ca^{2+} concentration profile under various conditions, expressed as fluorescence excitation ratios ($R_{340/380}$, proportional to $[Ca^{2+}]_i$) in Fura-2 AM-loaded aMØs. (a) Isolated aMØs loaded with Fura-2 AM were exposed to low pH stress *in vitro* and fluorescence ratios recorded at the indicated time points following low pH injury. (b) Fura-2 AM-loaded aMØs were stimulated with either LPS alone (black bar) or LPS preceded by transient low pH stress injury (Low pH + LPS, gray bar). (c) Fura-2 AM-loaded aMØs were stimulated with either LTA alone (black bar) or LTA stimulation preceded by a transient low pH stress injury (Low pH + LTA, gray bar). $^{*/\#}P < 0.05$ versus corresponding baseline, $^{\dagger}P < 0.05$ presence of low pH stress versus absence of low pH stress.

low pH stress prior to LPS stimulation resulted in increased $[Ca^{2+}]_i$ at 30 min after LPS exposure (Figure 3(b)), 0.89 ± 0.04 for LPS alone versus 2.86 ± 0.30 for low pH/LPS ($P < 0.001$). The increased $R_{340/380}$ persisted at the 1 hr ($P < 0.001$), 4 hrs ($P < 0.001$), and 24 hrs LPS exposure times ($P < 0.001$).

3.4. Role of Intracellular Ca^{2+} in Mediating Effects of Low pH Stress on LTA-Stimulated Responses. To examine the role of $[Ca^{2+}]_i$ in mediating the effects of low pH stress on LTA-mediated responses, we identified changes in $[Ca^{2+}]_i$ in response to LTA alone and following combined low pH stress and LTA stimulation (Figure 3(c)). In Fura-2 AM-loaded aMØs, LTA stimulation significantly increased $R_{340/380}$ from baseline levels of 0.40 ± 0.00 to 1.50 ± 0.06 ($P < 0.001$). This elevation persisted at 30 min and returned to baseline levels at 1 hr ($R_{340/380}$ = 0.40 ± 0.01, P = ns). After an initial return

to baseline levels, the $R_{340/380}$ ratio developed a second wave of increased $[Ca^{2+}]_i$ at 4 hrs and 24 hrs, 0.62 ± 0.02 ($P < 0.001$ versus baseline) and 0.58 ± 0.03 ($P < 0.001$), respectively.

The effect of low pH stress on LTA-induced Ca^{2+} profile was then investigated. LTA-stimulated aMØs were exposed to low pH conditions and changes in $R_{340/380}$ monitored. There was immediate suppression by low pH stress on the LTA-induced $[Ca^{2+}]_i$ increase, 1.50 ± 0.06 (LTA) versus 1.15 ± 0.07 (LTA/Low pH), $P < 0.01$, and the $[Ca^{2+}]_i$ suppression persisted at 30 min after LTA exposure. At 1 hr and 4 hrs, there was no difference in the LTA-stimulated response, with or without low pH stress. At 24 hrs, low pH stress resulted in a robust increase in $R_{340/380}$, 0.58 ± 0.03 (LTA) versus 1.45 ± 0.25 (LTA/Low pH), $P < 0.001$.

These findings indicate that complex changes in $[Ca^{2+}]_i$ are induced by LPS and LTA, and the effect of low pH stress on $[Ca^{2+}]_i$ appears unique to the specific ligand/TLR interaction. In LPS-stimulated cells, low pH stress resulted in low level augmentation of $[Ca^{2+}]_i$ while, in LTA-stimulated cells, low pH stress initially suppressed the $[Ca^{2+}]_i$ increase but was followed by a robust augmentation in $[Ca^{2+}]_i$.

3.5. Effects of Intracellular Ca^{2+} Sequestration on the Modulatory Role of Low pH Stress on LPS-Stimulated Cytokine Production. To further investigate the role of Ca^{2+} in LPS-stimulated aMØs, BAPTA-AM was utilized as a cell-permeable Ca^{2+} chelator to effectively sequester $[Ca^{2+}]_i$ [19]. The general hypothesis is that if elevated $[Ca^{2+}]_i$ is necessary as an intermediate signal for LPS responses, BAPTA-AM will effectively inhibit these responses. AMØs were stimulated with LPS in the presence or absence of BAPTA-AM and cytokine production quantified. BAPTA-AM alone (control) did not result in any significant change in measured cytokines (Figure 4). In LPS stimulated cells, BAPTA-AM pretreatment decreased CINC-1 production by 64% ($P < 0.001$), MIP-2 by 67% ($P < 0.001$), MCP-1 by 79% ($P < 0.001$), and IFN-β ($P < 0.001$) from their LPS treatment levels. TNF-α levels trended down but were not statistically significant ($P = 0.07$).

It was subsequently tested whether low pH stress can further modulate cytokine production in Ca^{2+}-chelated, LPS-stimulated aMØs (Figure 5). Low pH stress did not affect CINC-1, MIP-2, MCP-1, and IFN-β production in LPS/BAPTA-AM-treated macrophages but significantly reduced TNF-α production by 82% ($P < 0.001$).

3.6. Effects of Intracellular Ca^{2+} Sequestration on LTA-Mediated Cytokine Responses. A set of experiments were performed using LTA-stimulated aMØs with BAPTA-AM. BAPTA-AM, in LTA-stimulated macrophages (Figure 6), reduced levels of CINC-1 by 64% ($P < 0.001$), MIP-2 by 53% ($P < 0.01$), TNF-α by 65% ($P < 0.05$), and MCP-1 by 51% ($P < 0.05$), when compared with LTA treatment in the absence of BAPTA-AM. It was subsequently investigated whether low pH stress would further modulate cytokine production in Ca^{2+}-chelated, LTA-stimulated aMØs (Figure 7). Low pH stress suppressed levels of CINC-1 by 43% ($P < 0.001$), MIP-2 by 46% ($P < 0.01$), and TNF-α by 82% ($P < 0.001$) in LTA/BAPTA-AM-treated cells. Low pH stress tended to suppress MCP-1 ($P = 0.06$).

3.7. Effects of Low pH Stress on Bacterial Clearance In Vivo. To determine the effects of low pH stress on bacterial clearance *in vivo*, mice were exposed to an acidic pulmonary aspiration injury followed by intratracheal instillation of either *E. coli* or *S. pneumoniae* (mimicking LPS and LTA response, resp.) (see Section 2). In animals inoculated with *E. coli*, starting inoculum was $5.3 \times 10^6 \pm 0.5 \times 10^6$ cfu ($n = 17$). Twenty-four hours after intratracheal *E. coli* challenge, resulting lung bacterial loads in the absence of low pH injury were $5.3 \times 10^3 \pm 4.4 \times 10^3$ cfu ($n = 13$) versus $1.1 \times 10^5 \pm 0.3 \times 10^5$ cfu ($n = 17$) in the presence of low pH injury, demonstrating an increased *E. coli* load after low pH injury. Estimated *E. coli* clearance (see bacterial clearance estimation under Methods section) was 3.7 ± 0.2 versus 1.9 ± 0.1, respectively ($P < 0.001$). In animals inoculated with *S. pneumoniae*, starting inoculum was $4.5 \times 10^5 \pm 0$ ($n = 5$). Twenty-four hours after intratracheal *S. pneumoniae* challenge, resulting lung bacterial loads in the absence of low pH injury were $5.8 \times 10^3 \pm 2.9 \times 10^3$ cfu versus $8.4 \times 10^7 \pm 3.1 \times 10^7$ cfu in the presence of low pH injury, demonstrating a profound increase in total *S. pneumoniae* load, in excess of the starting inoculum. Estimated *S. pneumoniae* clearance was 2.4 ± 0.4 versus -2.19 ± 0.2, respectively ($P < 0.001$).

4. Discussion

The innate immune system recognizes a wide variety of bacterial pathogens through a series of PRRs including TLRs, C-type lectin, and NOD-like receptors. The TLRs are the most widely studied PRRs with at least 10 recognized subtypes, each recognizing specific microbial patterns. In this study we examined TLR4- and TLR2-cytosolic signal transduction pathways through the use of LPS and LTA, respectively, to identify detrimental effects of nonlethal low pH stress on aMØ function. Clinical consequences of gastric aspiration are well established, ranging from a self-limiting aspiration pneumonitis to life threatening lung injury as seen in ARDS. Superimposed bacterial pneumonia is a frequent and deleterious sequela of gastric aspiration. *In vivo* animal studies have demonstrated that gastric acid aspiration results in a severe heterogeneous lung injury [20] which involves both direct low pH stress and indirect, non-pH stress-induced inflammation. This inflammatory response involves mechanisms that include direct tissue damage [11], impairment of surfactant function [20], and augmented recruitment of inflammatory/immune cells [11].

aMØs are sentinel cells that act as the initial line of defense against bacterial infection and are exposed to the inhaled fluid during a gastric aspirate event. This fluid often includes a low pH component, partially digested food particles, and potentially different degrees of bacterial loading arising from oropharyngeal and/or gastric colonization. The role of low pH stress in modulating aMØs function contributing to susceptibility to bacterial infection is not completely understood. Our group used an *in vitro* model to mimic low

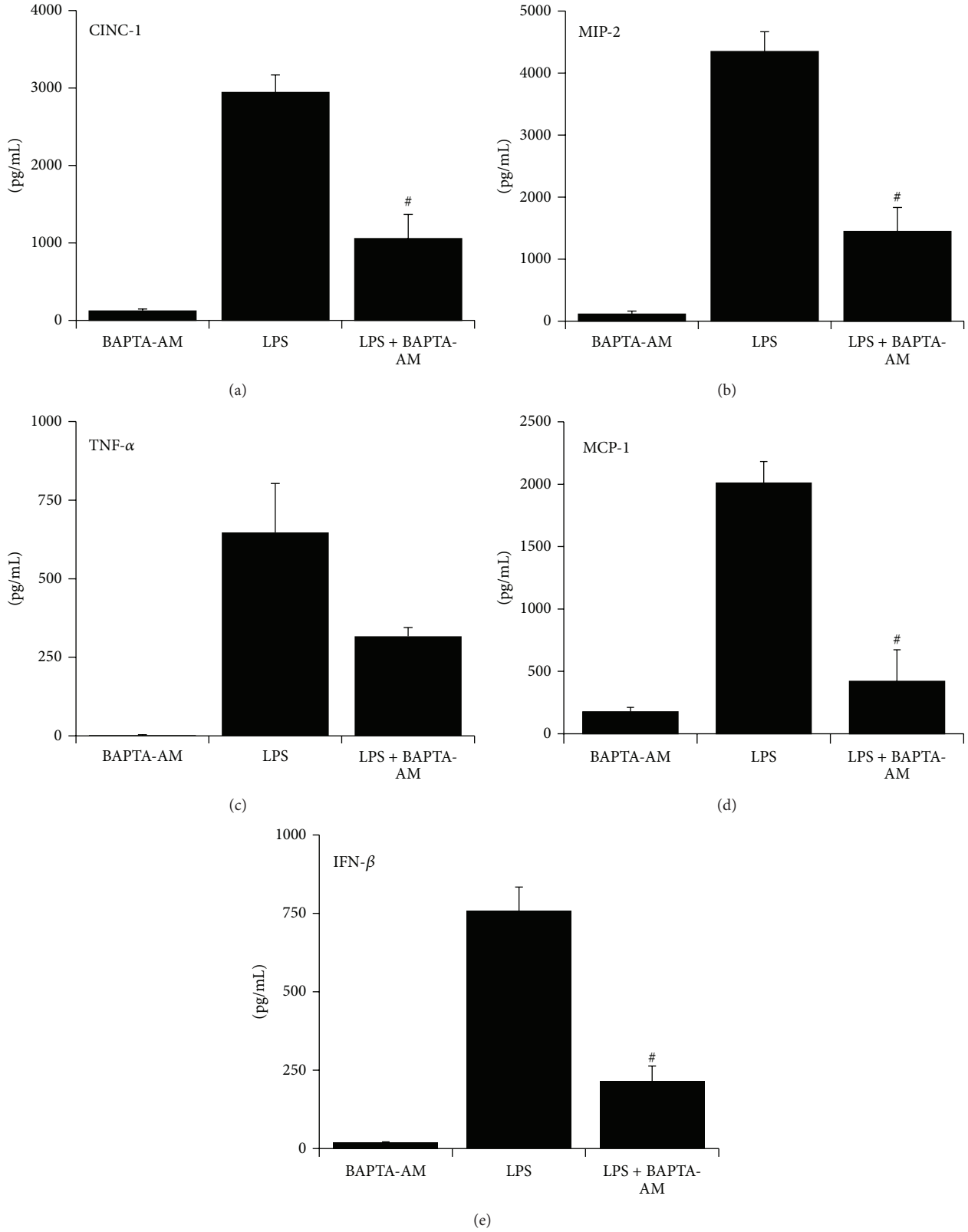

FIGURE 4: Effects of intracellular Ca^{2+} depletion (BAPTA-AM pretreatment) on LPS-stimulated cytokine production in aMØs. Isolated aMØs were stimulated *in vitro* with BAPTA-AM (5 μM for 30 min), LPS (1.5 g/mL for 24 hrs), or LPS in combination with preceding BAPTA-AM (LPS + BAPTA-AM). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, MCP-1, and INF-β) or cytotoxicity bioassay (TNF-α). $^{\#}P < 0.05$ LPS versus LPS + BAPTA AM.

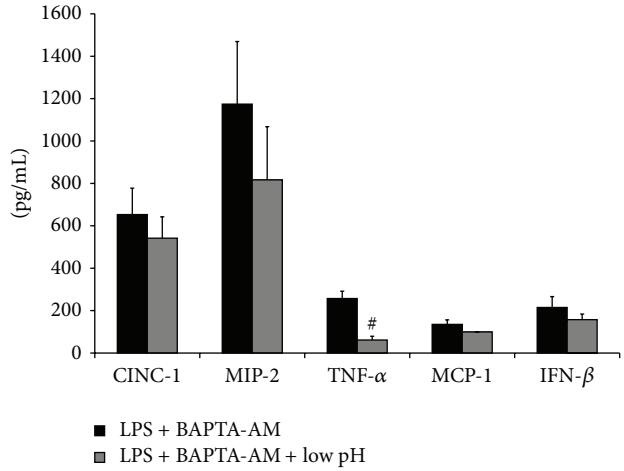

Figure 5: Effects of low pH stress on LPS-stimulated cytokine production in Ca^{2+}-depleted (BAPTA-AM pretreatment) aMØs. Isolated aMØs were exposed to LPS and BAPTA-AM alone (LPS + BAPTA-AM) or in combination with low pH stress (LPS + BAPTA-AM + pH). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, MCP-1, and INF-β) or cytotoxicity bioassay (TNF-α). $^{\#}P < 0.05$ LPS + BAPTA-AM versus LPS + BAPTA-AM + pH.

pH stress exposure of aMØs and identify direct changes in LPS- and LTA-mediated cytokine production.

Our primary findings demonstrated dramatic inhibition of proinflammatory cytokine secretion (i.e., CINC-1, MIP-2, TNF-α, and MCP-1) by aMØs exposed to a low pH stress. The model used in our experiment was specifically designed to achieve a level of acid exposure to induce the highest level of low pH stress without a decrease in cell viability. Our preliminary work on establishing cell integrity ensured that the reduced cytokine levels were not a result of changes in aMØs number but rather reflected a true alteration in aMØs signaling. The profound reduction in cytokine levels induced by low pH stress would be anticipated to negatively impact the aMØs sentinel response to bacterial infection. Our *in vivo* animal data supports this hypothesis demonstrating suppression of both *E. coli* and *S. pneumoniae* bacterial clearance (Figure 8).

As an early surveillance mechanism to detect infection, TLRs recognize specific microbial components or PAMPs. TLR2, which is activated by LTA, is a heterodimer in conjunction with TLR1 and TLR6 [21–23]. Other moieties reported to be recognized by TLR2 are lipoproteins, mycobacterial lipoarabinomannan [24], and rare lipopolysaccharide species [25]. On the other hand, TLR4, which principally acts as a homodimer, is important for recognition of LPS [22]. TLR4 has also been reported to recognize viral proteins [26, 27] and several damage-associated molecular patterns (DAMPs) which are products of tissue injury. DAMPs include ligands such as HMGB1, calgranulins, heat shock protein B8 [28], fibrinogen [29], and breakdown products of heparan sulfate polysaccharides [30]. Four adaptor proteins mediate TLRs signaling, and their differential recruitment partially affords pathogen response specificity; the adaptor proteins include MyD88, TRIF (toll-receptor-associated activator of interferon), MAL (MyD88 adapter-like), and TRAM (TRIF-related adapter molecule) [31]. Both TLR2 and TLR4 utilize MyD88 and are the prototypic pathway for TLRs signaling. Recruitment of MyD88 initiates a cascade involving sequential recruitment/activation of IRAK4 (IL-1R-associated kinase 4), IRAK1, tumor-necrosis-factor-receptor-associated factor 6 (TRAF6), and TAK1/TAB1/2/3. Ultimately, three important, well-characterized pathways are activated: nuclear factor-(NF-κB) [32], mitogen-activated protein kinases (MAP) [33], and phosphoinositide 3-kinases (PI3K) [34]. These distinct pathways result in induction of transcription, mRNA stabilization, and translation of a wide variety of proinflammatory cytokines and chemokines important for bactericidal activity and chemotaxis/recruitment of other immune competent cells [35–37]. TLR4, however, in addition to utilizing MyD88 pathway also utilizes MyD88-independent pathway, recruiting the adaptor protein TRIF/TRAM, leading to activation of interferon regulatory factor 3 (IRF3) and resulting in induction of IFN-β [38]. Consistent with previously reported data, our results demonstrated a robust LTA- and LPS-mediated induction of proinflammatory cytokines. Exposure to low pH stress dramatically inhibited LPS- and LTA-mediated CINC-1, MIP-2, TNF-α, and MCP-1 levels (Figures 1 and 2), as well as a parallel inhibition of LPS-mediated IFN-β levels (Figure 1(e)). Although the exact mechanism is unclear, this suggests that low pH stress may act upstream in the intracellular signaling involved in the MyD88 and TRIF pathways, possibly involving receptor binding and/or initial adaptor protein association.

Cytosolic Ca^{2+} is a ubiquitous second messenger important in many intracellular processes including induction of key transcriptional processes [39]. TLR2 and TLR4 ligand stimulation, in part, utilizes Ca^{2+} to generate increased cytokine production. The mechanism of Ca^{2+} flux continues to be investigated, but a mechanism that has been suggested involves receptor ligand binding causing receptor phosphorylation by c-Src [40, 41]. This leads to activation of PI3K and phospholipase Cγ2 (PLCγ2), affecting release of intracellular Ca^{2+} store through IP3R activation in the endoplasmic reticulum [41]. The exact Ca^{2+}-dependent step affecting NF-κB activity is still unclear, but several potential candidates may include traditional Ca^{2+}-sensitive protein kinases such as protein kinase Cα (PKCα), PKCβ, PKCγ, and calmodulin-dependent kinases that have been shown to modulate NF-κB activity [42, 43]. Cytosolic changes in $[Ca^{2+}]_i$ are also required for optimal TLR4 trafficking to the endosomes and the phosphorylation/nuclear localization of IRF3 [44]. Our data further confirms the essential role of Ca^{2+} in TLR2 and TLR4 signaling, through demonstration of $[Ca^{2+}]_i$ increases in response to LPS and LTA (Figure 3) and reduction in cytokine levels following intracellular Ca^{2+} depletion (Figures 4–6). By tracking the changes in $[Ca^{2+}]_i$ at different time points, we demonstrated the complex nature of the cytosolic Ca^{2+} profile generated by LPS with an early transient single peak followed by low level $[Ca^{2+}]_i$ (Figure 3(b)). LTA stimulation, on the other hand, resulted in a biphasic pattern with an early peak followed by return

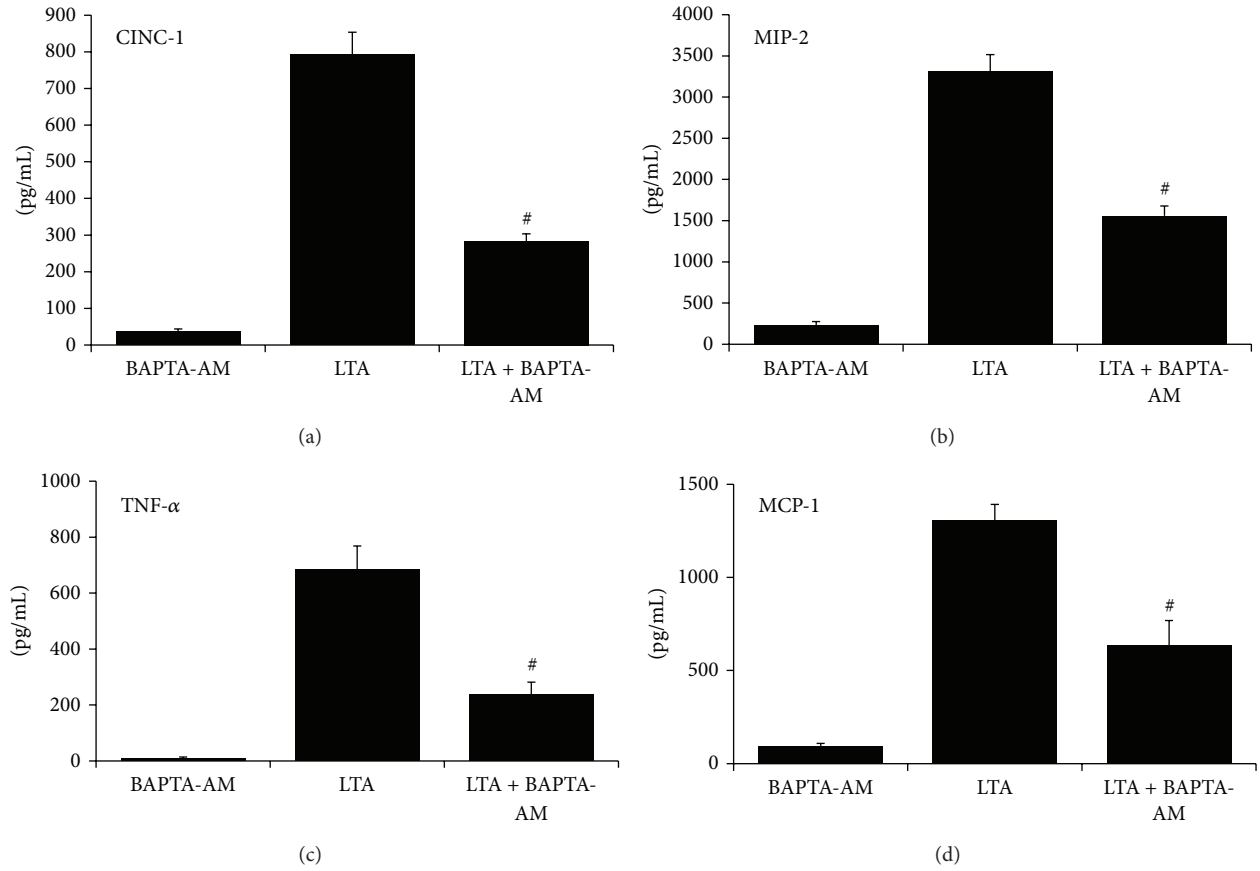

Figure 6: Effects of intracellular Ca^{2+} depletion (BAPTA-AM pretreatment) on LTA-stimulated cytokine production in aMØs. Isolated aMØs were stimulated *in vitro* with BAPTA-AM (5 μM for 30 min), LTA (30 mg/mL for 24 hrs), or LTA in combination with preceding BAPTA-AM treatment (LTA + BAPTA-AM). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, and MCP-1) or cytotoxicity bioassay (TNF-α). $^{\#}P < 0.05$ LTA versus LTA + BAPTA-AM.

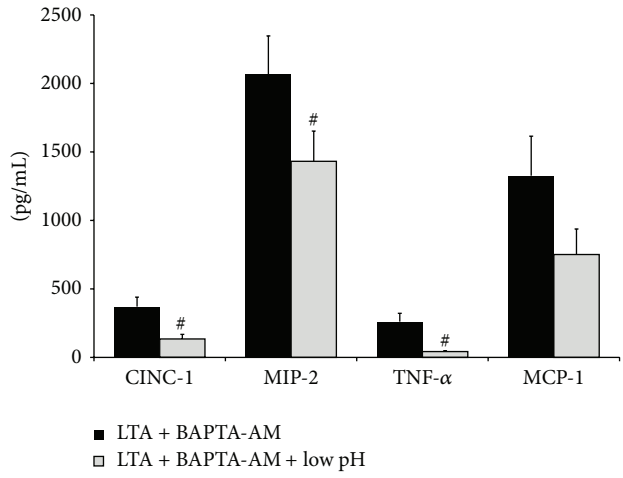

Figure 7: Effects of low pH stress on LTA-stimulated cytokine production in Ca^{2+}-depleted alveolar macrophages (BAPTA-AM pretreatment). Isolated aMØs were exposed to LTA and BAPTA-AM alone (LTA + BAPTA-AM) or in combination with low pH stress (LTA + BAPTA-AM + pH). Cytokines produced were assessed by ELISA (CINC-1, MIP-2, and MCP-1) or cytotoxicity bioassay (TNF-α). $^{\#}P < 0.05$ LTA + BAPTA-AM versus LTA + BAPTA-AM + pH.

to baseline and a secondary peak at a later time point (Figure 3(c)). Overall LPS, in contrast to LTA, resulted in a less robust Ca^{2+} response. The nature of this difference is unclear; however, this short-lived but biologically important Ca^{2+} response to LPS is consistent with other reported literature on LPS-stimulated Ca^{2+} response, demonstrating only transient increases in Ca^{2+}, predominantly in the range of 50–150 nM [45–47]. Combined exposures of LPS or LTA with low pH stress did not result in a simple summation of Ca^{2+} profile but modified LPS- and LTA-induced Ca^{2+} profiles in distinct patterns. In LPS-stimulated cells, low pH stress tended to generally increase $[Ca^{2+}]_i$ (Figure 3(b)). In contrast, low pH stress suppressed $[Ca^{2+}]_i$ in the early phase of LTA stimulation but augmented it at a later phase (Figure 3(c)).

To further investigate the role of Ca^{2+}, we also utilized BAPTA-AM to effectively sequester free intracellular Ca^{2+}. BAPTA-AM at the concentration used in our experiments has been used by different laboratories as a Ca^{2+}-selective sequestrant [19, 48–50]. Although other non-Ca^{2+}-dependent mechanisms can potentially be altered by BAPTA-AM, it appears to have a very high selectivity over other

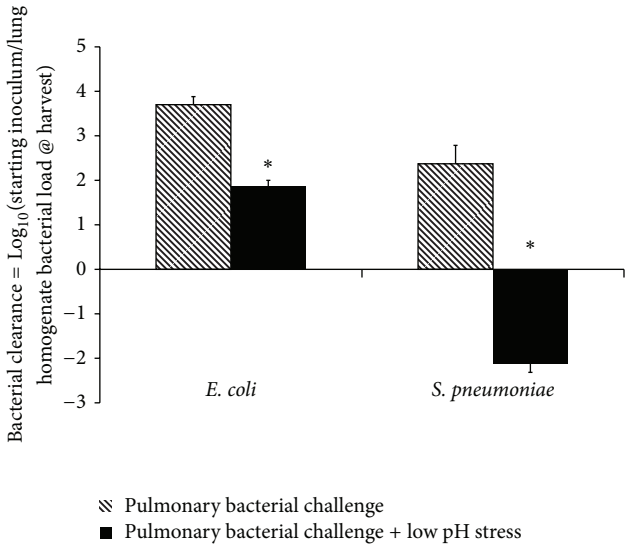

Figure 8: Effect of acid aspiration (low pH stress) on bacterial clearance *in vivo* was assessed using a mouse model of pulmonary bacterial infection. Pulmonary bacterial challenge was initiated using intratracheal administration of known titers (starting inoculum) of *E. coli* (left panel) or *S. pneumoniae* (right panel). Acid aspiration was modelled using intratracheal administration of acidified saline (pH 1.25) prior to bacterial challenge (pulmonary bacterial challenge + low pH stress). Lung homogenates were prepared 24 hr after intratracheal bacterial challenge and bacterial load quantified. $^{*}P < 0.05$ Bacterial pulmonary challenge alone versus bacterial pulmonary challenge + low pH stress. $^{*}P < 0.05$ versus bacterial inoculation alone.

bivalent cations, and its biologic activities have been linked predominantly to its ability to sequester Ca^{2+} [19, 48–50]. Under a $[Ca^{2+}]_i$-clamped state (BAPTA-treated cells), low pH stress failed to modify LPS-mediated production of proinflammatory cytokines (Figure 5) while continuing to suppress LTA-mediated cytokine production (Figure 7). This differential response suggests that the interaction of low pH stress on LPS/TLR4 and LTA/TLR2 pathways may be distinct with TLR4 involving predominantly Ca^{2+}-dependent mechanisms, while that of TLR2 may involve Ca^{2+}-independent mechanisms.

In summary, our findings demonstrate a strong suppressive direct effect of low pH stress on the ability of aMØs to mount an adequate antibacterial proinflammatory response following TLR2 and TLR4 activation. The site of action of the low pH stress appears to involve both the MyD88 and TRIF pathways. Ca^{2+} is an essential component for efficient elaboration of LPS- and LTA-mediated cytokine response but plays a differential role in mediating the suppressive effects of low pH stress. Changes in $[Ca^{2+}]_i$ appear to be required for LPS-stimulated responses but are less important for the LTA-mediated response. The mechanism for this differential role of cytosolic Ca^{2+} signaling needs further investigation, but the data presented in this study suggest that the mechanistic effect of low pH stress cannot be generalized across the various TLRs signaling cascades. These findings further our understanding of aMØs dysfunction following acute gastric aspiration and the role of this resident sentinel lung cell in the increased susceptibility to bacterial infection.

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Review Article

Potential Effects of Medicinal Plants and Secondary Metabolites on Acute Lung Injury

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Acute lung injury (ALI) is a life-threatening syndrome that causes high morbidity and mortality worldwide. ALI is characterized by increased permeability of the alveolar-capillary membrane, edema, uncontrolled neutrophils migration to the lung, and diffuse alveolar damage, leading to acute hypoxemic respiratory failure. Although corticosteroids remain the mainstay of ALI treatment, they cause significant side effects. Agents of natural origin, such as medicinal plants and their secondary metabolites, mainly those with very few side effects, could be excellent alternatives for ALI treatment. Several studies, including our own, have demonstrated that plant extracts and/or secondary metabolites isolated from them reduce most ALI phenotypes in experimental animal models, including neutrophil recruitment to the lung, the production of pro-inflammatory cytokines and chemokines, edema, and vascular permeability. In this review, we summarized these studies and described the anti-inflammatory activity of various plant extracts, such as *Ginkgo biloba* and *Punica granatum*, and such secondary metabolites as epigallocatechin-3-gallate and ellagic acid. In addition, we highlight the medical potential of these extracts and plant-derived compounds for treating of ALI.

1. Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), were first described in 1967 by Ashbaugh et al. [1] in patients with acute onset of tachypnea and hypoxia and the loss of compliance after a variety of stimuli [2–5]. According to the American-European Consensus Conference (AECC) ARDS was recognized as the most severe form of acute lung injury (ALI), a form of diffuse alveolar injury. In addition, the Berlin definition modified the AECC definition and divided ALI into the independent categories of ALI non-ARDS and ARDS alone [6, 7]. ALI is a life-threatening syndrome that causes high morbidity and mortality [8–12]; however, the worldwide incidence is variable, reaching, for example, 64.2 to 78.9 cases/100,000 person-years in the United States and 17 cases/100,000 person-years in Northern Europe, with an estimated 74,500 deaths annually [13]. Patients admitted to intensive care units are most affected by ALI (1 in 10) [14]. However, individuals with multiple comorbidities, chronic alcohol abuse, or chronic lung disease also present a high risk of developing ALI [15, 16]. The causes of ALI may be direct, such as *pneumonia*, inhalation injury, aspiration of gastric contents, inhalation injury, chest trauma, and near drowning, or indirect, such as sepsis, burns, pancreatitis, fat embolism, hypovolemia, and blood transfusion [8, 14]. The pathogenesis of ALI involves increased permeability of the alveolar-capillary membrane, accumulation of protein-rich fluid in the airspaces, pulmonary edema, and pulmonary infiltration of neutrophils, mainly bilateral, resulting in poor lung compliance, diffuse alveolar damage, and, consequently, acute hypoxemic respiratory failure [8, 17–23].

The inflammatory process of ALI can be classified into three stages: exudative, proliferative, and fibrotic stages [4,

9, 14]. The exudative stage is characterized by intense neutrophilic infiltrate, edema, and protein-rich fluid due to pulmonary capillary leakage [14]. The proliferative stage ensues as a consequence, the development of which is marked by proliferation and phenotypic changes in type II alveolar cells and fibroblasts [20]. In the absence of recovery, the fibrotic stage develops, which is characterized by diffuse fibrosis and modulation of the structural architecture remodeling of the lung. These stages characterize the chronic phase of ALI, leading to the formation of fibrotic scarring in the lung [9, 14].

Although inflammation is essential for the maintenance of tissue homeostasis and protection against infections, uncontrolled inflammation may contribute to lung damage, a characteristic phenomenon of several inflammatory disorders, including ALI [24–26]. In ALI airway inflammation, neutrophils are the first cells to be recruited and are the predominant cause of tissue damage [25, 27, 28], and their persistence is associated with a poor ALI prognosis [27–29]. The increased accumulation of neutrophils is associated with the exacerbation/amplification of inflammation and, consequently, of lung lesions due to the release of a complex network of proinflammatory mediators, such as cytokines (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8), chemokines chemokine (C-X-C motif) ligand (CXCL)-8, CXCL-1, CXCL-5, and chemokine (C-C motif) ligand (CCL)-2), proteases (elastases, collagenases, cathepsin G, and metalloproteinases), and oxidants (hydrogen peroxide and superoxide), and the accumulation of necrotic material [4, 24–26, 28–31]. Interestingly, an increase in such anti-inflammatory cytokines as IL-10 is also observed in ALI [32–34]. Thus, the balance of proinflammatory and anti-inflammatory mediators could coordinate the evolution or resolution of ALI. The resolution of inflammation is an active process and requires the activation of endogenous mechanisms, such as the biosynthesis of lipid mediators with proresolution activity, interaction between cells (hematopoietic and/or structural cells), and activation of cellular processes (e.g., apoptosis, phagocytosis) to maintain homeostasis [35–38]. Resolution includes the steps of (a) the inhibition of polymorphonuclear cell (neutrophil) infiltration, (b) return to normal vascular permeability, (c) clearance of polymorphonuclear cells (mainly by apoptosis), (d) infiltration of monocytes/alternatively activated macrophages, and (e) removal of apoptotic neutrophils, microorganisms, allergens, and foreign agents by macrophages [36, 38–42]. Clearly, resident and recruited macrophages play an important role in the clearance of injured tissues, debris, and apoptotic cells and are therefore important for the resolution of inflammation [36, 38]. Specifically, the resolution in ALI is characterized by the removal of neutrophils in the lung and the restoration of epithelial barrier function [38, 43]. Animal models have not been developed that fully to resemble human ALI but are quite useful for the better understanding of airway inflammation and the development of ALI [18, 44]. ALI experimental models in mouse, rat, rabbit, and guinea pigs are reported in the literature using different triggers, such as lipopolysaccharide (LPS), live bacteria, acid aspiration, and others. A more detailed description of the most commonly used ALI models and their characteristics can be found in Table 1.

The considerable progress made through the use of molecular and cellular assays together with knockout and transgenic animals has contributed significantly to the understanding of the genetic, tissue-specific, and immunological factors that contribute to the development of ALI [45–52]. Nevertheless, no therapeutic agents have demonstrated a clear benefit in ALI treatment [41], and corticosteroids have been used for treatment of ALI for many years [18, 53]. Besides, the disappointing results of a series of clinical trials treatment of ALI or patients at risk for ARDS using corticosteroids as well as the increase of the risk of infection and other adverse effects, the administration of corticosteroids might improve the injured tissue due to their anti-inflammatory effect [54]. Thus, the development of new compounds that exhibit similar therapeutic potential with reduced adverse effects is necessary for the continuous treatment of ALI. Furthermore, agents of natural origin that induce very few side effects should be considered for use as therapeutic substitutes or as complementary treatments to existing therapies. In addition, natural compounds may even form the basis of new drugs for the treatment of diseases [55–58]. In the course of a continued search for bioactive natural products derived from plants (secondary metabolites), several groups, including our own, have successfully employed experimental models to screen the pharmacologic activities of plant extracts and isolated compounds (secondary metabolites) [59–62]. Within this context, we and others have demonstrated that many plant extracts and secondary metabolites have the potential to be used in ALI treatment [8, 60, 63]. In a clinical trial in patients with severe pulmonary hypertension during extracorporeal circulation, Xu et al. [64] demonstrated that composite Rhodiolae (herbal plant) reduced the occurrence rate of acute lung injury and its mortality. In addition other studies were carried out with plant extracts (Table 2) and plant-derived substances (Table 3) in ALI experimental models.

Ginkgo biloba L. (Ginkgoaceae) is one of the most well-known plants in Chinese culture and has been used for therapeutic purposes for approximately 1,000 years. Its extracts are marketed worldwide to prevent or delay cognitive impairment associated with aging or neurodegenerative disorders [62, 65, 66]. In addition, *G. biloba* leaves have been used for the treatment of airway diseases, such as asthma and bronchitis [67]. In a bleomycin-induced acute lung injury rat model, a *G. biloba* leaf extract (EGb 761) reduced the responsiveness and diminished the occurrence of further reduction in the vasoconstrictor response of the pulmonary artery due to 5-hydroxytryptamine (5-HT). Furthermore, EGb 761 normalized bleomycin-induced alterations in the measured lung tissue biochemical markers [68]. Additionally, in another study, EGb 761 reduced protein leakage, neutrophil infiltration, myeloperoxidase (MPO, a heme enzyme present in the primary granules of neutrophils), and metalloproteinase (MMP)-9 activities in an LPS-initiated ALI rat model. These effects were associated with an inhibition of the activation of the nuclear factor-kappa B (NF-κB) pathway. In LPS-induced acute lung injury rat model, *G. biloba* extract reduced the recruitment of leukocytes to bronchoalveolar lavage fluid (BALF) and the pulmonary permeability. In addition, besides reducing other parameters, *G. biloba* extract also reduced the

Table 1: Animal models of lung injury.

Model	Characteristic inflammation	Animals	References
Acid aspiration	Rupture of the alveolar-capillary barrier with intense neutrophilic infiltrate [23, 56, 105, 106]	Mice Rats Rabbits	[42, 56, 107–110]
Bleomycin	Acute inflammatory injury, and reversible fibrosis [23, 111]	Mice Rats	[112–114] [20, 115]
Cecal ligation and puncture	Variable neutrophilic alveolar infiltrate and increased permeability [23, 43]	Mice Rats	[111, 116, 117] [118–120]
Hyperoxia	Epithelial injury and neutrophilic infiltration, followed by type II cell proliferation and scarring [23, 121–123]	Mice Rats	[124–127] [128]
Intrapulmonary bacteria	Increased neutrophilic alveolar infiltrate, interstitial edema, and permeability [23, 129]	Rabbits	[129]
Intravenous bacteria	Interstitial edema, neutrophils sequestration, and intravascular congestion [23, 130]	Mice	[131]
LPS	Neutrophilic inflammation with increased intrapulmonary cytokines [20, 23, 132]	Mice Rats Sheep	[20, 45, 59, 70, 132–135] [136]
Nonpulmonary ischemia/reperfusion	Increased microvascular permeability, neutrophils recruitment, edema, and sequestration in the lungs [23, 28]	Mice Rats	[28, 137–139]
Oleic acid	Neutrophilic inflammation, increased permeability, and edema [22, 23, 140]	Mice Rats	[141] [21, 22, 142]
Peritonitis by cecal ligation and puncture	Variable degrees Neutrophilic alveolar infiltrate and increased permeability [23, 143]	Rats Rabbits	[143, 144] [44]
Pulmonary ischemia/reperfusion	Increased pulmonary vascular permeability, neutrophil infiltration, and edema [23, 145]	Mice Rats Rabbits	[145–147] [148]

Table 2: Plants with anti-inflammatory effect on ALI.

Plant	Model of ALI	Doses	Relevant findings	Reference
Bathysa cuspidata	ALI in rats induced by Paraquat	200 and 400 mg/kg	↓ Lung edema	[149]
Ginkgo biloba	ALI in mice induced by LPS	10, 100, and 1000 mg/kg	↓ Leukocytes, PMN, MPO, and NF-κB	[150]
Panax notoginseng	ALI in rats induced by intestinal ischemia/reperfusion	100 mg/kg	↓ Leukocytes, PMN, MPO, IL-8, and TNF-α	[151]
Sho-seiryu-to	ALI in guinea pigs induced by oleic acid	3 and 0.75 g/kg	↓ Leukocytes and total protein	[152]
Viola yedoensis	ALI in mice induced by LPS	2, 4, and 8 mg/kg	↓ Leukocytes, total protein, lung edema, and MPO	[153]

blood TNF-α concentration and MPO in lung tissues [69]. Therefore, *G. biloba* appears to have potential to be used in the treatment of inflammation in ALI.

Another plant with antineutrophilic potential is *Lafoensia pacari* Jaumes St. Hilaire (Lythraceae), the extract of which is traditionally used by the population of Mato Grosso state, Brazil, to treat inflammation and gastric ulcers [70, 71]. In a clinical trial, however, *L. pacari* methanolic extract failed to eradicate *Helicobacter pylori* in dyspeptic urease-positive patients, even though the extract was well tolerated, and about 74% of patients had partial improvement of dyspnea, and 42% had full improvement of dyspnea in patients treated with extract of *L. pacari* [72]. Employing the asthma model induced by *T. canis* infection or the ovalbumin-induced asthma model, our group demonstrated that oral treatment with an ethanolic extract of *L. pacari* decreased the number of eosinophils and neutrophils recruited to BALF [73, 74]. In an attempt to identify the molecule(s) responsible for the antieosinophil and antineutrophil activity of the *L. pacari* extract, we used a mouse model of peritonitis induced by exposure to the F1 fraction of the *H. capsulatum* yeast wall [75]. This model of acute and localized eosinophilia and neutrophilia was suitable for the bioassay-guided fractionation of the *L. pacari* extract, and we were able to isolate and

Table 3: Secondary compounds with anti-inflammatory effects.

Active compound	Class	Models of ALI	Doses	Relevant findings	Reference
Matrine	Alkaloid	ALI in mice induced by LPS	25, 50, and 100 mg/kg	↓ Lung edema, MPO, TNF-α, IL-6, and NF-κB	[154]
Nicotine	Alkaloid	ALI in mice induced by LPS	0.2 and 0.4 mg/kg	↓ Lung edema, leukocytes, MPO, total protein, IL-1, IL-6, and TNF-α	[155, 156]
		ALI in mice induced by LPS	50, 250, and 500 mg/kg	↓ Lung edema, MPO, TNF-α, and IL-1β	[157]
Theophylline	Alkaloid	ALI in guinea pigs induced by LPS	3 and 30 mg/kg	↓ Leukocytes, and ↑ IL-10	[103]
Andrographolide	Diterpenoid	ALI in mice induced by LPS	1 and 10 mg/kg	↓ Lung edema, leukocytes, MPO, IL-6, TNF-α, IL-1β, and NF-κB	[59]
Astragalin	Flavonoid	ALI in mice induced by LPS	50 and 75 mg/kg	↓ Leukocytes, neutrophils, IL-1β, TNF-α, and IL-6	[158]
Caffeic acid phenethyl ester	Flavonoid	ALI in rats induced by phosgene	50 mg/kg	↓ NF-κB, and p38 MAPK	[159]
Cardamonin	Flavonoid	ALI in mice induced by LPS	10, 30, and 100 mg/kg	↓ Lung edema, TNF-α, IL-6, and p38 MAPK	[160]
Epigallocatechin-3-gallate	Flavonoid	ALI in rats induced by LPS	10, 50, and 100 mg/kg	↓ Lung edema, neutrophils, TNF-α, MIP-2, and ERK	[63]
	Flavonoid	ALI in rats induced by oleic acid		↓ Lung edema, TNF-α, and p38 MAPK	[87]
Kaempferol	Flavonoid	ALI in mice induced by LPS	100 mg/kg	↓ Leukocytes, neutrophils, MPO, TNF-α, IL-1β, IL-6, ERK, and NF-κB	[161]
Luteolin	Flavonoid	ALI in mice induced by LPS	35 and 70 μmol/kg	↓ neutrophils, MPO, MAPK, and NF-κB	[85]
		ALI in mice induced by LPS	70, 35, and 18 μmol /kg	↓ neutrophils, IL-6, TNF-α, and NF-κB	[84]
Naringin	Flavonoid	ALI in mice induced by LPS	15, 30, and 60 mg/kg	↓ Leukocytes, neutrophils, MPO, TNF-α, and NF-κB	[162]
		ALI in mice induced by Paraquat	60 and 120 mg/kg	↓ Leukocytes, TNF-α, TGF-β1, and MMP-9	[163]
Oroxylin A	Flavonoid	ALI in rats induced by LPS	15 mg/kg	↓ Neutrophils, TNF-α, and NF-κB	[164]
Quercetin	Flavonoid	ALI in rats induced by LPS	1, 5, and 10 mg/kg	↓ MPO, TNF-α, and IL-8	[165]
Geniposide	Iridoide	ALI in mice induced by LPS	20, 40, and 80 mg/kg	↓ Leukocytes, MPO, IL-6, TNF-α, and ↑ IL-10	[166]
Genistein*	Isoflavonoid	ALI in rats induced by LPS	50 mg/kg	↓ Lung edema, PMN, MPO, and ICAM-1	[167]
		ALI in rats induced by LPS	50 mg/kg	↓ MMP-9, NO, and NF-κB	[168]
Curcumin*	Polyphenol	ALI in rats induced by phosgene	50 and 200 mg/kg	↓ Leukocytes, Lung edema, total protein, MPO, TNF-α, and IL-8	[96]
		ALI in rats induced by sepsis	200 mg/kg	↓ TGF-β1	[94]
		ALI in rates induced by LPS		↓ Lung edema, neutrophils, and NF-κB	[169]
		ALI in rats induced by intestinal ischemia/reperfusion	100 mg/kg	↓ Leukocytes	[96]
		ALI in rats induced by oleic acid	5, 10, and 20 mg/kg	↓ Lung edema, TNF-α, and ↑ IL-10	[99]
Ellagic acid	Polyphenol	ALI in mice induced by acid	10 mg/kg	↓ Lung edema, neutrophils, IL-6, COX-2, NF-κB, and AP-1	[60]

Table 3: Continued.

Active compound	Class	Models of ALI	Doses	Relevant findings	Reference
Escin	Saponin	ALI in mice induced by LPS	0.9, 1.8, and 3.6 mg/kg	↓ MPO, TNF-α, and IL-1β	[170]
Glycyrrhizin	Triterpene	ALI in mice induced by LPS	10, 25, and 50 mg/kg	↓ Lung edema, neutrophils, MPO, COX-2, and iNOS	[171]
		ALI in mice induced by LPS	30, 10, and 3 mg/kg	↓ TNF-α and ↑ IL-10	[172]
Hydroxysafflor yellow A		ALI in mice induced by LPS	6, 15, and 37.5 mg/kg	↓ Leukocytes, lung edema, TNF-α, IL-1β, IL-6, p38 MAPK, and NF-κB	[173]
		ALI in rats induced by oleic acid and LPS	20, 40, and 60 mg/kg	↓ Leukocytes, lung edema TNF-α, IL-6, IL-1β, and ICAM-1	[174]

*No access to the entire manuscript, no answer from the corresponding authors.

chemically characterize ellagic acid (a polyphenol) as the major active component in the extract [61]. We showed that *L. pacari* extract as ellagic acid was able to reduce the number of eosinophils and neutrophils in this model [62]. We recently demonstrated that ellagic acid displayed anti-inflammatory properties by decreasing the severity of HCl acid-initiated ALI, accelerating the resolution of inflammation, and decreasing the cyclooxygenase-2 (COX-2) inhibitor-induced exacerbation of inflammation [60]. Ellagic acid reduced several inflammatory parameters, including vascular permeability alterations and neutrophil recruitment to BALF and the lung. In addition, ellagic acid reduced the proinflammatory cytokine IL-6 and increased the anti-inflammatory cytokine IL-10 in BALF without downregulating the NF-κB and activator protein 1 (AP-1) signaling pathways [60].

Pomegranate (*Punica granatum*) extracts, which have been used for centuries for medical purposes, contain also ellagic acid, and studies with pomegranate extract have demonstrated the anti-inflammatory effects in an experimental model of ALI (LPS-initiated) by reducing MPO in the lungs of mice [76]. Together, these findings suggest that ellagic acid has potential anti-inflammatory effects for the resolution of ALI inflammation.

Flavonoids are the best studied class of plant metabolites. Indeed, the search term "flavonoids" yielded more than 64,786 entries in the U.S. National Library of Medicine's Medline database accessed using PubMed in May 2013. Flavonoids occur naturally in fruits and vegetables, such as onions, apples, grapes, and nuts and are therefore commonly part of the human diet [77]. These compounds are also a component of disease treatment (phytotherapy), as they are present in the seeds, stems, barks, roots, and/or flowers of several medicinal plants [78]. Flavonoids have shown a wide range of therapeutic properties in clinical and preclinical studies, including, but not limited to, antioxidant, anticancer, antiinflammatory, and antiallergy activities [79–82]. Luteolin, a widely distributed flavonoid, has been reported to exhibit anti-inflammatory, antioxidant, and anticarcinogenic activities [83]. Luteolin was reported to reduce several hallmarks of ALI (LPS-initiated): leukocyte infiltration, histological changes, lung tissue edema, protein extravasation, MPO activity in lung tissue, TNF-α, keratinocyte-derived chemokine (KC), IL-6, and intercellular cell adhesion molecule-1 (ICAM-1) production, as well as inducible nitric oxide synthase (iNOS) and COX-2 expression in the lung [84]. Additionally, the expression of surface markers CD11b and Ly6G on neutrophils was reduced [85]. Luteolin also reduced N-Formylmethionyl-leucyl-phenylalanine (fMLP)-induced neutrophil chemotaxis and respiratory burst after LPS challenge and reduced LPS-induced activation of the NF-κB pathway, possibly via mitogen-activated protein (MAP) kinase (MAPK) and serine/threonine-protein kinases (AKT) [86]. These findings suggest that luteolin has potential anti-inflammatory effects for ALI treatment.

Green tea, from *Camellia sinensis* L. (Theaceae), is widely consumed around the world and is prepared by drying and steaming fresh tea leaves. Flavonoids are the major secondary metabolites found in green tea, with epigallocatechin-3-gallate being the most abundant. In acute lung injury induced by oleic acid in mice, epigallocatechin-3-gallate reduced the lung index, blood TNF-α concentration, and the phosphorylation of p38 MAPK [87]. In another study using LPS-initiated ALI in mice, epigallocatechin-3-gallate demonstrated an anti-inflammatory effect by reducing neutrophil recruitment in the lung and the production of TNF-α and macrophage inflammatory protein (MIP)-2, most likely via reduced extracellular-signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) phosphorylation in the lungs [63]. Therefore, epigallocatechin-3-gallate might constitute an attractive molecule with potential interest for the treatment of ALI.

The discovery of curcumin, the principal pigment of turmeric, dates from approximately two centuries ago when Vogel and Pelletier isolated a pigment of "yellow coloring matter" from the rhizomes of *Curcuma longa* (turmeric) [88–91]. Curcumin is present in the human diet and has been consumed for medicinal purposes for thousands of years [92]. This polyphenol has been shown to possess activities in the animal models of many human diseases. Curcumin modulates various molecules, including transcription factors, adhesion molecules, cytokines, and chemokines [92]. Curcumin demonstrated a significant anti-inflammatory effect with a reduction of the main ALI phenotypes, which included the reduction of neutrophil recruitment and activation, lung edema, inflammatory, and cytokines, most likely via a reduction of the NF-κB pathway in several ALI models. These models include sepsis-induced acute lung injury induced by cecal ligation and puncture surgery [93, 94], aspiration of polyethylene glycol and activated charcoal [95], intestinal ischemia/reperfusion (I/R) [96], bleomycin-induced lung injury [97], acute inflammation by *Klebsiella pneumonia* introduction [98], oleic acid-induced ALI [99], and LPS-induced acute lung injury [100]. These findings suggest that curcumin could be an interesting alternative for the ALI treatment.

The alkaloid theophylline is one of the oldest drugs in use in the management of obstructive airway diseases of diverse etiologies [101, 102], despite its weakness as a bronchodilator. However, the use of this alkaloid is often limited due to concerns regarding dose-related adverse effects, numerous drug interactions, and a narrow therapeutic index. In a chronic inflammatory lung injury model induced by LPS in guinea pigs, theophylline improved the airway injury and airway hyperreactivity induced by the repetitive exposure to LPS [103]. These findings suggest that theophylline has potential anti-inflammatory effects for the treatment of ALI inflammation.

In conclusion, ALI is a disease with high morbidity and mortality, and the current disease outcome has yet to be improved by pharmacologic treatment. Natural products and plant derivatives used in folk medicine are of vast medical importance due to their potential as a source of molecules with pharmacologic properties. Although active plant-derived secondary metabolites can be randomly discovered, the process is laborious, with a success rate on the order of 1 new product per 10,000 plants screened [62, 104]. In this review, we reviewed the effect of some plant extracts and their components on ALI experimental

models. The important benefits obtained with curcumin, ellagic acid, and *Ginkgo biloba* extract reveal powerful effects in reducing most ALI phenotypes, including inflammatory infiltrate, vascular permeability, and edema. As outlined in this review, we propose that there are several extracts of plants and compounds isolated from them with anti-inflammatory effects in ALI. So, they demonstrate potential to be used in the preliminary testing in humans which can provide a new alternative for ALI therapy.

Abbreviations

ALI: Acute lung injury
ARDS: Acute respiratory distress syndrome
BALF: Bronchoalveolar lavage fluids
CCL: Chemokine (C-C motif) ligand
COX-2: Cyclooxygenase-2
CXCL: Chemokine (C-X-C motif) ligand
EGb: *Ginkgo biloba* extract
HCl: Hydrochloric acid
IL: Interleukin
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein (MAP) kinase
MPO: Myeloperoxidase
NF-κB: Nuclear factor-kappa B
TNF-α: Tumor necrosis factor-alpha
SOD: Superoxide dismutase
MDA: Malondialdehyde
TBX: Thromboxane
ROS: Reactive oxygen species
ERK: Kinase activated by extracellular signal
MIP: Macrophage inflammatory protein
MMP: Matrix metalloprotease
GR: Glucocorticoid receptor
iNOS: Inducible nitric oxide synthase
PMN: Polymorphonuclear.

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Review Article

The Multifaceted Aspects of Interstitial Lung Disease in Rheumatoid Arthritis

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Interstitial lung disease (ILD) is a relevant extra-articular manifestation of rheumatoid arthritis (RA) that may occur either in early stages or as a complication of long-standing disease. RA related ILD (RA-ILD) significantly influences the *quoad vitam* prognosis of these patients. Several histopathological patterns of RA-ILD have been described: usual interstitial pneumonia (UIP) is the most frequent one, followed by nonspecific interstitial pneumonia (NSIP); other patterns are less commonly observed. Several factors have been associated with an increased risk of developing RA-ILD. The genetic background plays a fundamental but not sufficient role; smoking is an independent predictor of ILD, and a correlation with the presence of rheumatoid factor and anti-cyclic citrullinated peptide antibodies has also been reported. Moreover, both *exnovo* occurrence and progression of ILD have been related to drug therapies that are commonly prescribed in RA, such as methotrexate, leflunomide, anti-TNF alpha agents, and rituximab. A greater understanding of the disease process is necessary in order to improve the therapeutic approach to ILD and RA itself and to reduce the burden of this severe extra-articular manifestation.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory condition that mainly affects joints, in terms of pain, erosion, disability, and reduced survival [1–5]. RA may be complicated by several extra-articular manifestations (EAMs) [6–8]. The lung is among the most important targets of EAMs; the spectrum of lung involvement in RA includes manifestations such as pleural disease, rheumatoid nodules, Caplan's syndrome, bronchiectasis [9, 10] and, in particular, interstitial lung disease (ILD) [11–14]. The first report of a correlation between pulmonary fibrosis and RA was published in 1948 by Ellman and Ball [11], describing three patients with polyarthritis and interstitial pneumonitis with chronic fibrosing aspects on autopsy. Since this first description as a "curious chronic fibrosing bronchopneumonic lesion," it has become clear that RA associated interstitial lung disease (RA-ILD) actually includes a broad spectrum of disorders that vary greatly in their clinical presentation, pathology, and prognosis [9]. In the following years several authors pointed out the relevance of RA-ILD [15, 16], now widely accepted as an extra-articular complication with deep impact on prognosis and on therapeutic approach to RA [17–19]. Several histopathological patterns of ILD have been described [17] and differential diagnosis may be troublesome [20]. The etiopathogenesis of RA-ILD is not completely understood although genetic [21, 22], humoral [23], and environmental [24] factors seem to be involved. The picture is further complicated by the possible ILD-promoting effect of several drugs used to treat RA such as DMARDs (e.g., methotrexate and leflunomide) [25, 26] and biological agents (e.g., anti-TNF alpha and rituximab) [27, 28]. In this review we evaluated the main clinical characteristics of RA-ILD, the possible mechanisms underlying the occurrence of this EAM, and the current therapeutic approach.

2. Epidemiology and Prognosis

There is a great variation in the estimates of occurrence and in the general aspects associated with RA-ILD; this is partly due to the lack of acknowledged terminology and validated

Table 1: Histologic and clinical classification of IIPs, applicable to RA-ILD (adapted from [29–31]).

Histologic patterns	Clinical-radiological-pathologic diagnosis
Usual interstitial pneumonia (UIP)	(Idiopathic) pulmonary fibrosis/(cryptogenic) fibrosing alveolitis (IPF/CFA)
Nonspecific interstitial pneumonia (NSIP)	Nonspecific interstitial pneumonia (NSIP)
Organizing pneumonia (OP)	Organizing pneumonia (preferred definition) = Bronchiolitis obliterans organizing pneumonia (OP = BOOP)
Diffuse alveolar damage (DAD)	Acute interstitial pneumonia (AIP)
Respiratory bronchiolitis (RB)	Respiratory bronchiolitis interstitial lung disease (RB-ILD)
Desquamative interstitial pneumonia (DIP)	Desquamative interstitial pneumonia (DIP)
Lymphoid interstitial pneumonia (LIP)	Lymphoid interstitial pneumonia (LIP)

classification criteria, and to the different means of detection employed to diagnose ILD. In 2002, the American Thoracic Society and European Respiratory Society (ATS/ERS) redefined the nomenclature now used for acute and chronic diffuse parenchymal lung diseases [29]. Because of the lack of a dedicated classification, the consensus classification for idiopathic interstitial pneumonias (IIPs) has been adopted to define RA-ILD [30, 31]. RA-ILD can present as any of the seven idiopathic interstitial pneumonias according to the ATS/ERS consensus classification. Usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP) are the main patterns of ILD described in RA although also other forms, including lymphocytic interstitial pneumonia (LIP) and organizing pneumonia (OP), have been less commonly observed [14, 31, 32]. In Table 1 we summarized the different patterns of ILD that may be detected in RA.

The predominance of UIP distinguishes RA from the majority of other connective tissue diseases (CTDs), usually characterized by a prevalence of NSIP pattern [20, 33] although a bias related to diagnostic methodologies is possible. Diagnostic accuracy of high resolution computed tomography (HRCT) for UIP and NSIP with respect to histological diagnosis has been reported to be approximately 70% in various studies, with possible discordance in up to one-third of cases [34]. Despite these limits, the estimates of RA-ILD prevalence likely fall in the range from 4% to 30% [6, 14, 35], depending on detection methods and selection criteria, and the incidence may be as high as 4.1 per 1,000 people with RA [17].

Although RA is predominant in females, RA-ILD is frequently described in males [9, 10, 19, 31], with a 2 : 1 male to female ratio [10]. It is interesting to observe that even if the incidence of severe EAMs, such as vasculitis, has decreased in the last decades, fewer changes have been observed for ILD [36, 37]. ILD is frequently an early and asymptomatic finding in RA; in a recent study up to 27% of patients had HRCT findings of ILD within 2 years from disease onset [38], whereas in another study 25% of RA patients had ILD already diagnosed at presentation and another 25% developed ILD within 3 years from disease onset [17].

RA-ILD is a significant cause of mortality, with a median survival of 2.6 years versus 9.9 years of RA patients without ILD. The standardized mortality ratio for RA-ILD compared to RA alone is 2.86 [19]. The increased mortality in RA-ILD is mainly due to ILD progression with respiratory failure and direct RA complications [39]. Infectious processes, lung cancer, pulmonary embolisms, and other non-ILD pulmonary conditions, although reported in the setting of RA-ILD [40], are not so relevant on mortality rates as theoretically conceivable [39]. The literature data show that ILD contributes approximately to 6–13% of the excess mortality of RA patients when compared to the general population [19, 39, 41], being one of the most significant causes of death in these patients, together with cardiovascular complications [7, 42].

Despite these data, ILD associated with collagen vascular diseases, including RA, was reported to have a better prognosis than the idiopathic type of ILD [9, 31], even in cases of RA patients with biopsy proven UIP [42]. A median survival of 60 months in RA-ILD compared with 27 months in IPF has been reported [13]. However, this latter point is a matter of debate; according to different authors the prognosis of RA-UIP patients does not differ from that of IPF [11]. Possible factors responsible for these contradictory results could be the occurrence of subtle histologic differences not detectable by conventional radiographic techniques but influencing the correct diagnosis [34], the positive or detrimental influence of immunosuppressive therapies used in the treatment of RA, and the systemic consequences of a chronic autoimmune disease such as RA. Differences in prognosis among RA-ILD patients with different patterns of pulmonary involvement have also been described. Several studies have suggested a relationship of the UIP pattern to a shorter survival particularly when compared to NSIP [10].

3. Risk Factors

Several environmental, serologic, clinical, and genetic factors have been associated with the occurrence of RA-ILD. Occupational exposure to inhaled pollutants such as silica may lead to chronic lung inflammation and to the development of various autoimmune diseases such as RA [43]. In established RA, however, silica exposure is associated with the so called "Caplan's syndrome," first described in the early fifties [44]; this is a peculiar syndrome characterized by the occurrence of multiple well-defined rounded nodules on chest X-ray, of various diameters, distributed throughout the lungs but predominantly at the lung periphery [44, 45]. Lesions appear often in crops may coalesce and form a larger confluent nodule that often may cavitate or calcify [44, 45]. In subjects not affected by RA, silica exposure is associated with a large

amount of pathological lung conditions, such as pneumoconiosis, a peculiar form of interstitial lung disease that may lead to progressive massive fibrosis; exposure to silica and coal mine dusts may also result in pulmonary scarring in a pattern that mimics idiopathic pulmonary fibrosis [46]. To date, the pathogenetic link between exposure to silica, pneumoconiosis, Caplan's syndrome, and RA has not been clarified conclusively.

Smoking represents an independent risk factor for the occurrence of both autoantibody-positive [47, 48] and -negative RA [48]. The correlation is particularly evident for heavy (≥10 pack-years) and currently smoking individuals [48, 49]. Smoking is associated with several lung morbidities, including ILD [50–52]. The association with smoking is particularly evident for the UIP pattern [30, 53–55]. However, in a recent study involving 356 RA patients with lung involvement (either ILD or airway disease), the strong association between smoking history and ILD observed in the univariate analysis was not confirmed in the multinomial logistic regression analysis, thus suggesting that factors other than smoking may trigger ILD occurrence in RA [23]. According to this study, possible risk factors for RA-ILD are high titers of rheumatoid factor (RF), and to a lesser extent, anticyclic citrullinated peptide antibodies (ACPA), carriage of HLA-DRB1*1502, and older age [23]. The association of an increased risk of RA-ILD in RF positive patients has been confirmed by other studies [38, 56]. Data regarding the effect of ACPA on the development of lung disease are contradictory, with some authors confirming the association [57, 58] and others denying a possible role in the development of RA-ILD [59]. It is interesting to observe that both RF and ACPA can be present in smokers with ILD without clinical evidence of RA [60, 61]. Furthermore citrullination processes have been observed in lung tissue obtained from RA-ILD [62], and peculiar isoforms of citrullinated peptides, as, for example, the Hsp90 Isoforms [63], may be important antigen targets in RA-ILD. The increased citrullination of proteins and peptides in the lung is potentially smoke related due to peptidylarginine deiminase (PAD) increased activity and seems to play a role not only in ILD but also in RA occurrence [64]. The association with an allele connected with RA-ILD development, but not with occurrence of RA itself, HLA-DRB1*1502 has been reported in a different study [65]. As recently described, RA patients carrying DR2 serology (HLADRB1*15 and 16 alleles) and DQB1*06 have an increased risk of ILD, whereas HLA-DRB1 Shared Epitope (SE) appears to be protective against ILD development [66]. The protective role of HLA-DRB1 SE is indeed an unexpected finding, being generally associated with an increased risk of severe EAMs in RA [67, 68]. It should be underlined that these results have been observed in Japanese patients in whom the HLA allelic distribution is quite different from that of other ethnicities; therefore, these associations [23, 65, 66] should be confirmed by further studies. Older age has also been reported as a possible risk factor for the development of ILD [17, 19, 23]. Koduri et al. reported that risk estimates calculated on a 10-year difference in age are associated with a 64% increase in the likelihood of a RA patient of developing ILD over a shorter period of time [17]. Similar results were obtained by Bongartz et al. [19] and by Mori et al. [23]. Although the reason for this association is not completely clear, it is probably related to the established evidence of an increased incidence of EAMs in older RA patients (≥60 years) compared to younger ones [36, 69, 70].

Other risk factors associated with RA-ILD have been reported: high RA disease activity (e.g., DAS28 score) [19, 38], high grade functional impairment (measured using health assessment questionnaire) [17], and the presence of articular erosions and rheumatoid nodules [19].

4. DMARDs and Biological Agents Related ILD

In clinical practice, one of the most controversial issues is the possibility of ILD-promoting effects of some RA therapeutics. In fact, drugs such as methotrexate (MTX), leflunomide (LEF), antitumor necrosis factors (TNF) alpha agents, and rituximab have been associated with ILD occurrence or progression.

MTX may cause ILD/pneumonitis in 0.86%–6.9% of patients with morbidity and mortality rates reaching 20% [71]. As recently reported, this side effect may be triggered by genetic factors; HLA-A*31:01 allele has been reported to be a possible predictor of MTX induced ILD in Japanese patients [72]. This particular genetic subset is more common in Japanese population compared to the Caucasian one (8.7% versus 3.9%), possibly explaining the increased risk of this drug side effect in Japan [73]. Even if environmental factors may be involved [74], hypersensitivity is probably responsible for most cases of pneumonitis associated with MTX, particularly in the case established lung disease [55, 75].

Leflunomide is also associated with ILD [76], with an increased risk in the setting of preexisting lung disease and a potential relevant impact on survival [77, 78]. Similarly to MTX, hypersensitivity reactions may represent the main mechanism of LEF-induced pneumonitis [79]. The development of interstitial fibrosis is possibly connected with the effect of A771726, an active metabolite of LEF that may induce the transition of lung epithelial cells to myofibrasts [80], a phenomenon known as *"epithelial-mesenchymal transition"* (EMT). EMT is usually involved not only in abnormal wound repair and tissue remodelling, but also in organ fibrosis and reasonably in ILD [81]. However, EMT is not the only pathogenetic mechanisms in LEF-induced ILD. Experimental animal models demonstrated that the administration of LEF alone did not induce the EMT phenotype, but when LEF was administrated in the setting of a profibroting environment, such as bleomycin-induced pulmonary fibrosis, EMT was enhanced. This evidence suggests that the presence of other fibrosis-inducing stimuli, like in the case of preexisting lung fibrosis, may act as important risk factors for the development of LEF-induced ILD [80].

In recent years an increased number of reports described the new-onset or exacerbation of ILD after administration of biologic therapies, in particular anti-TNF alpha agents [82–84]. These drugs are pivotal in the treatment of several conditions, in particular RA [85, 86]. ILD may occur throughout the entire course of therapy with these agents, with reports ranging from few months to several years after treatment

is introduced, with a mean interval of approximately 26 weeks. A possible correlation with RA-ILD has been reported for all anti-TNF alpha agents approved for the treatment of RA (infliximab, etanercept, adalimumab, certolizumab, and golimumab) [83, 87, 88]. The mechanism of anti-TNF-alpha induced ILD is not well established. TNF-alpha is usually considered as a key cytokine in the pathogenesis of interstitial pneumonia [89–91]. Paradoxically, anti-TNF agents may actually have profibrotic effects on the lung. In fact, TNF-alpha promotes apoptosis of pulmonary inflammatory cells, thereby mediating tissue healing. Anti-TNF-alpha therapies may inhibit these apoptotic processes and promote persistence of inflammatory cells in the lung parenchyma and eventually the development of ILD [92]. Support for this hypothesis originates from experiments on TNF-alpha homozygous knockout mice in which intratracheal administration of bleomycin resulted in an accelerated form of pulmonary fibrosis. Exposing these mice to recombinant TNF-alpha effectively reduced inflammation and promoted lung tissue healing [93]. Anti-TNF therapy also promotes the expression of antiinflammatory cytokines, such as TGF-β1, potentially contributing to profibrotic states [94]. Finally, considering the role of TNF-alpha gene polymorphisms in determing the effectiveness of anti-TNF alpha therapy [95, 96], it is tempting to speculate that a similar mechanism may be involved in the susceptibility to drug-induced ILD, but further studies are needed to confirm this hypothesis.

Anti-TNF induced ILD may present with different patterns of interstitial involvement, most commonly UIP or NSIP; cases of organizing pneumonia, diffuse alveolar damage, and lymphoid interstitial pneumonia have also been described. Complete resolution may be observed in up to 40% after withdrawal of the biologic agent [83]. Mortality can be as high as 30% of cases, rising to about 60% in case of preexisting ILD [83]. Older age (>60–65 years) has been reported as a negative prognostic factor [83, 88]. According to data derived from one European biologics registry, general mortality in RA-ILD does not seem to be different between patients treated with conventional DMARDs or with anti-TNF-alpha agents although the proportion of deaths directly attributable to ILD is reported to be higher in this latter group [97]. Further studies on higher numbers of patients are required to confirm these results.

Despite the previously mentioned evidence, the association of anti-TNF alpha treatment and ILD development has been recently questioned. A large study including 8417 patients affected by RA, ankylosing spondylitis, psoriatic arthritis, psoriasis, and inflammatory bowel disease did not show an increased rate of ILD among the 4200 patients treated with anti-TNF agents, compared to those treated with non-biologic therapies, particularly in the RA group (adjusted hazard ratio, 1.03; 95% CI 0.51–2.07) [98]. Reports also suggest a possible improvement of RA-ILD following treatment with infliximab [99–101] and etanercept [102, 103]. The peculiar and nonunivocal link between anti-TNF alpha agents and ILD is clearly represented by a patient described by Komiya et al.; in this case report the administration of adalimumab first improved preexisting RA-ILD, and then induced its progression [104].

Finally, also other biological therapies with different mechanisms of action, including rituximab, have been involved in the occurrence of ILD in RA patients [10]. Some authors described the occurrence *exnovo* of ILD, following rituximab infusion [28]. ILD has also been reported during rituximab therapy for lymphoproliferative disorders [105]. The pathogenetic mechanism is not established. It is possible that by targeting CD20 positive cells, rituximab induces B-cell apoptosis, leading to antigen-presenting-cell maturation, cytotoxic T-cell activation, and subsequent vascular and alveolar damage [106]. In rituximab-ILD cytokine profile shows an increase of proinflammatory molecules such as IL-6 and TNF-alpha, with potential pathogenetic role in promoting interstitial fibrosis [107].

5. RA-ILD: Potential Pathogenic Cascade

The pathogenesis of RA-ILD is far from being completely clarified. Several multifactorial components and a large number of risk factors may be involved. Patients' genetic asset could be either predisposing (HLADRB1*15, HLADRB1*16, DQB1*06 [23, 66], and HLA-A*31:01 [72] alleles, this latter for MTX-related ILD) or protective (HLA-DRB1 SE [66]) for the development of RA-ILD. Environmental factors play a crucial role on a susceptible genetic background. Tobacco use has been recognized as a possible trigger in the development of RA-ILD. Smoking may directly injure respiratory epithelia and vascular endothelial cells [108, 109] and stimulate protein citrullination in the lung through local activation of PAD enzymes [64]. Citrullinated proteins may be key elements already at a preclinical level, acting as antigen targets for the local immune response, eventually leading to ACPA formation, RA occurrence [110], and finally lung interstitial involvement, characterized by particularly enhanced citrullination processes [23, 62, 63]. Furthermore, as previously mentioned, smoking may induce RF synthesis [111, 112] that has been associated with an increased risk of RA-ILD by several authors [19, 37, 41]. It is conceivable that smoking may trigger pulmonary immune response through direct lung damage, enhancement of protein citrullination, and autoantibodies synthesis (e.g., RF and ACPA). These events stimulate a local inflammatory response characterized by cellular infiltration and by the release of several mediators that further contribute to tissue damage [14, 113]. It must be considered that smoking is obviously not the only etiologic agent involved in the development of the disease as demonstrated by the fact that RA and RA-ILD often occur in nonsmokers. This suggests that other environmental [114] or infectious [14] factors are probably involved. Moreover, as analyzed in a previous section, drug-induced ILD is another relevant issue.

Pulmonary cellular infiltrate in RA-ILD is heterogeneous, frequently organized in lymphoid nodular aggregates [115], showing striking similarities with rheumatoid synovium [116]. Follicular B lymphocyte aggregates are mainly peribronchiolar in disposition [117], resembling bronchial-associated lymphoid tissue. These structures are involved in antigen retrieval directly from lung lumen and not through afferent lymphatics as in lymph nodes and are capable of

mounting adaptive immune responses [117]. Interestingly, an increased number of peribronchiolar B-cell follicles have been correlated with smoking [117], supporting once again not only the possible role of the lung and of environmental factors in promoting RA-ILD, but also RA itself [64, 117]. Follicular structures are generally accompanied by diffuse infiltration of pulmonary interstitium by plasma cells that may contribute to the humoral immune response through the production of ACPA [117]. These antibodies complex with citrullinated peptides through Fc receptors expressed on antigen presenting cells, ultimately leading to the production of proinflammatory cytokines, including TNF-alpha [64]. T-lymphocytes are essential to support B-cells activation and differentiation following antigen exposure. An increased CD4+ and, to a lesser extent, CD3+ T cells infiltrate has been demonstrated in RA-ILD, particularly compared to IIP, independently of the pattern of disease presentation (e.g., RA-UIP or RA-NSIP) [118]. Other authors suggested that CD8+ T cells may also be important in the development of pulmonary fibrosis in RA [119]. Although this hypothesis is supported by a report showing that smoking increases the number of CD8+ T cells in lungs [120], this plausible mechanism of injury should still be elucidated [119]. Furthermore, also neutrophils may be relevant in RA-ILD as suggested by the correlation of bronchoalveolar lavage neutrophilia and evidence of more advanced interstitial fibrosis [119, 121]. The complexity of lung cellular infiltrates has been confirmed in a recent model of RA-ILD in SKG mice: infiltrating cells observed in this model included CD4+ T cells, B cells, macrophages, and neutrophils [122]. Cytokines and chemokines play a crucial role in the interaction and crosstalk of the cellular network in RA-ILD. TNF-alpha is a key proinflammatory cytokine in the pathogenesis of interstitial lung involvement; it is mainly produced by activated macrophages, lymphocytes, epithelial, and endothelial cells. TNF-alpha plays a central role in the stimulation of cell-cell adhesion and transendothelial migration with a pivotal role in the early phases and in the maintenance of cytokines and chemokines production cascade [123]. TNF-alpha stimulates fibroblasts proliferation promoting their ability to degrade the extracellular matrix [124] and triggering the expression of growth factors such as platelet derived growth factor-β (PDGF-β) and transforming growth factor-β (TGF-β), cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13), and chemokines (e.g., CXCL5, CXCL8, CXCL12, and CXCL13) [14, 64, 125, 126] that further contribute to stimulate fibroblast differentiation and proliferation, thus potentially linking inflammatory and fibrotic processes [127]. PDGF-β is produced by a wide array of lung cells, including macrophages, fibroblasts, epithelial and endothelial cells, [127, 128]. PDGF-β plays a primary role in the pathogenesis of IPF, figuring among profibrotic and proinflammatory molecules known to be critical in the pathogenesis of ILD, such as TGF-beta and TNF-alpha [127, 129]. It is interesting to note that the inhibition of PDGF tyrosine kinase receptor significantly attenuates the development of ILD in an experimental mouse model of pulmonary fibrosis [130]. Further studies regarding the role of PDGF in the development of RA-ILD are needed.

TGF-beta is produced by several cell types: macrophages, epithelial, endothelial and dendritic cells, and fibroblasts [127]. The profibrotic action of TGF-beta is mediated by the recruitment and activation of monocytes and fibroblasts, and by the induction of extracellular matrix deposition [127, 131]. TGF-beta also induces fibroblasts' differentiation into myofibroblasts, that represent the main source of extracellular matrix in lung fibrogenetic processes [132]. The role of chemokines (CXCL5, CXCL8, CXCL12, CXCL13) in the recruitment and organization of lung interstitial inflammatory infiltrate in RA-ILD is still largely unknown. These chemokines are secreted by macrophages, fibroblast and epithelial cells and act trough fibroblast recruitment and activation [127]. CXCL13 is a B-cells chemoattractant known to regulate lymphoid follicules organization within rheumatoid synovial tissue [116]. The exact role of this chemokine in the lung parenchima still needs to be clarified although it has been demonstrated that CXCL13 expression correlates with the extent of inducible bronchus-associated lymphoid tissue (iBALT) in patients with RA-ILD. This finding introduces a possible fascinating connection of pathogenetic mechanisms linking arthritis to extra-articular manifestations of RA.

Other mediators involved in this multi-step pathogenic cascade include matrix metalloproteinases, originated from damaged epithelia, that perpetuate this crosstalk between inflammatory and fibrotic processes, by the enhancement of cellular recruitment (B and T cells, macrophages, and neutrophils) and by the production of additional profibrotic mediators. Angiogenesis, induced by vascular endothelial cell growth factor and by the proinflammatory *milieu,* is also strictly connected with different phases of the pathological processes of inflammation and fibrosis (Figure 1).

6. RA-ILD Diagnosis

Although some patients with proven RA-ILD can be asymptomatic, the majority present with exertional dyspnoea and dry cough. Pleuritic chest pain, fever, haemoptysis, and tachypnoea are also common. Bibasal crackles on chest examination are the most frequent finding [9, 31].

Plain chest radiography mainly reveals reticular and fine nodular opacities. These findings are usually more concentrated in the lower lung zones. Chest radiography has a low sensitivity for detection of ILD and can be normal in early stages [9]. In the majority of patients pulmonary function tests (PFT) demonstrate a restrictive defect with low forced vital capacity (FVC), low total lung capacity (TLC) with or without low diffusion capacity of the lung for carbon monoxide (DLCO), and hypoxemia at rest or on exertion [9, 31]. Decreased DLCO has been described in up to 40% of RA patients without signs or symptoms of lung disease [9] and is reported to be the most sensitive test for predicting the presence of ILD on HRCT [133].

Bronchoalveolar lavage (BAL) is not routinely used as a diagnostic modality in RA-ILD because BAL changes may be seen even in the absence of ILD. BAL characteristics in RA-ILD patients show a predominance of neutrophils and macrophages [31]. The analysis of BAL fluid may not be helpful to distinguish between different subtypes of

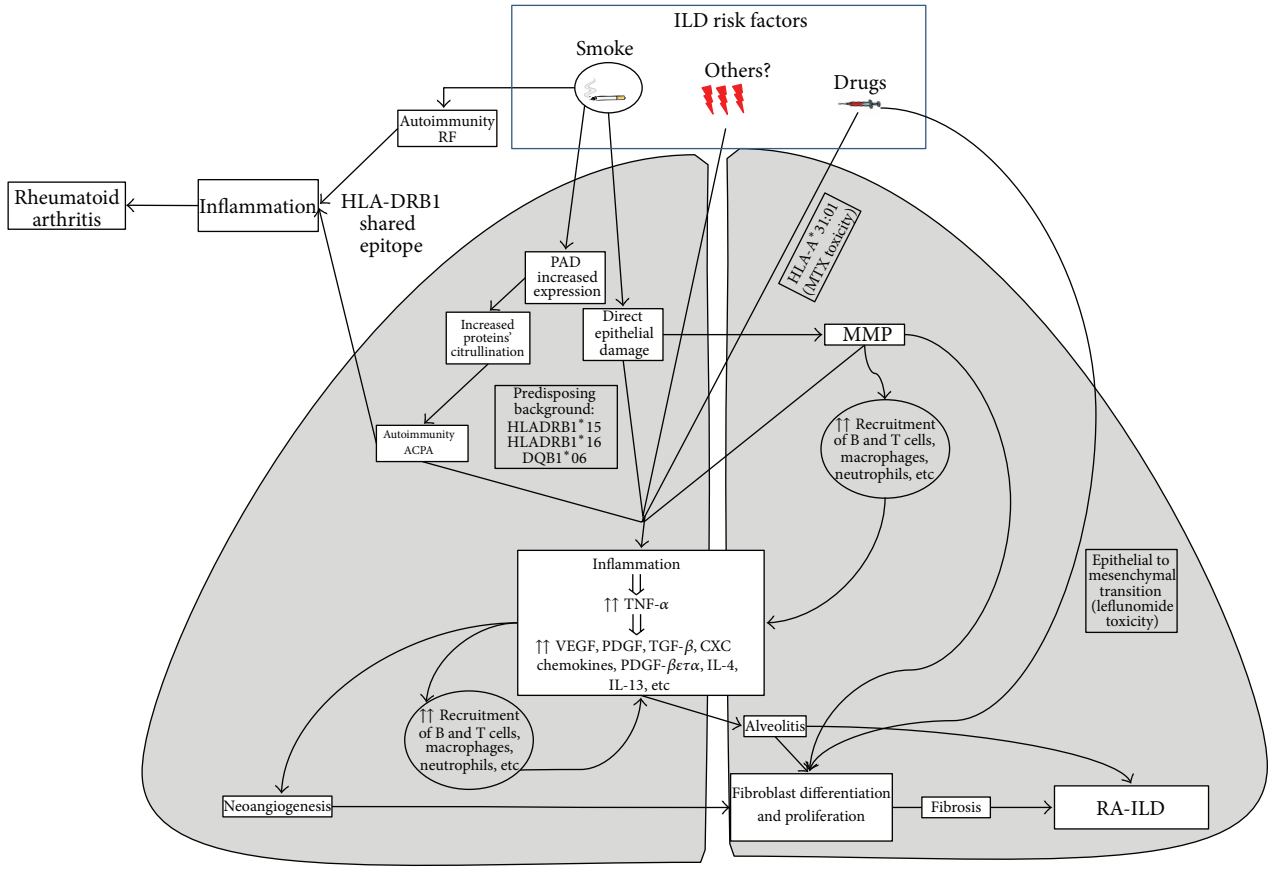

Figure 1: Possible pathogenetic mechanisms involved in the occurrence of interstitial lung disease in rheumatoid arthritis. ILD: interstitial lung disease, RF: rheumatoid factor, ACPA: anticyclic citrullinated peptide antibodies, VEGF: vascular endothelial growth factor, PDGF: platelet derived growth factor, PAD: peptidylarginine deiminase, and MTX: methotrexate, MMP: metalloproteinase.

RA-ILD; however, a slightly higher percentage of neutrophils (>4%) [134] may be more frequent in UIP. Lymphocytosis (>18%) is more common in NSIP and OP [31]. Nevertheless, the results of BAL cellularity may play a prognostic role, with neutrophilia associated with more advanced disease involvement and decreased response to therapy [134, 135]. BAL may also be useful in excluding infectious processes.

HRCT has been accepted as the standard noninvasive method of diagnosing and following ILD in patients with RA [136]. The results of HRCT have been shown to correlate closely with those of open lung biopsy [13, 135]. The most frequent HRCT findings detected in different subtypes of RA-ILD are presented in Table 2. High prevalence of enlarged mediastinal lymphnodes in patients with ILD is described [136].

Surgical lung biopsy provides the best means of establishing a histopathological diagnosis. However, because of the potential risks associated with this procedure, many patients are diagnosed without pathological confirmation. Usually, video-assisted thoracoscopic surgery (VATS) is preferred to open-lung biopsy. It is important to note that transbronchial biopsies are not useful in the diagnosis, with the exception of DAD/AIP, and occasionally organizing pneumonia OP/COP [29]. Histological changes in RA-ILD are very similar to those seen in IPF [9] and are summarized in Table 2.

The individuation of biomarkers to be used in the clinical setting to guide diagnosis and response to treatment is still ongoing. Increasing attention has been paid to a serologic marker of pulmonary disease, KL-6 (Krebs von den Lungen-6, a high molecular weight glycoprotein expressed on proliferated type 2 alveolar pneumocytes and epithelial cells), that is known to occur in patients with interstitial pneumonia, hypersensitivity pneumonitis, tuberculosis, sarcoidosis, and pulmonary alveolar proteinosis. It has also been reported that KL-6 is elevated in RA-ILD [30, 137]. KL-6 can be used as a useful marker reflecting the severity of pulmonary fibrosis, the grade of alveolitis, and the extent of HRCT lesions and for detecting active and progressive lung disease in RA-ILD; however, KL-6 may not be very sensible in detecting early stages of lung disease [137]. Oyama et al. [138] demonstrated that KL-6 is elevated in 88.9% of patients with active ILD and only in 0.6% of RA patients without active interstitial disease. RA-ILD patients present high levels of proliferative potential colony-forming cells (HPP-CFCs) in peripheral blood compared to those without ILD [139]. High levels of anti-cytokeratin 19 [140], IL-1, anti-IL1 antibodies, and serum

Table 2: Histological and radiological patterns of RA-ILD [9, 10, 29–31].

Pattern	Histology	CT features	CT differential diagnosis
UIP	Subpleural and peripheral fibrosis. Fibroblastic foci, lymphoid aggregates with germinal centres and honeycombing are characteristic. Mild inflammation; architectural destruction.	Peripheral, subpleural, basal reticulation, *and* honeycombing Traction bronchiectasis, architectural distorsion, GGO (less diffuse). Subpleural *lines*	IPF, other collagen vascular diseases, hypersensitivity pneumonitis (micronodules and sparing of lung bases), sarcoidosis, asbestosis (pleural thickening).
NSIP	Uniform interstitial involvement; various degrees of fibrosis and/or inflammation. Lymphoid aggregates. Rare honeycombing	Bilateral, symmetrical, patchy, mainly basal *GGO*, possible reticulation, traction bronchiectasis, irregular lines, or consolidation. Little or no honeycombing (in fibrosing NSIP).	UIP, DIP, COP, hypersensitivity pneumonitis, and HIV-associated interstitial lung disease.
OP	Connective tissue plugs within small airways and air spaces (Masson bodies). Little or no inflammation or fibrosis.	*Patchy and multiple airspace consolidation*, mainly basal, peripheral, or peribronchovascular. Air bronchograms can be seen. Possible associated GGO or centrilobular nodules.	Infections, vasculitis, sarcoidosis, alveolar carcinoma, lymphoma, eosinophilic pneumonia, NSIP, and COP.
DAD	(i) Acute phase: hyaline membranes, edema. (ii) Organizing phase: airspace and interstitial organization	(i) Acute phase: progressive, patchy, or diffuse *GGO* and dependent consolidation, often with lobular sparing (ii) Organizing phase: reticulation, traction bronchiectasis, and architectural distorsion.	Hydrostatic edema, pneumonia, eosinophilic pneumonia, and ARDS (but more symmetrical and lower lung zones)
DIP	Extensive macrophage accumulation in the distal air spaces. Mild interstitial involvement.	Patchy *GGO*, basal, and peripheral. Microcystic changes within GGO, reticular lines.	RB-ILD, hypersensitivity pneumonitis, sarcoidosis, and *Pneumocystis jiroveci* pneumonia.
RB-ILD	Bronchiolocentric macrophage accumulation. Mild bronchiolar fibrosis	Diffuse/upper lobes distribution, centrilobular nodules, bronchial wall thickening, and patchy GGO.	DIP, NSIP, and hypersensitivity pneumonitis
LIP	Bronchiolocentric lymphoid tissue hyperplasia	Diffuse, GGO, centrilobular nodules, septal and bronchovascular thickening, thin-walled *cysts,* and lymph node enlargement.	Sarcoidosis, lympangitic carcinoma, and Langherans' cell histiocytosis

GGO: ground glass opacities; UIP: usual interstitial pneumonia, NSIP: non-specific interstitial pneumonia, OP: organizing pneumonia; COP: cryptogenic organizing pneumonia; DAD: diffuse alveolar damage; DIP: desqumative interstitial pneumonia; RB-ILD: respiratory bronchiolitis-associated interstitial lung disease; LIP: lymphoid interstitial pneumonia; IPF: idiopathic pulmonary fibrosis.

LDH are reported [141], but their clinical utility still has to be better defined.

7. Differential Diagnosis

Differential diagnosis can be very challenging considering that interstitial lung involvement is not exclusive of RA but can be a complication of several conditions in the setting of connective tissue diseases (CTD). Antisynthetase syndrome (ASS) is the prototypical example of these conditions. ASS is characterized by the occurrence of peripheral arthritis, myositis, interstitial lung disease (ILD), typical cutaneous manifestations (e.g., mechanic's hands), and Raynaud's phenomenon, together with the positivity of antisynthetase antibodies [20, 142]. In these patients, ILD is described in up to 95% [20]. Arthritis is frequently symmetrical, and joint erosions and ACPA positivity have been described also in ASS patients [143], making the differential diagnosis between RA and ASS particularly challenging. Some reports indicate that the onset of different manifestations may be spread out [144, 145] with patients presenting first with arthritis and then with ILD. In our experience on 18 ASS, arthritis was the first symptom in 5 cases; all patients developed ILD in a time interval ranging from 3 months to 13 years.

Opportunistic infections, often connected with immunosuppressive treatments, particularly with biologic therapies, can mimick RA-ILD and can represent a real diagnostic challenge. Differential diagnosis in this setting can be even more troublesome in the case of patients with preexisting ILD [40]. Pathogens to consider as potential ILD mimickers are *Pneumocystis jiroveci,* fungal [40] and viral infections such as Epstein-Barr [146] and cytomegalovirus [147]. The main differential diagnosis based on radiological aspects is presented in Table 2. From the previously mentioned evidence it is clear that differential diagnosis is fundamental in distinguishing RA-ILD from different conditions that can be great mimickers such as ASS or infectious diseases. This process is fundamental in order to promptly start the appropriate treatment, with significant influence on patients' prognosis [20, 148].

8. RA-ILD Treatment

To date, little is known about optimal treatment for RA-ILD and whether the pattern of ILD may influence the response to

immunosuppression [10]. For newly diagnosed patients, first-line treatment is generally based on high dose corticosteroids [149] that may be associated with immunosuppressants such as cyclophosphamide [14, 150] and azathioprine [14, 151]. Mycophenolate mofetil should be considered as a potentially useful treatment, considering its additional action on fibroblasts, endothelial cells, and smooth muscle cells [151, 152]. Similarly to ASS [20, 148], good results have been obtained with cyclosporine [153–156]. Despite the previously mentioned lung toxicity, cases of MTX and LEF efficacy in RA-ILD have been reported [157]. As stated in a previous section, the relationship between anti-TNF alpha agents and ILD is far from being elucidated, and the role of rituximab has been recently questioned after the first reports of drug-induced ILD. Data are scarce regarding other biological therapies such as abatacept or tocilizumab. This latter improved RA-ILD in one single case report [158], whereas other authors addressed ILD occurrence or exacerbation following tocilizumab therapy [159, 160].

9. Conclusions

Although ILD is a well-established EAM of RA with a substantial impact on prognosis, it is evident that several aspects are far from being fully elucidated and are still widely discussed. In particular, we should clarify the cascade of events underlying the occurrence of ILD, by linking all etiologic and pathogenetic steps, starting from established risk factors (such as smoking) and resulting in the ultimate pulmonary manifestation (lung fibrosis). The comprehension of these passages is mandatory in order to improve the therapeutic approach of RA-ILD and to minimize the risk of ILD development in potentially predisposed patients.

Glossary

ACPA: Anticyclic citrullinated peptide antibodies
AIP: Acute interstitial pneumonia
ASS: Antisynthetase syndrome
ATS/ERS: American Thoracic Society/European Respiratory Society
BAL: Bronchoalveolar lavage
BOOP: Bronchiolitis obliterans organizing pneumonia (recently classified as OP)
CFA: Cryptogenic fibrosing alveolitis
COP: Cryptogenic organizing pneumonia
CTDs: Connective tissue diseases
DAD: Diffuse alveolar damage
DIP: Desquamative interstitial pneumonia
DLCO: Diffusion capacity of the lung for carbon monoxide
DMARDs: Disease modifying antirheumatic drugs
EAMs: Extra-articular manifestations
EMT: Epithelial-mesenchymal transition
FVC: Forced vital capacity
GGO: Ground glass opacities
HRCT: High resolution computed tomography
IIPs: Idiopathic interstitial pneumonias
IL-13: Interleukin-13
IL-4: Interleukin-4
IL-6: Interleukin-6
ILD: Interstitial lung disease
IPF: Idiopathic pulmonary fibrosis
KL-6: Krebs von den Lungen-6
LEF: Leflunomide
LIP: Lymphoid interstitial pneumonia
MMP: Metalloproteinase
MTX: Methotrexate
NSIP: NonSpecific Interstitial Pneumonia
OP: Organizing pneumonia (preferred definition to BOOP)
PAD: Peptidylarginine deiminase
PDGF-β: Platelet derived growth factor-β
PFT: Pulmonary function tests
RA: Rheumatoid arthritis
RA-ILD: Interstitial lung disease in rheumatoid arthritis
RB-ILD: Respiratory bronchiolitis interstitial lung disease
RF: Rheumatoid factor
SE: Shared epitope
TGF-β: Transforming growth factor-β
TNF: Tumor necrosis factor
TLC: Total lung capacity
UIP: Usual Interstitial Pneumonia
VEGF: Vascular endothelial growth factor.

Conflict of Interests

The authors declare they have no conflict of interest.

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Research Article

Modulation of the Effects of Lung Immune Response on Bone Marrow by Oral Antigen Exposure

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Allergic airway inflammation is attenuated by oral tolerization (oral exposure to allergen, followed by conventional sensitization and challenge with homologous antigen), which decreases airway allergen challenge-induced eosinophilic infiltration of the lungs and bone marrow eosinophilia. We examined its effects on bone marrow eosinophil and neutrophil production. Mice of wild type (BP-2, BALB/c, and C57BL/6) and mutant strains (lacking iNOS or CD95L) were given ovalbumin (OVA) or water (vehicle) orally and subsequently sensitized and challenged with OVA (OVA/OVA/OVA and H_2O/OVA/OVA groups, resp.). Anti-OVA IgG and IgE, bone marrow eosinophil and neutrophil numbers, and eosinophil and neutrophil production ex vivo were evaluated. T lymphocytes from OVA/OVA/OVA or control H_2O/OVA/OVA donors were transferred into naïve syngeneic recipients, which were subsequently sensitized/challenged with OVA. Alternatively, T lymphocytes were cocultured with bone marrow eosinophil precursors from histocompatible sensitized/challenged mice. OVA/OVA/OVA mice of the BP-2 and BALB/c strains showed, relative to H_2O/OVA/OVA controls, significantly decreased bone marrow eosinophil counts and ex vivo eosinopoiesis/neutropoiesis. Full effectiveness in vivo required sequential oral/subcutaneous/intranasal exposures to the same allergen. Transfer of splenic T lymphocytes from OVA/OVA/OVA donors to naive recipients prevented bone marrow eosinophilia and eosinopoiesis in response to recipient sensitization/challenge and supressed eosinopoiesis upon coculture with syngeneic bone marrow precursors from sensitized/challenged donors.

1. Introduction

The immunoregulatory effects of allergen exposure at the digestive tract, the major mucosal interface between the immune system and the antigens in environment, have received considerable attention over several decades [1, 2]. In the so-called *oral tolerization* models, feeding variable amounts of allergenic proteins to experimental animals, including mice, predictably changes their ability to subsequently respond to conventional sensitization and challenge with the same antigens, in a way consistent with attenuated, rather than exacerbated, hypersensitivity reactions [1–8]. Along with a variety of other strategies to change the course of allergic and autoimmune disease through immunomodulation, rather than through avoidance of environmental antigen exposure, oral tolerization holds promise for treatment as well as prophylaxis, and a better understanding of the mechanisms involved is likely to increase its practical value in management of immunological diseases [1, 2].

One important, unexplored issue in this field is the mechanism through which oral tolerance affects eosinophilic inflammation, a hallmark of asthma as well as experimental models of allergic airway inflammation [9]. Eosinophils are recruited in large numbers to sites of allergen challenge in sensitized animals, and believed to participate in complex ways in the pathogenesis of asthma [9–11]. However, because

they have a limited lifespan in tissues, which can be extended to some degree by a variety of inflammatory mediators [9], their relevance to the chronic manifestations of asthma depends on the ability of the bone marrow to continuously replace eosinophils that eventually undergo apoptosis, followed by degradation of apoptotic rests inside lung resident phagocytes [12]. Accordingly, the evidence from different models shows that an early consequence of airway challenge is upregulated eosinophil production in the bone marrow [13], paralleled by accumulation of eosinophil progenitors (colony-forming cells) in the lung tissue [14, 15].

While the impact of oral tolerization on bone marrow eosinophils has been examined by previous investigators, this effort has been limited, to our knowledge, to determining the percentage of eosinophils in bone marrow samples [3, 6]. This is, however, an unreliable indicator, because it can falsely increase or decrease following changes in the frequency of other bone marrow cell populations, regardless of any change in the numbers of eosinophils themselves.

On the other hand, we have recently characterized two distinct mechanisms, operative in vivo, which effectively prevent the stimulatory effect of lung immune responses on bone marrow eosinophil production. The first requires both the inducible isoform of nitric oxide synthase (iNOS) and the cell surface-associated ligand for the so-called death receptor CD95 (Fas), CD95L [16]. The second involves suppression of eosinophil progenitors, paralleled by stimulation of neutrophil progenitors [17]. While the first mediates the effects of diethylcarbamazine in an experimental asthma model [16], the second is mobilized by G-CSF, a cytokine selectively stimulatory for neutrophils that has multiple immunoregulatory effects, including the ability to prevent upregulation of bone marrow eosinophil production by aerosol challenge of sensitized mice [17]. One, or both, of these mechanisms might be operative during oral tolerization and contribute to a reduction in eosinophil production, indirectly reducing eosinophilic inflammation.

Given the complexity and interest of these interrelated issues, we have here examined whether (a) oral tolerization suppresses eosinophil production in the bone marrow, (b) it has additional effects on production of bone marrow neutrophils, or (c) a role for regulatory lymphocytes can be demonstrated.

2. Methods

Experimental Design and Groups. Pilot studies established that tolerization only had a demonstrable effect on the bone marrow when oral exposure was followed by conventional sensitization and challenge. Accordingly, *oral tolerization* is hereafter defined as an initial oral exposure reinforced by conventional s.c. sensitization and by i.n. challenge, totaling 3 consecutive exposures in distinct anatomical sites (oral/subcutaneous/airway). In the *direct model* of oral tolerization, the full sequence of exposures (OVA/OVA/OVA) is both necessary and sufficient for characterization of hematological effects in vivo (see *Results*). Controls (nontolerized) are given only water in the first step (H_2O/OVA/OVA) and thereafter are sensitized and challenged. Challenge controls receive saline (SAL) in the last step (OVA/OVA/SAL, H_2O/OVA/SAL). In the *transfer (indirect) model*, the immunoregulatory mechanisms could be explored by inducing oral tolerization as above in a donor (OVA/OVA/OVA) mouse and by transferring this donor's splenic T lymphocytes into a naive syngeneic recipient, which subsequently underwent sensitization and challenge bypassing oral exposure. Recipient groups for the indirect model are termed T/OVA/OVA or OVA/T/OVA, indicating that T cell transfer occurred before sensitization or after sensitization, respectively. For OVA/T/OVA, transfer was done either *before* or *after* the boost injection.

Mouse Strains and Animal Procedures. Six- to 8-week-old mice of the BP-2, BALB/c (wild-type and CD95L-deficient, naturally occurring *gld* mutants, [16]), and C57BL/6 backgrounds (both wild-type and iNOS-deficient knockout mice, [16]), from CECAL-FIOCRUZ/RJ, were lodged and handled following institutionally approved (CEUA#L-010/04, CEUA#L-002/09) protocols. The original observations were made in BP-2; BALB/c and C57BL/6 mice were further used to examine the effect of different genetic backgrounds and of mutations in CD95L and iNOS. Where indicated, heparinized blood was collected for plasma antibody quantitation from the abdominal vena cava of mice anesthetized with an injection of xylazine (12 mg/kg) and ketamine (100 mg/kg), in a total 0.2 mL volume of saline.

Immunological Reagents and Procedures. For *oral tolerization*, mice were given dehydrated Hen Egg White (Salto's, São Paulo, Brazil) as a 1% (w/v) solution in drinking water for 5 consecutive days [3], changed once daily. Where indicated, bovine serum albumin or human serum albumin (BSA or HSA, Sigma-Aldrich, St. Louis, MO, both 1% w/v in drinking water) was given instead, to control for the antigen-specificity of oral tolerization.

For *sensitization/boost*, mice were injected s.c. with 400 μL saline containing 100 μg Ovalbumin (OVA grade V, ICN Biomedicals, USA), the major antigen of Hen Egg White [18], and 1.6 mg alum, receiving two injections 7 days apart. For orally tolerized mice, sensitization and boost correspond to days 8 and 15, with oral exposure beginning at day 1. For *challenge*, they were given 25 μL OVA 0.04% (w/v) in the nostrils at day 22.

Anti-OVA IgG and IgE antibodies were quantified by ELISA as follows: flat bottom 96 well plates were sensitized overnight with 25 μg OVA in 100 μL PBS (1 h at 37°C followed by 4°C overnight). After 2 PBS washes, quenching was carried out for 1 h at 37°C with 5% Molico nonfat milk (for IgG) or BSA 1% (for IgE). Serum (100 μL) diluted 1/800~1/25.600 (for IgG) or 1/500~1/16.000 (for IgE) in the respective quenching buffer was added for 1 h at 37°C, followed by 3 washes. Anti-murine IgG (Horseradish peroxidase-conjugated goat anti-mouse IgG, Southern Biotechnology Cat # 1031-05, 1/2000) or anti-murine IgE (rat anti-mouse IgE, Southern Biotechnology Cat # 1130-01, 1/1200, followed by biotinylated rabbit anti-rat IgG, Dako A/S, Denmark, Code # E0467, 1/1500, and avidin-HRP, eBioscience Lot E022539, 1/1500) antibodies

were added for 1 h at 37°C for each step, with 3 PBS washes between incubations. The reaction was developed for 10 minutes with 100 μL 0.1 M citrate-sodium phosphate buffer, pH 5.1, containing benzene-1,2-diamine (o-Phenylenediamine) 5 mg/10 mL and 4 μL/10 mL 30% H_2O_2 (v/v) and stopped with 50 mL 4 N H_2SO_4. Absorbance was read at 490 nm. Where indicated, spleen cells were collected from tolerized and control donors, submitted to red cell lysis in Tris-NH_4Cl for 2 minutes at 20°C, washed, and separated on Lymphoprep (density (20°C) 1.077 ± 0.001 g/mL, Nycomed Pharma AS, Oslo, Norway) cushions (300 ×*g*, 30 minutes, 20°C). Mononuclear cells from the interface were collected, washed, and filtered through nylon wool columns to yield T lymphocyte-enriched populations, recovered as 25–30% of the input. Where indicated, 10^7 purified T cells were injected i.v. in a 100 μL volume of PBS per recipient mouse through the tail vein. Sensitization of the recipients was started 24 h after transfer. Alternatively, T cells were used for coculture with hemopoietic precursors (see below) at a 1 : 10 ratio.

Bone Marrow Assays. Bone marrow was collected from both femurs of individual mice by flushing with RPMI-1640 medium containing 1% FCS. Total nucleated cell counts (in haemocytometer) and differential counts (on cytocentrifuge smears, stained for eosinophil peroxidase/EPO according to [19]) of EPO+ (eosinophil-lineage, encompassing both mature and immature) cells and neutrophils were carried out to determine the impact of oral tolerization on bone marrow in vivo. The ex vivo effects of oral tolerization were initially evaluated in *liquid* (nonclonal) bone marrow cultures, which allow the study of terminal eosinophil and neutrophil differentiation from committed precursors, established from 5×10^5 freshly harvested bone marrow cells in 0.5 mL RPMI-1640 medium, with 10% FCS in the presence of IL-5 (eosinophil-selective) or GM-CSF (active on both the eosinophil and neutrophil lineages) and incubated at 37°C, 5% CO_2/95% air, for 6 (GM-CSF) or 7 (IL-5) days, before counting total cells in a hemocytometer and determining the frequency of EPO+ cells and/or neutrophils in cytocentrifuge smears. These conditions were shown previously to support eosinophil proliferation and terminal differentiation and to allow detection of enhancing and suppressive effects [20, 21]. In the absence of exogenous IL-5 or GM-CSF, eosinophil differentiation does not occur, and cultures present virtually no EPO+ cells by day 7, containing only macrophages and endothelial/stromal cells, which survive from the bone marrow inoculum.

Where indicated, bone marrow cells were separated (100 ×g, 20 minutes, 20°C) on Percoll (Sigma-Aldrich ref. St. Louis, MO) discontinuous gradients (40%/60%/75% isotonic Percoll in medium with 10% FCS). Cells in the 40%–60% interface (termed the P2 layer), enriched in hemopoietic precursors, including those of the eosinophil lineage, and depleted in mature eosinophils [13, 22] were washed and cultured as above, alone or together with T lymphocytes in a 10 : 1 ratio.

Further characterization involved *semisolid* (clonal) cultures, which allow definition of an impact on lineage-committed progenitors (colony-forming cells). These were established by seeding 2×10^5 cells in 1 mL in 35 mm triplicate culture dishes in a mixture of IMDM with 20% FCS and agar Noble (0.3% final concentration) in the presence of GM-CSF (2 ng/mL). Colonies (defined as the progeny of a single progenitor, totaling at least 50 cells) were scored at day 7 under an inverted microscope [13, 20]. The frequency of eosinophil-containing colonies was determined on agar layers dried, mounted on microscope slides, stained for EPO, and scored under high magnification (400x). We have previously confirmed that these conditions were adequate for counting total myeloid colonies and for accurate differential counts of myeloid colony types on dried agar layers [13, 20].

Statistical Procedures. Comparisons between two groups were made by the two-tailed *t*-test; where indicated, multiple comparisons were made with ANOVA with the Tukey's (HSD) test or with Bonferroni's test.

3. Results

We initially examined the effect of oral OVA preexposure on subsequent bone marrow and antibody responses to sensitization and challenge in mice of different strains. In BP-2 mice (Figure 1(a)), the numbers of EPO+ cells in freshly harvested femoral bone marrow, which reflect ongoing eosinopoiesis in vivo, were significantly different in tolerized-sensitized-challenged (OVA/OVA/OVA) mice relative to those of both sensitized (H_2O/OVA/SAL) and sensitized-challenged (H_2O/OVA/OVA) control groups. By contrast, tolerized-sensitized-unchallenged (OVA/OVA/SAL) controls, despite a trend towards lower eosinophil counts, did not significantly differ from either of the preceding control groups. Upon ex vivo exposure to IL-5 (Figure 1(b)), eosinopoiesis in bone marrow cultures from OVA/OVA/OVA mice was significantly different from the H_2O/OVA/OVA controls at all concentrations of IL-5 tested. In this assay, response in OVA/OVA/SAL was significantly different from the respective H_2O/OVA/SAL controls in the same IL-5 concentration range. In addition, challenge itself had a significant effect, at IL-5 10 ng/mL only (compare H_2O/OVA/SAL and H_2O/OVA/OVA). Hence, both in vivo and ex vivo assays demonstrate a significant effect of oral tolerization on subsequent responses of the bone marrow to S/C. Although the ex vivo assay may be more sensitive, full effectiveness in vivo seems to require sequential oral/subcutaneous/intranasal exposure. Oral tolerization did not prevent production of anti-OVA IgG (Figure 1(c)) or IgE (Figure 1(d)) antibodies. However, it shifted antibody class production towards increased IgG and decreased IgE production. As a consequence, the highest IgG/IgE ratios among all experimental groups were found in OVA/OVA/OVA mice. This coordinate shift in Ig class was considered evidence of immune deviation, rather than classical tolerance due to a failure of B lymphocyte activation.

We further examined the effects of this protocol in BALB/c mice, because a number of mutant strains of this background carry selective defects which can provide

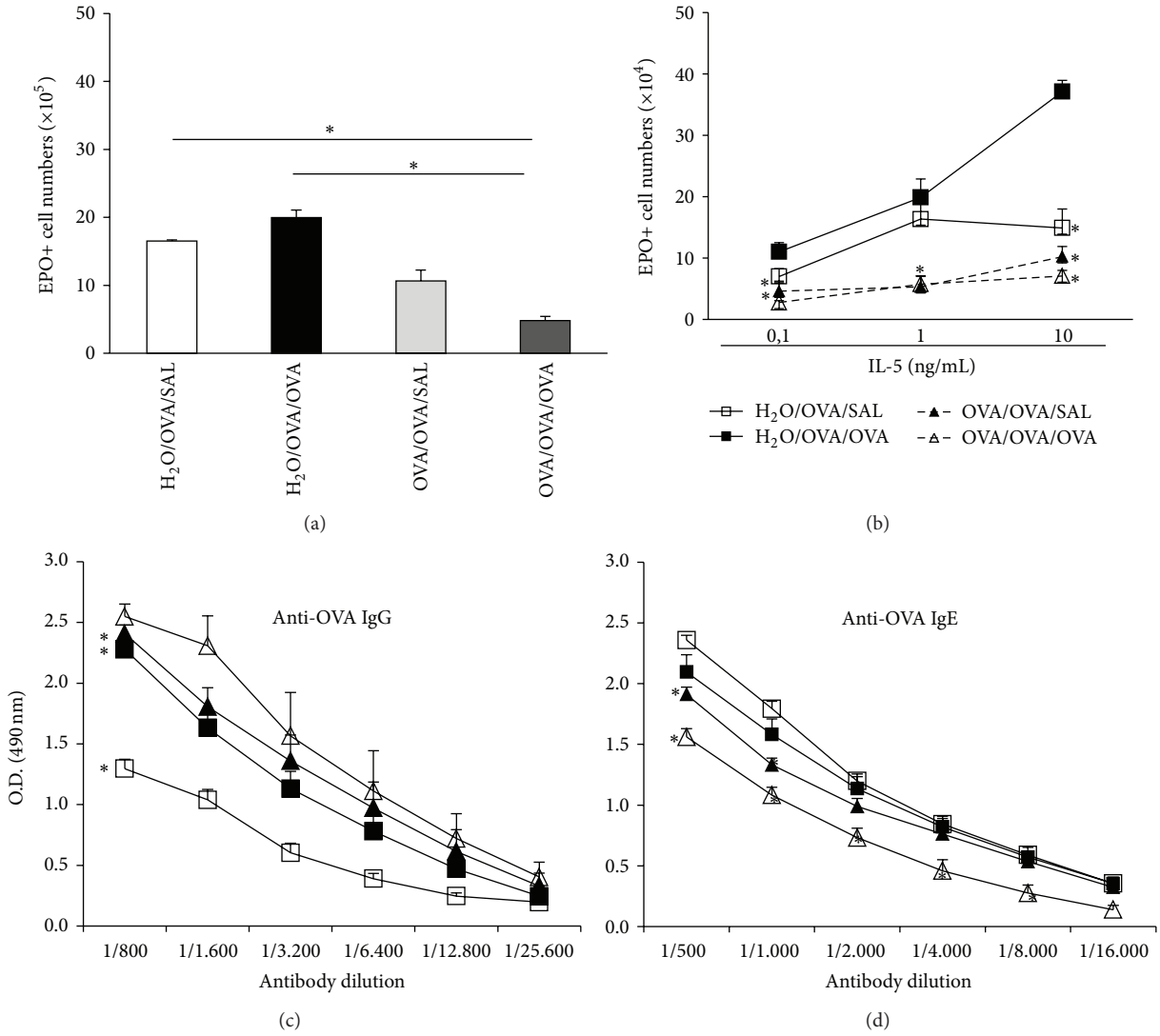

Figure 1: *Effects of oral tolerization on eosinopoiesis and anti-OVA antibody*. BP-2 mice, orally preexposed to OVA and further subcutaneously sensitized/boosted with OVA, were either saline challenged (OVA/OVA/SAL) or OVA challenged (OVA/OVA/OVA) by the intranasal route. Controls were given water, sensitized/boosted, and either saline challenged (H_2O/OVA/SAL) or OVA challenged (H_2O/OVA/OVA). Bone marrow ((a), (b)) and plasma ((c), (d)) were collected 24 h after-challenge. Data are Mean ± SEM. (a) EPO+ cell numbers in freshly collected BM ($n = 6$) ($^*P \leq 0.004$ for the indicated differences). (b) EPO+ cell numbers in bone marrow cultured with the indicated concentrations of IL-5 for 7 days. ($n = 4$) ($^*P \leq 0.015$ for the indicated differences relative to the respective control groups). ((c), (d)) Titration curves for IgG (c) and IgE (d) anti-OVA antibodies ($n = 6$) ($^*P < 0.01$ for the differences relative to the respective control groups).

information on the mechanisms of bone marrow regulation by oral tolerance. Like BP-2 mice, BALB/c submitted to oral tolerization followed by sensitization and challenge (OVA/OVA/OVA) presented significantly reduced EPO+ cell counts in freshly harvested femoral bone marrow, compared with nontolerized H_2O/OVA/OVA controls (Figure 2(a)). We also examined whether oral tolerization affected further the neutrophil lineage, which is closely related to eosinophils [23] but usually present in larger numbers in bone marrow and blood and stimulated by infection rather than by allergen exposure. In BALB/c mice, freshly harvested bone marrow from OVA/OVA/OVA donors presented significantly increased neutrophil counts relative to the H_2O/OVA/OVA controls (Figure 2(b)). Furthermore, OVA/OVA/OVA BALB/c mice presented diminished ex vivo responses to IL-5 in bone marrow culture, as shown by significantly reduced EPO+ cell recovery relative to the same controls (Figure 2(c)). Importantly, tolerization to unrelated antigens (Figure 2(c); BSA and HSA, resp., third and fourth columns), followed by sensitization and challenge with OVA, failed to suppress eosinophil production in IL-5-stimulated cultures, highlighting the need for sequential oral/subcutaneous/respiratory exposure to the *same* antigen. Taken together, these observations pointed to a broad effect of oral tolerization on bone marrow, affecting neutrophils as well as eosinophils, and prompted further examination of granulopoiesis in vivo and ex vivo in the same strain originally used (BP-2).

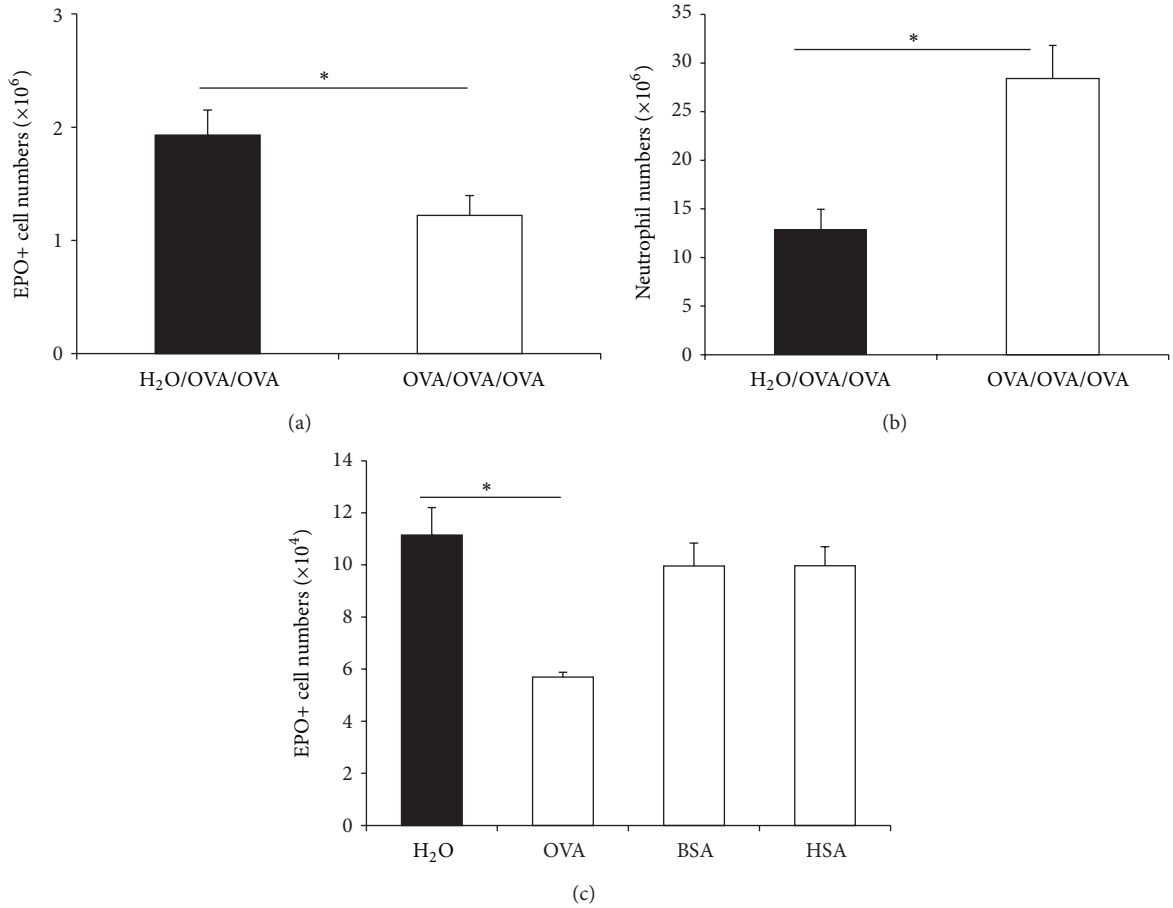

Figure 2: *Target- and stimulus-specificity of the effects of oral tolerization on BALB/c bone marrow*. BALB/c mice were orally preexposed to OVA ((a), (b)) or to the control antigens, BSA and HSA (c), before subcutaneous sensitization and airway challenge with OVA (open bars). Controls received only water before sensitization/challenge (black bars). Data (Mean ± SEM) are (a) EPO+ cell counts and (b) neutrophil counts, in freshly harvested bone marrow ($n = 5$) ($^*P \leq 0.03$); (c) EPO+ cell numbers in bone marrow cultured with 1 ng/mL IL-5 for 7 days ($n = 3$) ($^*P \leq 0.001$).

In BP-2 mice, freshly harvested bone marrow from OVA/OVA/OVA donors presented significantly increased neutrophil counts relative to the H_2O/OVA/OVA controls, along with significantly decreased EPO+ cell counts (Figure 3(a)). Hence, in BP-2 as well as in BALB/c mice (Figures 2(a) and 2(b)), bone marrow eosinophil and neutrophil counts were both affected in vivo by oral tolerization, although in opposite senses. We next examined whether these changes would be paralleled by changes observable ex vivo, when bone marrow was cultured with GM-CSF, a hemopoietic cytokine that, unlike IL-5 (see Figure 1(b)), supports differentiation of both eosinophils and neutrophils (Figures 3(b)–3(d)). Contrary to expectations, GM-CSF-stimulated neutrophil production in liquid culture was significantly reduced in cultures from OVA/OVA/OVA BP-2 mice, relative to the H_2O/OVA/SAL and H_2O/OVA/OVA nontolerized controls (Figure 3(b)). A less marked reduction, which did not reach statistical significance, was also observed in the OVA/OVA/SAL unchallenged controls, relative to the preceding nontolerized control groups. We further explored the effects of oral tolerization on GM-CSF-stimulated neutropoiesis, using colony formation assays to evaluate the responses of myeloid progenitors, which include both neutrophil (GM-, G-) and eosinophil (Eos-) colony forming cells (CFC), as well as mixed (GMEos [23]) CFC. Separate effects of oral tolerization could be detected in these conditions, depending on whether scores were of total myeloid colony numbers (Figure 3(c)) or of eosinophil-, neutrophil-, and macrophage-containing colonies (Figure 3(d)), determined by differential counts on stained agar layers. Myeloid colony formation of all types responsive to GM-CSF was significantly reduced in OVA/OVA/OVA bone marrow relative to the nontolerized control groups (Figure 3(c)). Myeloid colony formation was as effectively suppressed in OVA/OVA/SAL bone marrow as in mice submitted to the full sequence of allergen exposures (OVA/OVA/OVA). This clearly contrasts with observations of neutrophil production in liquid culture (Figure 3(b)) from the same animals, a discrepancy that may reflect the different sensitivity of the liquid versus semisolid culture conditions to the oral tolerization protocol. Importantly, differential counts showed the formation of eosinophil-containing (i.e., the sum of those formed by Eos- and GMEos-CFC) colonies to be significantly reduced, relative to the nontolerized control groups, in

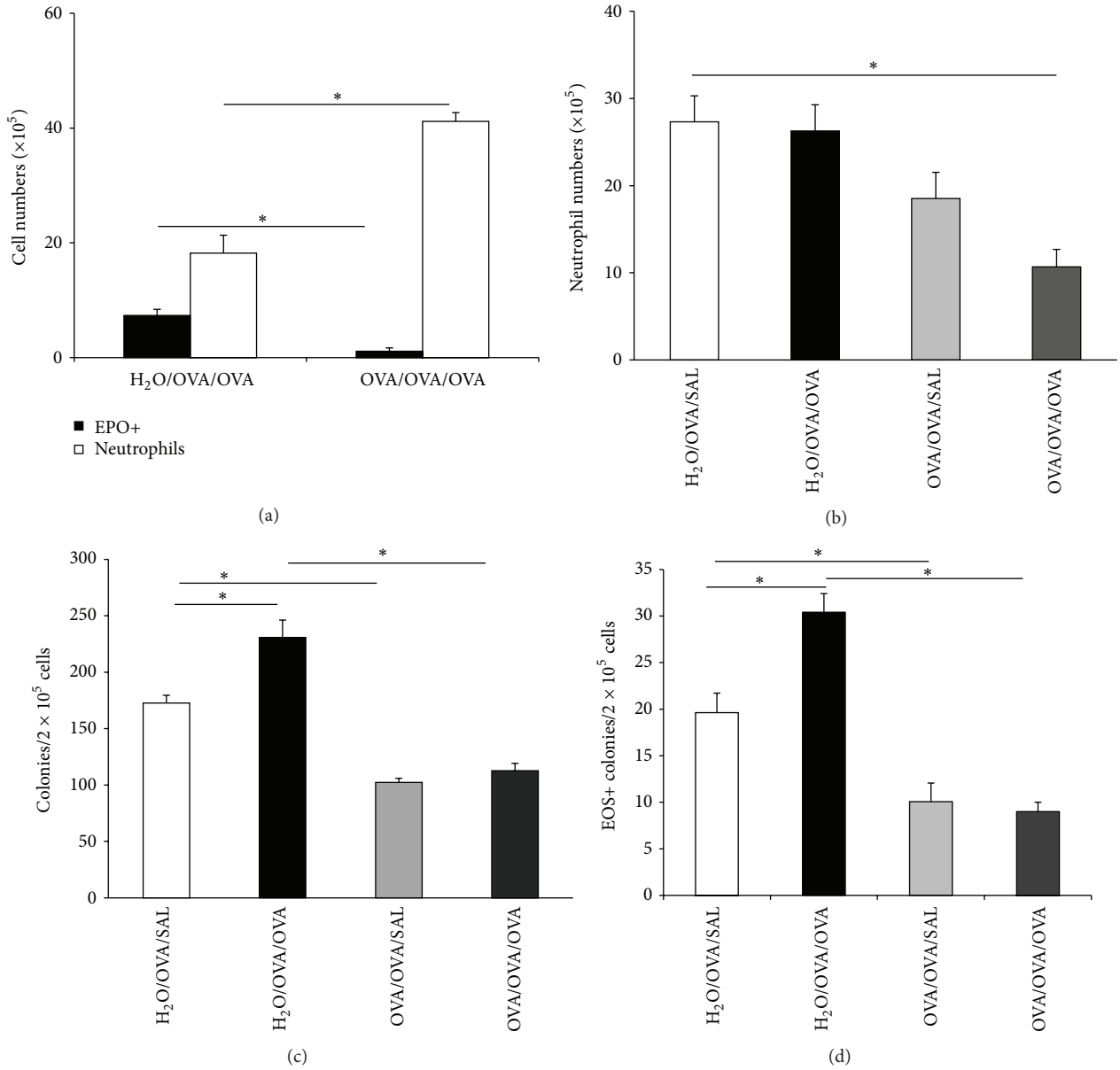

Figure 3: *Coordinate regulation of eosinophil and neutrophil numbers by oral tolerization.* Experimental groups of BP-2 mice were the same as in Figure 1. Data (Mean ± SEM) are (a) numbers of EPO+ cells (black bars) and neutrophils (white bars) in freshly harvested femoral bone marrow of OVA/OVA/OVA and H_2O/OVA/OVA mice ($n = 3$); $^*P \leq 0.003$ for the indicated differences. (b) Numbers of neutrophils recovered after 6 days from liquid bone marrow cultures established with GM-CSF from the indicated groups $n = 4$; $P = 0.005$. (c) Total numbers of colonies formed by bone marrow from the indicated groups in the presence of GM-CSF ($n = 6$); $^*P \leq 0.02$ for the indicated differences. (d) Counts of colonies containing EPO+ cells from the experiment shown in (c), $n = 6$; $P \leq 0.009$ for the indicated differences.

cultures from OVA/OVA/OVA as well as OVA/OVA/SAL BP-2 mice (Figure 3(d)). On the other hand, the formation of neutrophil-containing (i.e., the sum of G-, GM-, and GMEos-CFC) colonies was also reduced in the OVA/OVA/OVA and OVA/OVA/SAL groups, relative to nontolerized controls, mostly accounted for by halving of GM-CFC counts (not shown). Because these effects of oral tolerization on neutropoiesis and eosinopoiesis in semisolid culture do not require the full OVA/OVA/OVA sequence, they clearly contrast with the reduction of eosinophil counts in freshly harvested bone marrow, which was observed in this strain only in OVA/OVA/OVA mice (Figure 1(a)).

One mechanism for blocking the stimulatory effects of lung allergen challenge on eosinophil production, both in vivo and in bone marrow culture, requires iNOS and CD95L, acting sequentially [16]. Because such blockade resembles that achieved by oral tolerization in the present study, insofar as both prevent the impact of airway challenge on the bone marrow, we evaluated the relevance of iNOS and CD95L to the phenomena described above. In a separate set of

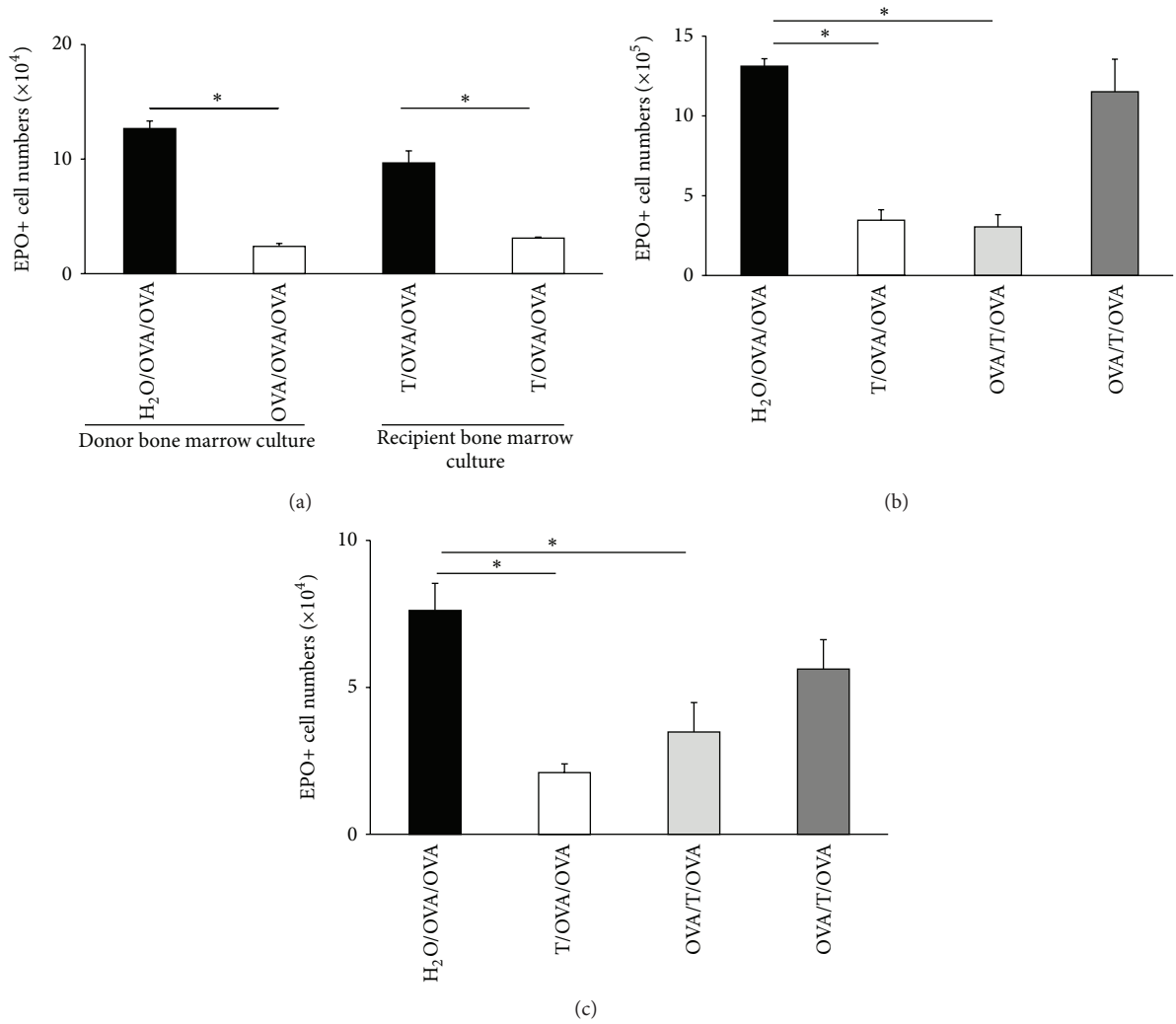

Figure 4: *Effect of splenic T lymphocytes on bone marrow eosinopoiesis in a transfer protocol*. T lymphocytes were isolated from spleens of BP-2 *donor* mice (either OVA/OVA/OVA tolerized or H_2O/OVA/OVA controls) and used for i.v. transfers into syngeneic recipients. *Recipient* mice were either naïve and subsequently sensitized and challenged (T/OVA/OVA) or sensitized before transfer (OVA/T/OVA), challenge occurring after transfer. Data are Mean + SEM of EPO+ cell counts in 7-day IL-5 stimulated bone marrow cultures ((a), (c)) or freshly harvested bone marrow (b). (a) Eosinophil production ex vivo from donor (left) and recipient mice (right). T/OVA/OVA mice received T cells from control (black column) or tolerized (white column) donors. $n = 6$; $P \leq 0.025$ for the indicated differences; (b), (c) EPO+ cell counts in bone marrow (b) and eosinophil production ex vivo (c) in control H_2O/OVA/OVA mice (black columns) or in recipients of T cells from OVA/OVA/OVA donors, with transfer before sensitization (T/OVA/OVA, white bars), after sensitization but before boost (OVA/T/OVA, light grey bars), or after sensitization and boost (OVA/T/OVA, dark grey bars). $n = 3$; $P \leq 0.002$ (b); $P \leq 0.036$ (c) for the indicated differences.

control experiments (not shown), CD95L-deficient mice of the BALB/c background, submitted to the OVA/OVA/OVA protocol, presented downregulation of eosinophil production in vivo that was comparable to that observed in the respective wild-type controls in the same conditions (Figure 2(a)); total myeloid colony counts from semisolid clonal cultures were significantly reduced in OVA/OVA/OVA iNOS-deficient mice of the C57BL/6 background, relative to H_2O/OVA/OVA controls of the same strain; suppression of eosinopoiesis in liquid culture from iNOS-deficient OVA/OVA/OVA mice, as compared to H_2O/OVA/OVA controls, was also observed. Together, these argue against the involvement of iNOS or CD95L in the hemopoietic effects of oral tolerization.

On the other hand, the increase in OVA-specific IgG/IgE ratios induced by oral tolerization, suggestive of class-specific regulation of antibody production by T lymphocyte subpopulations, prompted us to examine a possible contribution of T cells to the complex changes in bone marrow from OVA/OVA/OVA mice. This was examined in transfer experiments, in which T lymphocytes from OVA/OVA/OVA donors were injected i.v. into naïve syngeneic recipients, which were subsequently sensitized and challenged (Figure 4). This experimental design allowed us to separate the *induction* of oral tolerization from the *expression* of its immunoregulatory effects, because oral allergen exposure only occurred in the donor, while its effects mediated by

T lymphocytes were detectable in the recipients, which were themselves never orally exposed to ovalbumin.

As shown in Figure 4, splenic T lymphocytes from OVA/OVA/OVA donors, upon transfer into syngeneic (BP-2) recipients, duplicated the effects of oral tolerization on the bone marrow. Confirming previous observations (Figure 1(b)), oral tolerization in the *direct model* significantly reduced eosinophil production ex vivo in IL-5-stimulated cultures established from bone marrow of tolerized and control donor mice (Figure 4(a), left) . When spleen T cells from donors of these two groups were transferred into naïve recipients, which were themselves subsequently sensitized and challenged ((Figure 4(a), right) , a significant reduction in eosinophil production ex vivo was seen in bone marrow from recipients of T cells from OVA/OVA/OVA donors, relative to H_2O/OVA/OVA controls, showing that this effect of oral tolerization can be duplicated in mice that were never orally exposed to OVA, by merely providing the appropriate T cell population. Importantly, the same effect was observed in freshly harvested bone marrow (Figure 4(b)), confirming its relevance to hemopoiesis in vivo. As further shown (Figures 4(b) and 4(c)), T cell transfer from the appropriate donor retained its effectiveness in the recipient even after the sensitization injection (compare the light grey columns to the white columns in Figures 4(b) and 4(c)). By contrast, transfer after the boost injection (dark grey columns in Figures 4(b) and 4(c)) was no longer able to significantly decrease in vivo (Figure 4(b)) or ex vivo (Figure 4(c)) EPO+ cell counts relative to the respective H_2O/OVA/OVA controls.

Finally, we examined whether splenic T lymphocytes would be able to influence eosinopoiesis ex vivo when co cultured with hemopoietic precursors. As shown in Figure 5, eosinophil production by hemopoietic precursors (fraction P2) isolated on Percoll discontinuous gradients [22] was significantly reduced in bone marrow from OVA/OVA/OVA donors relative to H_2O/OVA/OVA controls. Addition of T lymphocytes from control H_2O/OVA/OVA donors to the culture did not restore the eosinopoietic activity of precursors isolated from tolerized OVA/OVA/OVA donors. By contrast, addition of T lymphocytes from tolerized donors significantly reduced the eosinopoietic activity of precursors isolated from control H_2O/OVA/OVA mice. Together, these observations suggest that oral tolerization induces long-term modification of eosinophil precursors, which may be directly evoked by coculture of nontolerized cells with T lymphocytes from tolerized donors.

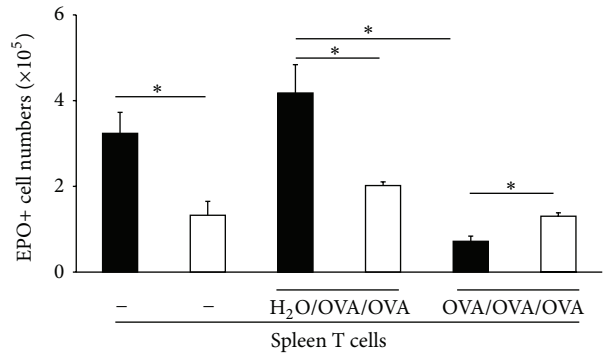

Figure 5: *Splenic T lymphocytes from tolerized donors suppress eosinopoiesis from bone marrow of sensitized/challenged mice in coculture*. Bone marrow and spleen cells were collected from OVA/OVA/OVA donors (white columns) and H_2O/OVA/OVA (black columns) controls. Eosinophil precursors were separated on Percoll discontinuous gradients and cultured in the presence of IL-5 alone (−) or in coculture with splenic T lymphocytes from either experimental group as indicated, in a 10 : 1 ratio. Data are Mean + SEM of EPO+ cell numbers in 7-day cultures ($n = 3$, $^*P \leq 0.02$ for the indicated differences).

4. Discussion

This is, to our knowledge, the first detailed description of the effects of oral tolerization on bone marrow hemopoietic function, both in vivo and ex vivo. It clearly establishes a parallelism between the previously reported modulation of lung immune responses by oral allergen preexposure [3] and a corresponding decrease in the production of eosinophils by the bone marrow. It further documents unexpected effects of oral tolerization on neutrophil numbers and neutrophil production. These effects suggest that shared immunoregulatory mechanisms coordinately regulate both the eosinophil and neutrophil lineages, even though the changes observed are not necessarily in the same direction in both lineages.

We will address below a number of issues that warrant further investigation and the reasons thereof are as follows.

(a) *The role of oral, subcutaneous, and respiratory exposures to the same antigen*. In vivo, the eosinophil lineage was significantly suppressed only in animals which underwent three consecutive exposures to ovalbumin. This is important, because it shows that merely feeding allergen, without further sensitization and challenge, has no detectable impact on bone marrow composition. This is consistent with everyday experience in the consumption of food that contains variable amounts of antigenic proteins, which have no known impact on basic hematological parameters. Equally important is the observation that the antigen used for tolerization must be the same as that used for sensitization and challenge, in order to have a detectable effect in vivo. The need for identity of stimuli at the first and second step strongly suggests that the hematological effect is mediated by antigen-specific (clonal) cell populations and consistent with the evidence that this effect can be duplicated by T lymphocytes, both in transfer protocols and in coculture. The simplest interpretation is that the oral exposure primes a cell population that does not become active effector at this step, but becomes so by further encountering the same antigen at the sensitization and challenge steps. The implication is that such cell population does not stay restricted to the site of priming (presumably the gut-associated lymphoid tissue [1]) but rather circulates freely, as required for a restimulation with antigen injected

subcutaneously in the dorsum. Evidence available so far suggests that it does not become, by this restimulation, able to suppress eosinophil production in vivo, but it may be already capable of interfering with the response of eosinophil precursors to IL-5 ex vivo. The implication would be that the effector cell, even before an airway challenge, is able to affect bone marrow cells. As a nonspecific, systemically active mechanism of action is unlikely, in view of the requirement for specific antigen, we suggest that after restimulation by conventional immunization, we assume that the effector cell does so in close proximity to its targets, which requires the ability to home to the bone marrow, where it can act on eosinophil precursors and progenitors, as shown by data on liquid and semisolid culture, respectively. This is consistent with evidence that T lymphocytes from the appropriate donors can, in coculture with the susceptible targets (precursors from nontolerized donors), effectively suppress eosinophil production. Overall, both the magnitude and apparent selectivity of the suppressive effects in vivo are consistent with the mechanism proposed to account for a decreased eosinophilia of the lung in tolerized animals [3]. They are equally consistent with observations on bone marrow eosinophilia in other models of oral tolerance involving peripheral allergen challenge at sites distinct from the lungs [6].

(b) *The nature and relevance of the effects on the neutrophil lineage*. Two kinds of effects were detected in the bone marrow neutrophils: (a) an in vivo increase in neutrophil numbers, consistent with descriptions of previous studies [6]; (b) an ex vivo decrease in neutrophil production, but in liquid and semisolid culture conditions. The latter could not have been detected in previous studies, which did not examine hemopoiesis, but rather focused on the percent eosinophils and neutrophils in bone marrow samples, which reflect not only production of these granulocyte types, but their rates of emigration from the bone marrow reserve pool as well as their longevity. Percent data can also be artifactually changed by increases or decreases in other bone marrow subpopulations. These pitfalls were avoided by the experimental approach we have taken, and we were able to clearly distinguish between an increase in absolute neutrophil numbers in vivo and a reduced production of neutrophils ex vivo in response to a standard cytokine (GM-CSF) stimulus. We favor the view that the in vivo data are more biologically relevant, and therefore can rule out that neutropenia and abnormal susceptibility to infection result from the oral tolerization protocol. We think, nevertheless, that the ex vivo data on reduced responses to GM-CSF are informative on important cellular events and might shed some light on the mechanisms of bone marrow regulation by oral tolerance.

(c) *The relationship of the effects on the eosinophil lineage to previously described mechanisms*. The observations in this study are inconsistent with the previously described mechanism involving sequential activation of iNOS and CD95L, since oral tolerance was very effective in mutant bone marrow lacking either iNOS or CD95L. By contrast, the effects of oral tolerance involve a decrease in eosinophil-containing colony formation, along with an increase in neutrophil numbers in vivo. This is consistent with observations in mice exposed to G-CSF in vivo, in which eosinophil production and eosinophilic inflammation were reduced, while the neutrophil lineage was stimulated [17]. A possible role for G-CSF in our experimental models, both direct and indirect, should be explored in future studies.

(d) *Ex vivo events can be linked to defective responses through βc-associated receptors*. One of the puzzling observations of this study is that neutrophils are increased in number in vivo, but neutrophil colony formation in the presence of GM-CSF ex vivo is reduced by oral tolerization. These apparently incompatible observations can be reconciled if one takes into account that GM-CSF is not required for neutrophil production in vivo, although it is a powerful stimulus for neutrophil colony formation in culture and most likely plays a role in the stress neutropoiesis associated with infection [24]. Therefore, both sets of observations can be reconciled if oral tolerization decreased the effectiveness of exogenous GM-CSF as a stimulus in culture, but not the production of neutrophils in vivo, which goes unabated even when bone marrow is insensitive to GM-CSF [24]. Interestingly, formation of eosinophil colonies in the presence of GM-CSF and eosinophil differentiation in the presence of IL-5 were both decreased. In this case, reduction of eosinopoiesis ex vivo was paralleled by decreased eosinophil numbers in bone marrow. Again, this requires understanding of the relationship of eosinopoiesis to both IL-5 and GM-CSF. These cytokines signal through distinct receptors, which share a common signaling β chain (βc; [24]). Cells in the eosinophil lineage express both IL-5 and GM-CSF receptors, signaling through which is abolished in βc-deficient mice. Although GM-CSF is not essential for neutrophil nor eosinophil production, IL-5 signaling is a major determinant of eosinophil production in vivo, and βc-deficient mice lack normal numbers of eosinophils [24]. All of our observations would therefore be consistent with a mechanism through which oral tolerization prevented expression or interfered with function of the βc chain. The predictions of this hypothesis would be the following: (a) eosinophils would be similarly reduced both in vivo and ex vivo, since βc-dependent signaling through IL-5 receptors is required in both settings; (b) βc expression would be reduced or absent from cells in both the eosinophil and neutrophil lineages, recovered from bone marrow

of OVA/OVA/OVA mice but not of H_2O/OVA/OVA controls.

(e) *The contribution of T lymphocytes to the regulation of bone marrow eosinophils*. In many studies of oral tolerance, an important role for T lymphocytes has been demonstrated [1, 2, 7, 8, 25–28]. In many cases, these have been isolated from gut-associated lymphoid tissue, including mesenteric lymph nodes, but regulation by oral tolerization-induced splenic T lymphocytes has also been demonstrated by others [26] as well as in the present study. There is considerable variability among different published studies as to the precise phenotype and properties of the immunoregulatory T cells involved [25–28], and at this point there is no obligate profile for a regulatory T cell in an oral tolerance model. As a consequence, the phenotype of a T cell responsible for suppressing eosinopoiesis and possibly increasing neutropoiesis must be established experimentally, rather than theoretically predicted on the basis of findings in other models and laboratories. The limiting factor, in this respect, is the availability of an assay that can accurately and reproducibly detect the relevant cells. While a T cell transfer protocol (Figure 4) is very useful and should be used for addressing questions that can only be answered in vivo (such as, for instance, whether or not they migrate to bone marrow, and which activating steps must occur before this happens), it is unfortunately limited, from a practical point of view, by the requirement for relatively large numbers of cells (10^7 per recipient). We suggest that a coculture assay (Figure 5) circumvents these limitations, since it reproducibly detects activity with a much smaller number of T cells (5×10^4) and allows parallel testing of many variables in a short time and with use of fewer animals. This is the assay that we are currently using to determine the phenotype and mechanism of action of the oral tolerization-induced T cells on bone marrow eosinopoiesis.

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Research Article

Antileukotriene Reverts the Early Effects of Inflammatory Response of Distal Parenchyma in Experimental Chronic Allergic Inflammation

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Aims. Compare the effects of montelukast or dexamethasone in distal lung parenchyma and airway walls of guinea pigs (GP) with chronic allergic inflammation. *Methods*. GP have inhaled ovalbumin (OVA group-2x/week/4weeks). After the 4th inhalation, GP were treated with montelukast or dexamethasone. After 72 hours of the 7th inhalation, GP were anesthetised, and lungs were removed and submitted to histopathological evaluation. *Results*. Montelukast and dexamethasone treatments reduced the number of eosinophils in airway wall and distal lung parenchyma compared to OVA group ($P < 0.05$). On distal parenchyma, both treatments were effective in reducing RANTES, NF-κB, and fibronectin positive cells compared to OVA group ($P < 0.001$). Montelukast was more effective in reducing eotaxin positive cells on distal parenchyma compared to dexamethasone treatment ($P < 0.001$), while there was a more expressive reduction of IGF-I positive cells in OVA-D group ($P < 0.001$). On airway walls, montelukast and dexamethasone were effective in reducing IGF-I, RANTES, and fibronectin positive cells compared to OVA group ($P < 0.05$). Dexamethasone was more effective in reducing the number of eotaxin and NF-κB positive cells than Montelukast ($P < 0.05$). *Conclusions*. In this animal model, both treatments were effective in modulating allergic inflammation and remodeling distal lung parenchyma and airway wall, contributing to a better control of the inflammatory response.

1. Introduction

Asthma plays an important role as a major cause of mortality and morbidity all over the world. Data from the World Health Organization estimates that 100 millions to 150 millions people worldwide have a diagnosis of asthma [1], and around 180.000 people around the world die, every year, from this disease. Moreover, asthma affects people in school age and economically active people, having a high social and economic impact with lost productivity and reduced participation in family life. Therefore, it is considered a public health problem with high cost medications and hospitalizations of patients, enhancing the importance of continuing studying asthma.

Bronchial asthma is a chronic inflammatory disorder involving chronic airway inflammation, tissue remodeling, and declined airway function. The asthma physiopathology involves mediators and many cells, mainly eosinophils which plays important role through the release of specific mediators [2], and the degree of eosinophilia has been related with the disease severity in some asthmatic patients [3].

Eosinophil recruitment at the airway tissue is considered a multifaceted mechanism which involves chemokines such as eotaxins and RANTES. Besides that, a wide array of growth

factors can be involved in airway inflammation, including insulin-like growth factor-1 (IGF-I) which is upregulated during this event [4], contributing with the remodeling process [5]. Furthermore, we may also consider the extracellular matrix (ECM) proteins, as fibronectin for example. Increased deposition of fibronectin and collagen into the subepithelial space of the airways is observed in all forms of asthma and appears very early in the disease [6].

Much of the subjacent inflammation of asthma can be mediated by the transcription nuclear factor-kappa B (NF-κB), which is involved in the regulation of many of the inflammatory proteins that are expressed in asthmatic airways [7].

The importance of distal lung responses to the global pulmonary alterations enhancing asthma symptoms, particularly on severe asthma, has been recently addressed [8–10]. The inflammation process in the peripheral airways has a highaffinity to cysteinyl leukotriene-1 receptors ($CysLT_1Rs$), and it is known that inhaled therapies are insufficient to achieve the small airways [11]. It is also important to highlight that important features of remodeling seem to occur mainly in the small airways [12], emphasizing the importance of targeting peripheral inflammation to achieve the disease control.

Our group had previously shown that in the ovalbumin-induced guinea pig asthma model, repeated allergen exposure leads to chronic allergic airway and distal lung parenchyma inflammation, characterized by an influx of eosinophils, mast cells, and allergen-specific T cells, a Th2-type cytokine pattern and airway and tissue remodeling [8, 13, 14].

Corticosteroids are the first-line therapy to threat asthma patients and were considered the most important drugs to improve hyperreactivity and bronchial hyperresponsiveness. However, antileukotrienes may also be considered to control asthma patients since cysteinyl leukotrienes (CysLTs) have been known to play an important role in bronchoconstriction and airway inflammation. CysLTs are synthesized from arachidonic acid and most of their actions are mediated by the $CysLT_1Rs$, a G protein-coupled receptor. CysLTs have many pulmonary actions, including human airway smooth muscle contraction, chemotaxis, mucous secretion, smooth muscle proliferation, and increase in vascular permeability [15–18].

Clinical trials with anti-leukotriene drugs in mild and moderate persistent asthmatics have shown improvement in pulmonary function, symptoms, and need for rescue medications and a reduction in asthma exacerbations [19, 20]. It has also been shown that antileukotrienes reduce sputum and mucosal eosinophils in subjects with asthma [21]. However, recent long duration trials have evaluated the impact of antileukotrienes in comparison to glucocorticoids and showed that symptoms, spirometry, β_2-agonist use, and also quality of life were improved to a greater extent with glucocorticoids [22, 23].

Using the same experimental model described in the present study, we observed that both montelukast or dexamethasone treatments diminished collagen fiber content, metallopeptidase inhibitor-1 (TIMP-1), matrix metallopeptidase-9 (MMP-9) and transforming growth factor (TGF-β) in distal lung parenchyma. Nonetheless, concerning elastic fiber content, dexamethasone's treatment did not reduce this response [24]. These foundings are in agreement with the results observed by Goleva et al. [25], in the evaluation of asthmatic patients resistant or not to corticosteroids.

Concerning these aspects, we considered pertinent to evaluate the effects of corticosteroids (dexamethasone) and antileukotrienes (montelukast sodium) in the eosinophilic inflammation and remodeling process modulated by fibronectin and IGF-I in an animal model of chronic allergic inflammation, studying both compartments, airway walls, and distal lung parenchyma.

2. Material and Methods

Guinea pigs (GP) were maintained in an animal facility with a 12-hour light/dark cycle and fed water and chow *ad libitum*. GP received humane care in compliance with the "Guide for care and use of laboratory animals" [26], and all experiments described in this study were previously approved by the Institutional Review Board of the University of São Paulo (São Paulo, Brazil/CAPpesq number 0276/09).

2.1. Experimental Groups. Four groups of GP were studied: (a) saline group (SAL, $n = 8$); (b) ovalbumin-sensitized GP (OVA, $n = 8$); (c) ovalbumin-sensitized GP treated with montelukast (OVA-M, $n = 8$); (d) ovalbumin-sensitized GP treated with dexamethasone (OVA-D, $n = 8$).

2.2. Induction of Chronic Pulmonary Allergic Inflammation. Male Hartley GP, weighing 300–400 g, were placed in a plexiglass box (30 × 15 × 20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of ovalbumin (OVA, Grade V, Sigma Chemical Co., Saint Louis, MO, USA) diluted in 0.9% NaCl (sterile saline solution) was prepared. The animals received seven inhalations during four weeks with increasing concentrations of OVA (1~5 mg/mL) in order to avoid tolerance. Control animals received aerosolized normal saline. The solution was continuously aerosolized into the environment until respiratory distress occurred, or until 15 minutes had elapsed, as previously described [13, 14, 27]. The observer who made the decision to withdraw the guinea pig from the inhalation box was blinded to the treatment status of the animal.

2.3. Montelukast and Dexamethasone Treatments. In order to avoid interference with sensitization, only after twenty-four hours of the fourth inhalation guinea pigs started to receive either daily oral montelukast sodium 10 $mg{\cdot}kg^{-1}$ (four hours before inhalations of ovalbumin and at the same hour on the other days) or dexamethasone (5 $mg{\cdot}kg^{-1}$/day i.p.) [13, 28].

2.4. Morphometric Studies. After 4 weeks, guinea pigs were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), tracheostomized, and ventilated at 60 breaths/min (Harvard Apparatus South Natick, MA). Ten minutes after OVA challenge, a positive end-expiratory pressure of 5 cm H_2O was applied to the respiratory system, and the airways

were occluded at the end of expiration. GP were exsanguinated via the abdominal aorta, and lungs were removed *en bloc*.

Lungs were fixed with buffered 4% formaldehyde. Sections representing peripheral areas were processed for paraffin embedding. Histological sections of 3–5 μm in thickness were stained and evaluated by persons blinded to the protocol design. Morphometric analysis was performed with a light microscope (Nikon, E200, City, Japan), with an integrating eyepiece (composed of 100 points and 50 lines) using a point-counting technique [13, 14, 29].

Three to five airways randomly selected from each lung slide were focused at a magnification of ×1,000. The number of eosinophils and positive cells for IGF-I, eotaxin, RANTES, fibronectin, and NF-κB was determined as numbers of points of the integrating eyepiece falling on areas of cells or positive cells in three randomly selected areas of each airway wall divided by the number pointing in the airway wall area (10^4 μm^2) [13, 14, 29].

To measure the number of positive cells in distal lung parenchyma, we counted in 10 fields per slide the number of eosinophils and positive cells for IGF-I, eotaxin, RANTES, fibronectin, and NF-κB in each field divided by alveolar tissue area. Measurements were expressed as cells/10^4 μm^2, at ×1,000 magnification (10^4 μm^2), divided by the number pointing in the parenchyma area (10^4 μm^2) [29, 30].

2.5. Measurement of Eosinophil Density. Five μm thick slides of lung were stained with Luna [13, 31]. We analyzed the density of eosinophils within the alveolar septa and in airway walls as described above. Measurements were expressed as cells/10^4 μm^2.

2.6. Immunohistochemistry. Immunohistochemistry was performed with anti-IGF-I (1:75; Santa Cruz—Sc 9013), anti-NF-κB (1:50; Santa Cruz—Sc 109), anti-fibronectin (1:400; DAKO 0245), anti-eotaxin (1:100; Santa Cruz—Sc 6181), and anti-RANTES (1:400; Santa Cruz—Sc 1410) antibodies, by peroxidase method [24, 29].

Using a 100-point grid (area: 10^4 μm^{2}at ×1,000 magnification), the number of the positive cells for IGF-I, eotaxin, RANTES, fibronectin, and NF-κB was counted as mentioned above in Section 2.4.

2.7. Data Analysis. Values are expressed as mean ± standard error (SEM). Statistical analysis was performed using *SigmaStat* software (SPSS Inc, Chicago, IL, USA). Data were evaluated by one-way analysis of variance (ANOVA) and multiple comparisons were made using *Holm-Sidak* method. The P value < 0.05 was considered significant [32].

3. Results

3.1. Measurements of Eosinophils Density. Figure 1(a) presents the mean and SEM values of eosinophilic recruitment in distal lung parenchyma. We observed a significant increase in the number of eosinophils (cells/10^4 μm^2) in OVA group (9.91 ± 0.70) compared to control (SAL group: 2.62 ± 0.26). There was a decrease of eosinophils in OVA-M (3.03 ± 0.28) and OVA-D (4.17 ± 0.36) compared to OVA($P < 0.05$). There were no significant differences between OVA-M group and OVA-D group.

Figure 1(b) presents the mean and SEM values of eosinophilic recruitment in airway walls. We observed a significant increase in the number of eosinophils (cells/10^4 μm^2) in OVA group (17.72±3.79) compared to control (SAL group: 4.89 ± 0.97). There was a decrease of eosinophils in OVA-M (11.55±0.87) and OVA-D (8.05±0.67) compared to OVA($P < 0.05$). There were no significant differences between OVA-M group and OVA-D group.

3.2. Measurements of Eotaxin Expression. Considering eotaxin expression in inflammatory cells (Figure 2(a)), we observed a significant increase in the number of eotaxin positive cells (10^4 μm^2) in distal lung parenchyma in OVA group (19.38 ± 0.79) compared to control (SAL group: 3.00 ± 0.39, $P < 0.001$). There was a decrease of eotaxin positive cells in OVA-M (5.65 ± 0.38) and OVA-D (9.9 ± 0.54) compared to OVA($P < 0.001$). There was a significant reduction of eotaxin positive cells in OVA-M compared to OVA-D ($P < 0.001$).

Figure 2(b) shows eotaxin expression in inflammatory cells in airway walls. We observed a significant increase in the number of eotaxin positive cells (10^4 μm^2) on airway walls in OVA group (13.23 ± 1.18) compared to control (SAL group: 6.92 ± 0.99, $P < 0.001$). There was a significant decrease of eotaxin positive cells in OVA-M (9.29 ± 0.86) and in OVA-D (7.03 ± 0.70) compared to OVA ($P < 0.001$). There were no differences between OVA-D and OVA-M groups.

3.3. Measurements of RANTES Expression. The RANTES expression in distal lung parenchyma is shown in Figure 3(a). There was a significant increase in RANTES expression (positive cells/10^4 μm^2) in OVA group (19.84 ± 1.25) compared to control (SAL group: 2.40 ± 0.35, $P < 0.001$). There was a significant decrease of RANTES positive cells in OVA-M (6.44 ± 0.66) and in OVA-D (5.47 ± 0.54) compared to OVA ($P < 0.001$). There were no differences between OVA-D and OVA-M groups.

Figure 3(b) shows RANTES expression in airway walls (positive cells/10^4 μm^2). There was a significant increase in RANTES expression in OVA group (27.56 ± 1.83) compared to control (SAL group: 18.88 ± 1.31, $P < 0.05$). There was a significant decrease of RANTES positive cells in OVA-M (18.14 ± 1.30) and in OVA-D (20.05 ± 1.24) compared to OVA ($P < 0.05$). There were no differences between OVA-D and OVA-M groups.

3.4. Measurements of IGF-I. Considering IGF-I expression in inflammatory cells (Figure 4(a)), we observed a significant increase in the number of IGF-I positive cells (10^4 μm^2) in distal lung parenchyma in OVA group (22.89 ± 1.16) compared to control (SAL group: 4.87 ± 0.93, $P < 0.001$). There was a decrease of IGF-I positive cells in OVA-M (11.83 ± 0.51) and OVA-D (6.21 ± 0.56) compared to OVA

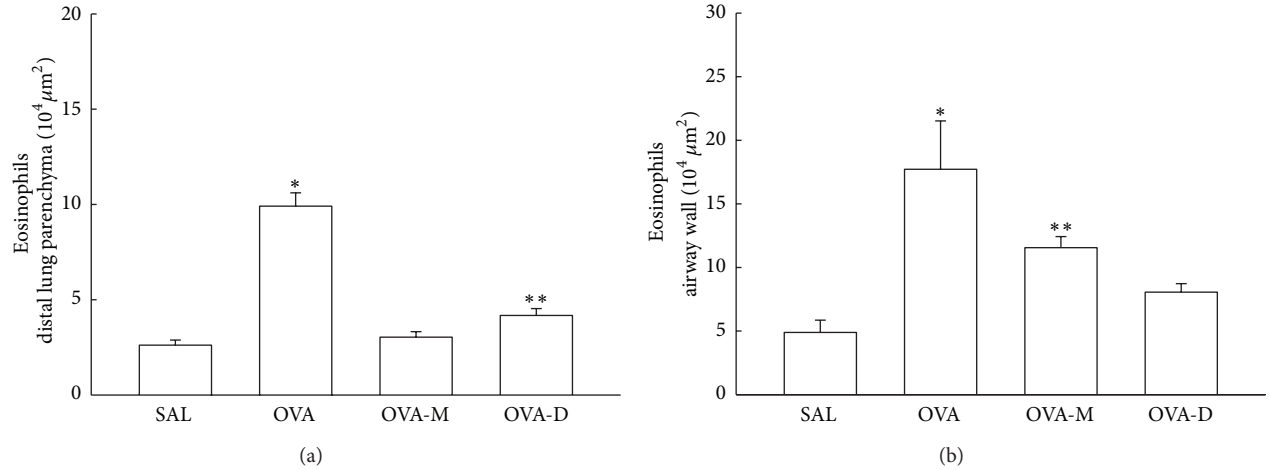

Figure 1: (a) Mean and SEM values of eosinophil in distal lung of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.05$ compared to the other groups. $^{**}P < 0.05$ compared to SAL group. (b) Mean and SEM values of eosinophil in airway wall of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.05$ compared to the other groups. $^{**}P < 0.05$ compared to SAL group.

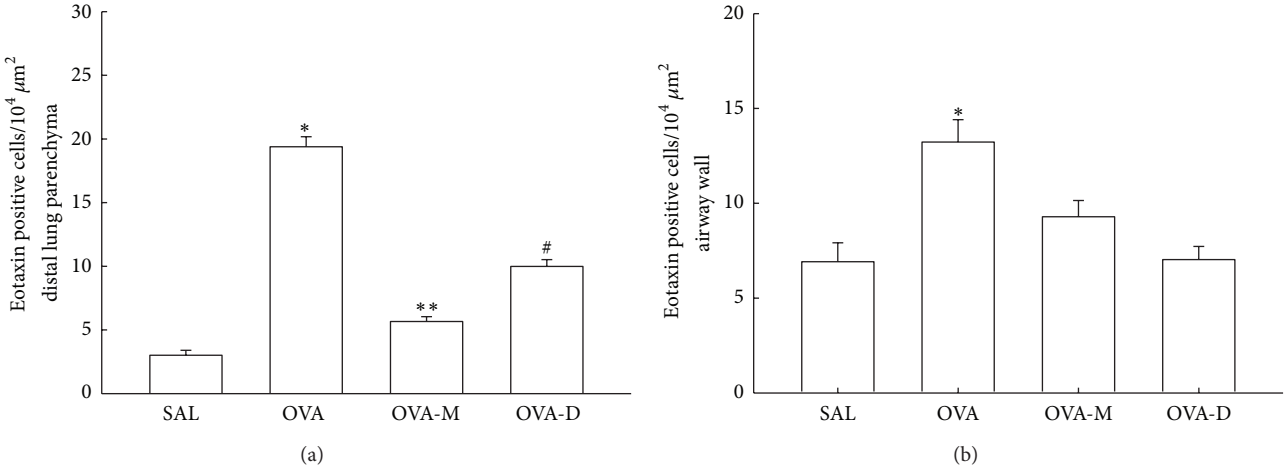

Figure 2: (a) Mean and SEM values of eotaxin positive cells in distal lung of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups. $^{**}P < 0.001$ compared to SAL and OVA-D groups. $^{\#}P < 0.001$ compared to SAL group. (b) Mean and SEM values of Eotaxin positive cells in airway wall of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to SAL, OVA-M and OVA-D groups.

($P < 0.001$). There was a significant reduction of IGF-I positive cells in OVA-D compared to OVA-M ($P < 0.001$). There were no differences between OVA-D compared to SAL group.

Figure 4(b) presents the mean and SEM values of IGF-I positive cells (10^4 μm^2) in airway walls. We observed a significant increase in expression of IGF-I positive cells in OVA group (50.70 ± 1.43) compared to control (SAL group: 25.70 ± 1.79). There was a decrease of IGF-I positive cells in OVA-M (25.53 ± 1.36) and OVA-D (20.90 ± 4.35) compared to OVA ($P < 0.001$). There were no differences between OVA-M and OVA-D groups.

3.5. Measurements of Fibronectin Positive Cells. Figure 5(a) shows the number of fibronectin positive cells (10^4 μm^2) in distal lung parenchyma. We observed a significant increase in the number of fibronectin positive cells in OVA group (25.25 ± 0.90) compared to control (SAL group: 8.61 ± 0.70, $P < 0.001$). There was a significant decrease in the number of fibronectin positive cells in OVA-M (13.41 ± 0.47) and OVA-D (14.61 ± 0.82), compared to OVA group ($P < 0.001$). There were no significant differences between OVA-D and OVA-M groups.

Figure 5(b) shows the number of fibronectin positive cells (10^4 μm^2) in airway walls. We observed a significant increase

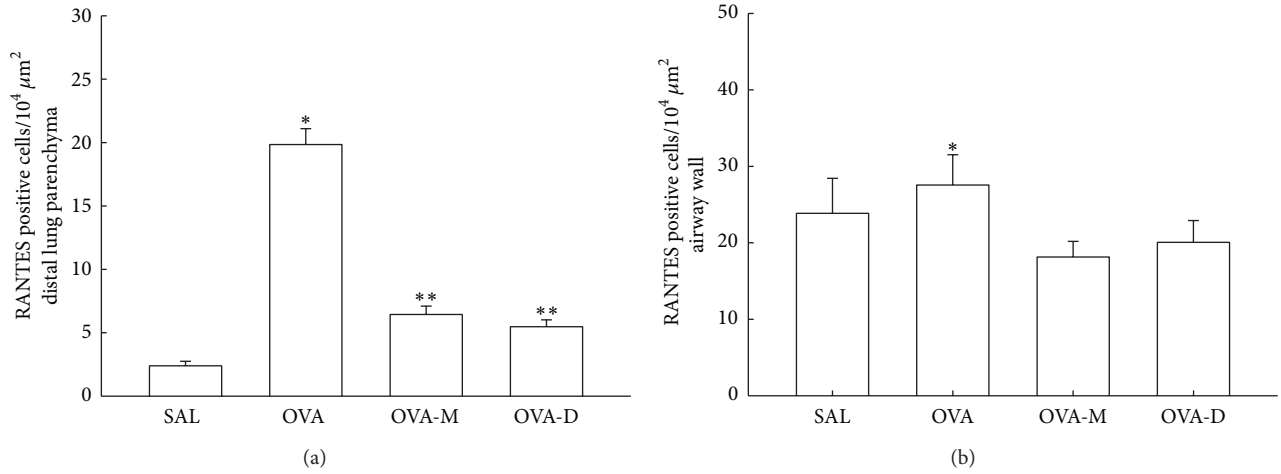

Figure 3: (a) Mean and SEM values of RANTES positive cells in distal lung of GP that previously inhaled normal saline or ovalbumin and after the 4th inhalation GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups. $^{**}P < 0.001$ compared to SAL group. (b) Mean and SEM values of RANTES positive cells in airway wall of GP that previously inhaled normal saline or ovalbumin and after the 4th inhalation GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.05$ compared to the other groups.

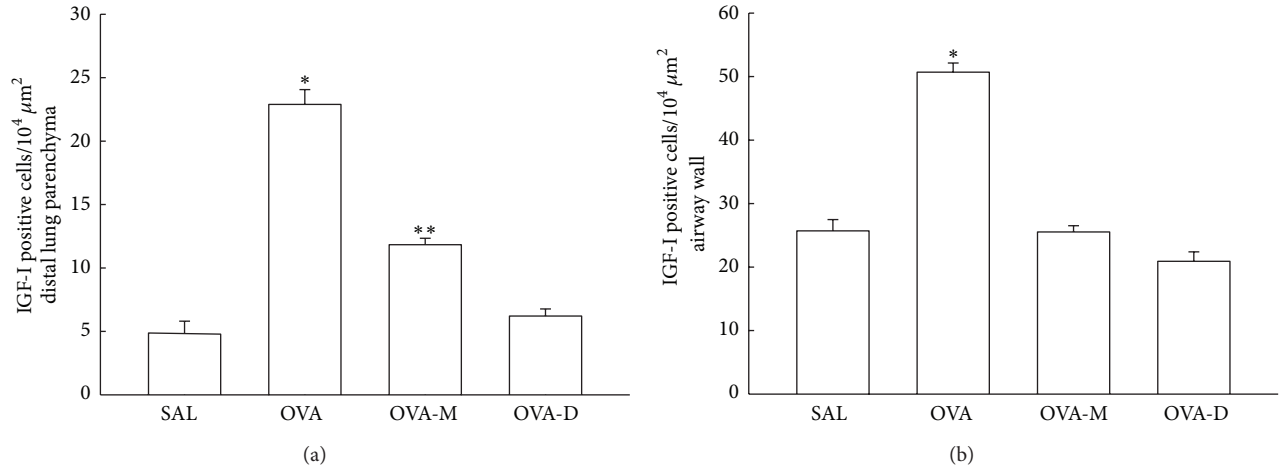

Figure 4: (a) Mean and SEM values of IGF-I positive cells in distal lung of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups. $^{**}P < 0.001$ compared to SAL and OVA-D groups. (b) Mean and SEM values of IGF-I positive cells in airway wall of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups.

in the number of fibronectin positive cells in OVA group (33.69 ± 1.66) compared to control (SAL group: 10.35 ± 5.98, $P < 0.001$). There was a significant decrease in the number of fibronectin positive cells in OVA-M (5.21 ± 2.47) and OVA-D (10.88 ± 3.33) animals compared to OVA group ($P < 0.001$). There were no significant differences between OVA-D and OVA-M groups.

3.6. Measurements of NF-κB. Figure 6(a) shows the NF-κB expression in distal parenchyma. We observed a significant increase in the number of NF-κB (positive cells/$10^4\,\mu m^2$) in OVA group (31.38 ± 5.21) compared to control (SAL group: 5.38 ± 0.79, $P < 0.001$). There was a significant decrease in the number of NF-κB positive cells in OVA-M (11.04 ± 0.86) and OVA-D (8.06 ± 0.70) compared to OVA group ($P < 0.001$). There were no differences between OVA-M and OVA-D groups.

Figure 6(b) shows the NF-κB expression in airway walls. We observed a significant increase in the number of NF-κB (positive cells/$10^4\,\mu m^2$) in OVA group (26.20 ± 1.34) compared to control (SAL group: 11.95 ± 0.96, $P < 0.05$). There was a significant decrease in the number of NF-κB positive cells in OVA-M (12.84 ± 1.09) and OVA-D (8.95 ± 0.78) compared to OVA group ($P < 0.05$). Nevertheless, dexamethasone group (OVA-D) was significantly reduced in comparison to montelukast group (OVA-M). There were

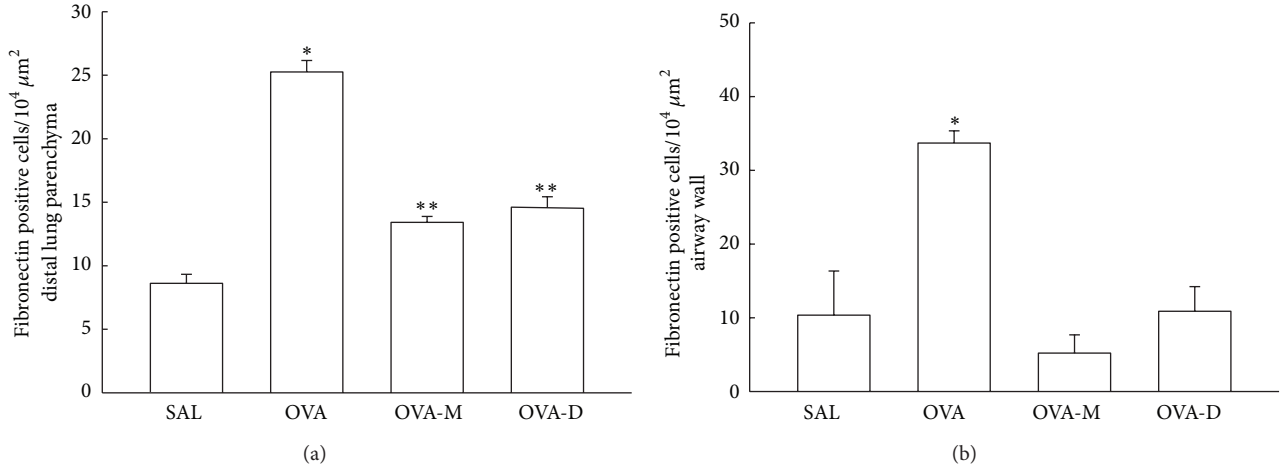

Figure 5: (a) Mean and SEM values of fibronectin positive cells in distal lung of GP that previously inhaled with normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups. $^{**}P < 0.001$ compared to SAL group. (b) Mean and SEM values of Fibronectin positive cells in airway wall of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups.

no differences between OVA-M and OVA-D groups when compared to SAL group.

3.7. Representative Photomicrographs of Airway Walls. Figure 7 shows representative photomicrographs of guinea pigs airway wall samples. We observed a significant increase in the number of eosinophils, eotaxin positive cells, RANTES positive cells, IGF-I positive cells, fibronectin positive cells, and NF-κB positive cells in OVA group compared to control (SAL group). There was a significant decrease in eosinophils, eotaxin positive cells, RANTES positive cells, IGF-I positive cells, fibronectin positive cells, and NK-κB positive cells in OVA-M and OVA-D compared to OVA group.

3.8. Representative Photomicrographs of Distal Lung Parenchyma. Figure 8 presents representative photomicrographs of guinea pigs distal parenchyma samples. We observed a significant increase in the number of eosinophils, eotaxin positive cells, RANTES positive cells, IGF-I positive cells, fibronectin positive cells, and NF-κB positive cells in OVA group compared to control (SAL group). There was a significant decrease in eosinophils, eotaxin positive cells, RANTES positive cells, IGF-I positive cells, fibronectin positive cells, and NK-κB positive cells in OVA-M and OVA-D compared to OVA group.

It is important to mention that two more control groups were performed: saline groups treated either with montelukast (SAL-M) or dexamethasone (SAL-D). There were no statistical significant differences among the three control groups, so we showed only one of them.

4. Discussion

Although corticosteroids are highly recommended as a first line therapy for asthma, it is important to highlight that montelukast can have a consistent benefit in controlling asthma symptoms and can be used as an alternative for those patients who have difficulties using inhaled corticosteroids (especially young children) or have some kind of intolerance with dexamethasone therapy [33]. Furthermore, a reduced adherence with inhalants for asthma compared to those therapies which are orally administrated has been addressed [34, 35].

To elucidate the role of antileukotrienes and corticosteroids in the control of changes in asthma remodeling, we evaluated the effects of montelukast or dexamethasone treatments on eosinophilic recruitment, including its activation by detecting eotaxin and RANTES positive cells in airways and lung parenchyma in guinea pigs (GP), with chronic allergic lung inflammation. Furthermore, to better comprehend the mechanisms involved in the remodeling process, we evaluated IGF-I and fibronectin in distal lung parenchyma and airway walls. Considering the importance of NF-κB, one of the most important transcriptional factors involved in asthmatic responses, we also analyzed the expression of this protein in both pulmonary compartments.

We initiated these treatments only one day after the fourth inhalation, because in a previous study, using passive cutaneous anaphylaxis technique (PCA) [10], we observed an increase in specific immunoglobulin G1 (IgG1) homocytotropic anaphylactic antibodies in GP that received four inhalations with ovalbumin (at the end of the second week of this protocol).

Dexamethasone is considered a very potent anti-inflammatory therapy available for asthma. Its effects are probably related to a broad anti-inflammatory actions, including a decrease in airway infiltration of lymphocytes and eosinophils [36]. However, it is important to emphasize that one of the major challenges in using corticosteroids is related with their side effects [37], corroborating the need for better

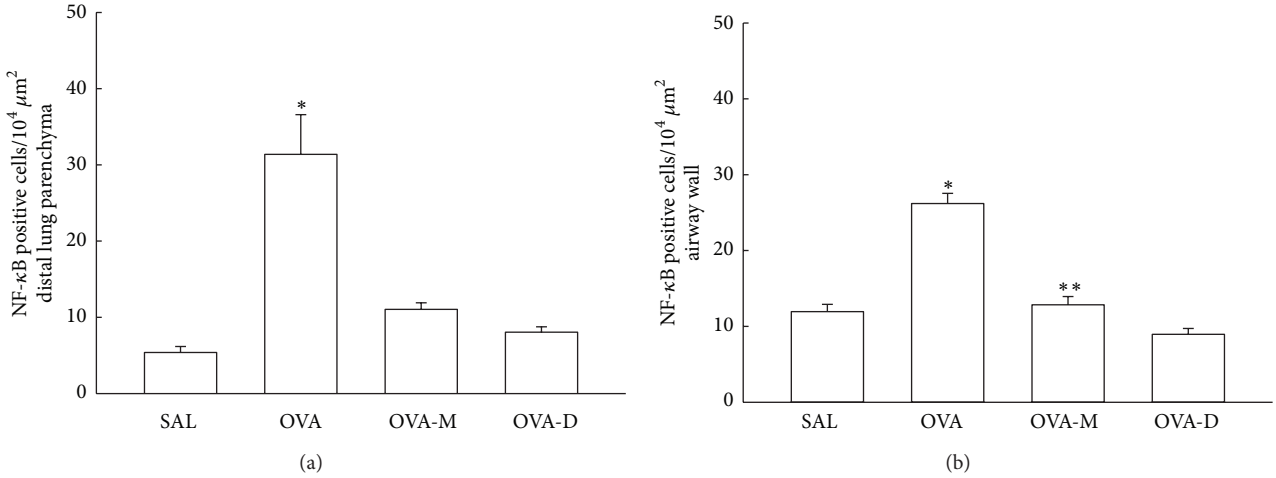

Figure 6: (a) Mean and SEM values of NF-κB positive cells in distal lung of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.001$ compared to the other groups. (b) Mean and SEM values of NF-κB positive cells in airway wall of GP that previously inhaled normal saline or ovalbumin, and after the 4th inhalation, GP were treated with montelukast (OVA-M group) and dexamethasone (OVA-D group). $^{*}P < 0.05$ compared to the other groups. $^{**}P < 0.05$ compared to OVA-D group.

treatments that can improve the quality of life of asthmatic patients.

CysLTs play a major role in the pathophysiology of asthma, activating and recruiting some inflammatory cells, promoting airway remodeling, and increasing bronchial hyperreactivity and vascular permeability [38, 39].

Antileukotrienes are a new class of anti-inflammatory drugs, which includes, Zafirlukast, Montelukast, Pranlukast, and Zileuton. Montelukast is known to be a highly selective, pharmacological antagonist of type 1 cysteinyl leukotriene receptors ($CysLT_1Rs$) which can recognize the cysteinyl leukotrienes (CysLTs) LTD_4 and LTC_4/LTE_4 expressed on some inflammatory cells such as basophils, neutrophils, and lymphocytes and also on some structural cells such as fibroblasts, myofibroblasts, smooth muscle cells, and epithelial cells [38].

Since montelukast acts as a potent antagonist of the proinflammatory aspects of the CysLTs, it reduces asthmatic inflammation and airway resistance and also prevents bronchoconstriction, controlling asthma inflammation and improving inflammatory and pulmonary responses [40, 41].

Many studies in patients with asthma demonstrated a decrease in the number of eosinophils in the airways after treatment with corticosteroids [42]. Corticosteroids can cause the induction of eosinophils apoptosis by the mechanism known as programmed cell death, acting as a target therapy in asthmatic patients [43]. McMillan et al. [21] showed in mice chronically exposed to ovalbumin that chronic administration of budesonide was able to decrease airway hyperreactivity, as well as leukocyte infiltration, and decreased production of Th2 mediators such as interleukin 4 (IL-4), interleukin 12 (IL-12), and eotaxin-1.

Concerning eosinophil recruitment, we observed that both treatments tested were efficient in reducing this infiltration in distal lung and in airway walls.

Not only cysteinyl leukotrienes can act as a chemotactic for eosinophils, but also RANTES, eotaxin and eotaxin 2 are chemotactic substances that are involved during eosinophilic recruitment [44–46].

Eotaxin and RANTES are known as members of the C-C family of chemokines. The most important characteristic of the C-C chemokines is their facility to chemoattract and activate inflammatory leucocytes, particularly lymphocytes, monocytes, eosinophils, and basophils, and also some stromal cells such as endothelial and smooth muscle cells [47]. Another effect of eotaxins is their ability to cause immunoglobulin E(IgE)-independent degranulation of basophils [48].

A family of receptors mediates the activities of C-C chemokines; in general, any C-C chemokine can bind to several C-C receptors. Eotaxin is exceptional and binds only to the chemokine receptor type 3 (CCR3). Furthermore, eotaxin can chemoattract and activate eosinophils specifically, while RANTES are less specific to eosinophils, chemoattracting and activating other inflammatory cells as monocytes and lymphocytes [49].

Montelukast has mainly antieosinophil properties [50, 51]. This could be one of the possible explanations in our study suggesting why montelukast showed a better performance when compared to dexamethasone treatment while reducing eotaxin positive cells in distal parenchyma. Another possibility is that eotaxins are considered to be more specific than RANTES when activating eosinophils during the inflammation process.

A huge number of cells expressing eotaxin mRNA and protein in the bronchial mucosa area of atopic asthmatics compared with controls have been demonstrated by Ying et al. [52] and Mattoli et al. [53]. Furthermore, Ying et al. [52] showed CCR3 expression on local infiltrating eosinophils. These studies reinforce the theory that eotaxin, by the action

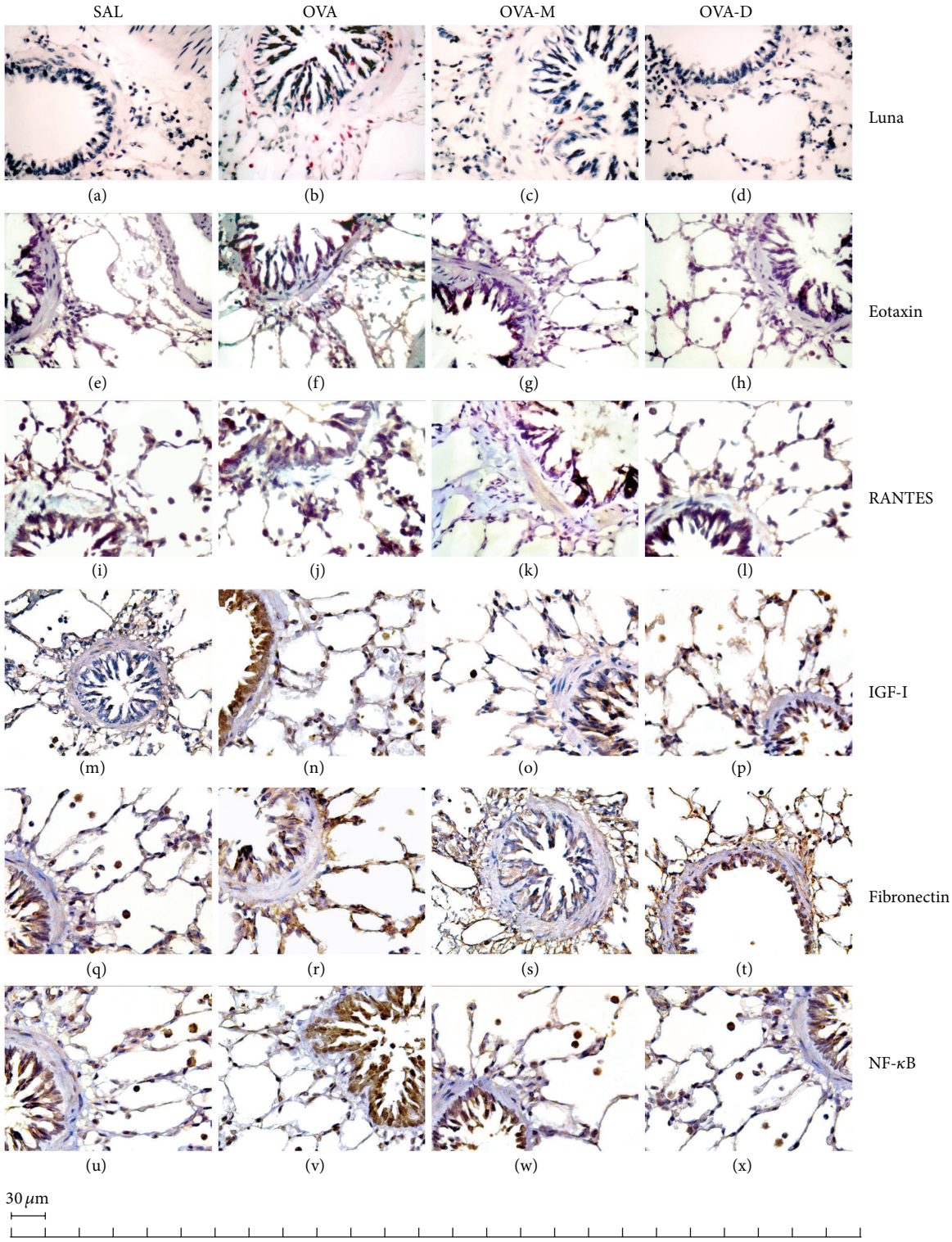

Figure 7: Representative photomicrographs showing guinea pig airway wall samples obtained from controls ((a), (e), (i), (m), (q), and (u)), OVA-exposed ((b), (f), (j), (n), (r), and (v)), OVA-exposed MK treated ((c), (g), (k), (o), (s), and (w)), and OVA-exposed D treated ((d), (h), (l), (p), (t), and (x)) stained with LUNA ((a) to (d)—×1000) and immunohistochemistry for eotaxin detection ((e) to (h)—×1000), RANTES detection ((i) to (l)—×1000), IGF-I detection ((m) to (p)—×1000), fibronectin detection ((q) to (t)—×1000), and NF-κB detection ((u) to (x)). Control groups show a scarce number of eosinophils, eotaxinpositive cells, RANTES positive cells, IGF-I positive cells, fibronectin positive cells, and NF-κB positive cells. In contrast, the airway wall of OVA-exposed animals shows intense eosinophilic infiltration (b), a large number of eotaxin positive cells (f), RANTES positive inflammatory cells (j), IGF-I positive inflammatory cells (n), fibronectin positive inflammatory cells (r), and NF-κB positive inflammatory cells. Both treatments (MK and D) in ovalbumin-exposed animals reduced all these parameters.

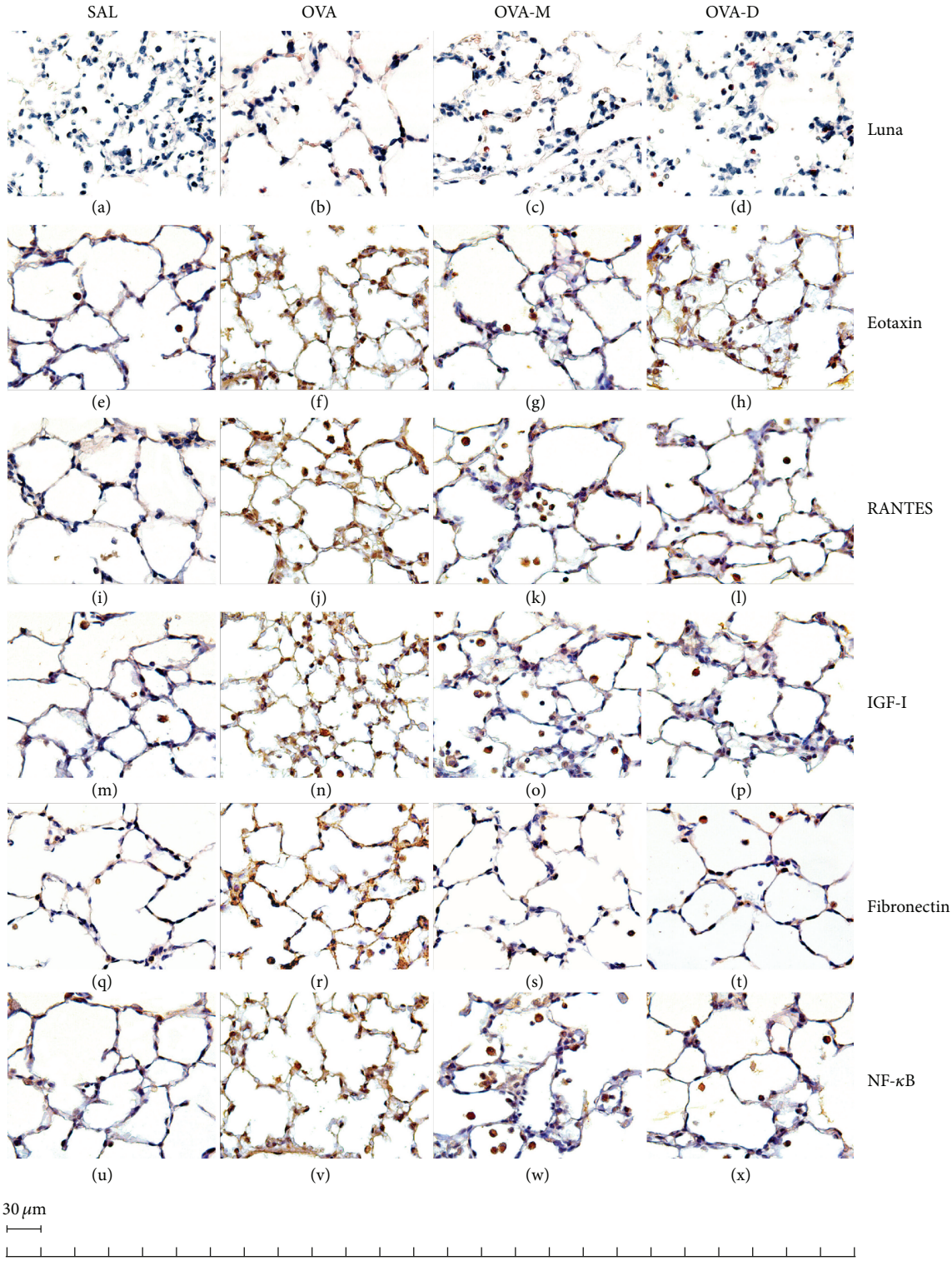

Figure 8: Representative photomicrographs of guinea pigs distal lung samples obtained from controls ((a), (e), (i), (m), (q), and (u)), OVA-exposed ((b), (f), (j), (n), (r), and (v)), OVA-exposed MK treated ((c), (g), (k), (o), (s), and (w)), and OVA-exposed D treated ((d), (h), (l), (p), (t), and (x)) stained with LUNA ((a) to (d)—×1000) and immunohistochemistry for eotaxin detection ((e) to (h)—×1000), RANTES detection ((i) to (l)—×1000), IGF-I detection ((m) to (p)—×1000), fibronectin detection ((q) to (t)—×1000), and NF-κB detection ((u) to (x)). Control groups show a scarce number of eosinophils, eotaxin positive cells, RANTES positive cells, IGF-I positive cells, Fibronectin positive cells and NF-κB positive cells. In contrast, the distal lung parenchyma of OVA-exposed animals shows intense eosinophilic infiltration (b), a large number of eotaxin positive cells (f), RANTES positive inflammatory cells (j), IGF-I positive inflammatory cells (n), fibronectin positive inflammatory cells (r), and NF-κB positive inflammatory cells. Both treatments (MK and D) in ovalbumin-exposed animals reduced all these parameters.

of binding only to CCR3, plays a role in the specific recruitment of eosinophils to the asthmatic bronchial mucosa, a phenomenon which in turn regulates asthma severity.

In many asthmatic patients, the addition of antileukotriene can be a valuable approach for uncontrollable asthma regardless of treatment with inhaled corticosteroid despite the fact that little is known about its molecular mechanism.

Wu et al. [54] studying to the effect of a 3-day course of high-dose montelukast on mediators of airway inflammation induced by a single allergen challenge in sensitized mice showed that eotaxin protein and mRNA expression in the lung remained unchanged.

Uguccioni et al. [55] using pranlukast (another antileukotriene) in vitro, evaluated eosinophil transmigration across human umbilical vein endothelial cells in response to LTD4, eotaxin, RANTES, and platelet-activating factor (PAF). They showed that pranlukast did not modify eosinophil transmigration in response to eotaxin, RANTES, or PAF.

Nevertheless, in the present study, both treatments tested were efficient in reducing eotaxin positive cells in distal lung and in airway walls. Although montelukast treatment showed more efficiency in reducing eotaxin positive cells in distal lung parenchyma compared to dexamethasone treatment, both of them reduced these parameters in airway walls.

Some studies have shown the chemoattractant power activity of RANTES for eosinophils in airway allergic inflammation. Gonzalo et al. [56] showed that treatment with methylated RANTES (a CCL5 antagonist) decreased eosinophilia after antigen challenge by blocking the activation of RANTES and its binding to the receptor.

Regarding the evaluation of RANTES positive cells, we noted that both treatments have shown effectiveness either in distal lung parenchyma or in the airway wall contributing to a better control of asthma.

Lung remodeling and chronic inflammation are important characteristics of asthma. It has been shown that modulation of fibroblasts into myofibroblastic phenotype, with addition of particular contractile aspects, indispensable for connective tissue remodeling during the wound healing process in asthmatics airways [57]. Nonetheless, the connection between fibroblasts, myofibroblasts, and smooth muscle cells needs to be better investigated [58].

Fibroblasts are responsible for producing a wide array of extracellular matrix components like collagen, reticular and elastic fibers, laminin, fibronectin, hyaluronic acid, glycoproteins, and proteoglycans amorphous extracellular substance [59, 60]. Nam et al. [61] showed that primary bronchial fibroblasts were susceptible to mechanical stimuli during differentiation into myofibroblasts. Furthermore, they have addressed that fibroblasts from asthmatics showed higher potential for tissue fibrosis when compared to control group.

Myofibroblasts are known to be contractile cells with biochemical and morphologic aspects that stay somewhere between fibroblasts and smooth muscle cells [62, 63]. TGF-β is the major regulator that promotes myofibroblast differentiation through its capacity to accumulate intracellular contractile proteins, fibronectin containing extra type III domain A (EDAcFN), and collagen density [57].

Through inflammatory mesenchymal and epithelial cells, a multimeric form of cellular fibronectin is produced, and it is deposited in the fibrils of the extracellular matrix, containing variable portions of the extra type III domain A and B (EDA and EDB) sequences [64]. These domains are known to play an important role in the contribution of EDAcFN to fibroblast activation [65] and wound healing process [66].

Fibronectin matrix formation is a dynamic, cell-dependent process that is tightly regulated. The loss of this regulation promotes an increase in the deposition of fibronectin and other extracellular matrix (ECM) molecules in the subepithelial matrix in patients with asthma. A specific site of fibronectin modulates cell contractility, collagen gel contraction, and cell migration. Furthermore, the increased or inappropriate exposure of this site in matrix fibronectin may lead to abnormal tissue remodeling by enhancing and/or prolonging cell contractility and by altering the rate of reepithelialization. The development of new strategies to control the extent and duration of the exposure of cells to matrix-specific epitopes can supply a functional method to structure normal tissue remodeling in asthma [6].

The cysteinyl leukotriene LTD4 causes a potentiation of fibronectin-induced migration of human lung fibroblasts [67], contributing to the airway remodeling process. Furthermore, Tokuriki et al. [68] observed that the leukotriene D_4 acts as a precipitating factor during the remodeling mediated by endothelin-1, and antileukotrienes have a role in preventing aberrant extracellular matrix degradation. An experimental mice model of asthma showed that the antileukotrienes inhibit the process of airway remodeling, including the influx of eosinophils into the lungs, eosinophil degranulation, the release of Th2 cytokines, hyperplasia of mucous glands, hypersecretion mucus, hyperplasia of smooth muscle cells, collagen deposition, and pulmonary fibrosis [69].

In this study, we observed an increase in fibronectin positive cells in chronic allergic lung inflammation model. Both treatments tested were able to decrease the number of fibronectin positive cells in distal lung parenchyma and in distal airway walls.

Fibrosis and asthma are highly linked by the increased deposition of extracellular matrix at specific places in the airway wall. Growth factors may be considered during asthma remodeling. Increased expression of TGF-β [70] has been shown in lung fibrosis and plays an important role in the excessive matrix deposition and also in cell proliferation. IGF-I is known to be a highly potent mitogen for fibroblasts and smooth muscle cells, acting on fibroblasts as a progression factor pushing the cells from a G1 phase to mitosis in the cell cycle, activating them to make the collagen synthesis [71]. It is important to emphasize that collagen deposition at the lamina reticularis contributes to the remodeling process, an asthma characteristic, enhancing the role that IGF-I can play as an important mediator of inflammation and remodeling in asthmatic airways.

Muz et al. [72] showed that airway collagen deposition/fibrosis was reduced after montelukast treatment, suggesting that cysteinyl-leukotrienes have an important role

in collagen deposition/lung fibrosis. In addition, Henderson Jr. et al. [73] showed that montelukast could significantly reduce airway remodeling in a mouse model of chronic asthma, addressing montelukast effect in some aspects of the remodeling process.

Veraldi et al. [5] showed that insulin-like growth factor binding protein-3 IGFBP-3 levels are increased in bronchoalveolar lavage (BAL) fluid of asthmatic patients 48 hours after allergen challenge, suggesting that this is the most important protein that IGF binds, developing airway remodeling in asthma through this modulation. Considering this, the creation of an IGFBP-3 antagonist could be a promise therapeutic target for asthma treatment.

Rajah et al. [74] using cultured airway smooth muscle cells addressed the synergism between IGF and inflammatory agents such as leukotriene D_4 and interleukin 1-beta, showing that these agents induce the secretion of an IGFBP protease which cleaves the IGFBPs secreted by airway smooth cells. This event allows IGF to stimulate proliferation, emphasizing that IGFBP proteases act as a critical element in asthma and other diseases. Rajah et al. [75] also reported that antimatrix metalloproteinase (MMP-1) acts as an IGFBP protease induced by leukotrienes that plays an important role in modulating IGF action in airway smooth muscle cells. Our findings showed that the anti-leukotriene montelukast reduced IGF levels in distal lung parenchyma and airway walls in experimental models of chronic allergic inflammation.

Hoshino et al. [76] have investigated the effects of inhaled corticosteroids in asthmatic patients. They observed a significant decrease in thickness of the subepithelial collagen and also an important reduction in the number of fibroblasts and the expression of IGF-I, suggesting that the decrease in collagen thickness after the treatment with inhaled corticosteroid could be a result of blocking the transcription of the IGF-I gene. In our study, we observed a decrease in IGF with both treatments. However, dexamethasone treatment was more efficient in reducing IGF-I positive cells in distal parenchyma when compared to montelukast treatment.

Corticosteroids are known to play an important role in the therapy of a large number of diseases, especially diseases of the gastrointestinal tract and liver [77]. Gayan-Ramirez et al. [78] showed that corticosteroid treatment in rats was highly linked with a reduction in IGF-I serum levels mainly after triamcinolone treatment that resulted from a decrease in IGF-I manifestation in the liver.

Our findings showed that dexamethasone was more efficient when compared to montelukast treatment in reducing the expression of IGF-I in distal lung parenchyma. This could be explained since this growth factor is synthesized and released by the liver and can be mediated by the action of corticosteroids [79].

Further studies are needed to ascertain the molecular mechanisms involving IGF-I and the current therapies.

NF-κB is a transcription factor, and it plays an important role in expression of lots of proinflammatory genes, which leads to the synthesis of growth factors, adhesion molecules, and chemokines [80].

Lin et al. [81] showed that total lung extracts from brownNorway rats exhibit enhanced NF-κB activity after the ovalbumin (OVA) sensitization and challenge model. There is also a relation between NF-κB and nitric oxide (NO). When inducible nitric oxide synthase (iNOS) is activated, it generates a high concentration of NO through the activation of some inducible nuclear factors, including the NF-κB [82].

Our results showed a higher efficacy of corticosteroids compared to montelukast treatment in reducing NF-κB in airway walls. This can be explained by the broad mechanism that corticosteroids play, by switching off the expression of multiple genes, including adhesion molecules and chemokines [83].

NF-κB activity is highly regulated by its interaction with IκB proteins. One possible explanation to corticosteroids higher efficacy in reducing NF-κB is that they can be involved in regulating the expression of IκB proteins. Another possibility is that NF-κB is regulated during the interaction with other coactivators, such as transcriptional co-activator proteins (CBP/p300), which are highly known to interact with NF-κB promoting transcription [83].

Arachidonate 5-lipoxygenase (ALOX5) is an enzyme that synthetizes the cysteinyl leukotrienes LTC_4, LTD_4, LTE_4, and LTB_4. ALOX5 inhibition can be associated with an improvement in asthma outcome, and it is known that the ALOX5 gene promoter has binding-sites for some transcription factors, including NF-κB. However, not all asthmatic patients will respond to antileukotrienes since those who have mutant alleles at the ALOX-5 should not have clinical benefits [84]. This could be a possible explanation of the relative lack of response by montelukast treatment in reducing NF-κB in airway walls.

Considerable attention has been given to the signaling pathways related with the NF-κB activation. These signaling protein molecules can be, in the near future, a potential therapeutic target to block NF-κB activation, interrupting the asthma process. Further studies are needed to better understand how these molecules interact with each other [85].

Despite the fact that there is current evidence of the importance of these treatments on airways regarding the control of main morphofunctional alterations present on asthma, the present study enlightens that isolated treatment with these drugs was chiefly capable of controlling also distal lung parenchyma alterations.

It is worthy to mention that, in the present study, all of the drugs were systemically administered, not through inhalation that probably contributed to the efficacy of the treatment on distal lung parenchyma.

Despite all the scientific and ethical care involved in the insertion of a new form of treatment for any disease, only after some time of use we can adequately characterize its efficacy's spectrum. This motivates the need for practical applications that help people. In other words, translational medicine, research that aims to move "from bench to bedside" or from laboratory experiments through clinical trials to point-of-care patient applications or dissemination to population-based community interventions. Thus, this study used and experimental model of chronic allergic inflammation to

better understand the mechanisms involved in the pathophysiology of asthma. We observed important results on the relation of the available treatments for allergic diseases and the immune response on the asthmatic patient. Our goal is a continuous feedback loop to accelerate the translation of data into knowledge from the basic sciences into the development of new treatments, translating the findings from clinical trials into everyday practice.

5. Conclusion

In this animal model, both corticosteroid and montelukast treatments were effective in the control of the inflammatory response in distal lung parenchyma and distal airway walls. But it is noteworthy to emphasize the contribution of montelukast in the resolution of some inflammatory and remodeling aspects in this experimental model, mainly in the control of distal lung parenchyma functional and histopathological alterations.

In conclusion, asthma is a chronic disease and corticosteroids are considered to be a gold standard medical therapy. However, since corticosteroids can have a wide range of side effects, it is essential that studies of therapeutic efficacy consider alternative treatments. Antileukotrienes are an orally, safe administered, nonsteroidal therapy for treating mild persistent asthma, especially in young children. They are an option for the treatment of asthma, based on many of its biological effects described above.

Therefore, our data contributes to a better understanding of the inflammatory and remodeling processes in both treatments in this experimental model of asthma and may assist researchers to further studies based on their expected outcomes.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Nathália Brandão Gobbato designed and performed the major part of the experiments and morphometric analysis, performed the statistical analysis and drafted the manuscript. Flávia Castro Ribas de Souza and Stella Bruna Napolitano Fumagalli assisted in performing the experiments. Carla Máximo Prado helped with the statistical analysis and in the preparation of the manuscript. Fernanda Degobbi Tenório Quirino dos Santos Lopes assisted in the interpretation of the manuscripst. Iolanda de Fátima Lopes Calvo Tibério and Milton Arruda Martins participated in the design and discussion of the study. Edna Aparecida Leick supervised the study, participated in its design and in elucidation of results as well as in the elaboration of the manuscript. All authors read and approved the final manuscript.

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Research Article

Intracellular Secretory Leukoprotease Inhibitor Modulates Inositol 1,4,5-Triphosphate Generation and Exerts an Anti-Inflammatory Effect on Neutrophils of Individuals with Cystic Fibrosis and Chronic Obstructive Pulmonary Disease

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Secretory leukoprotease inhibitor (SLPI) is an anti-inflammatory protein present in respiratory secretions. Whilst epithelial cell SLPI is extensively studied, neutrophil associated SLPI is poorly characterised. Neutrophil function including chemotaxis and degranulation of proteolytic enzymes involves changes in cytosolic calcium (Ca^{2+}) levels which is mediated by production of inositol 1,4,5-triphosphate (IP_3) in response to G-protein-coupled receptor (GPCR) stimuli. The aim of this study was to investigate the intracellular function of SLPI and the mechanism-based modulation of neutrophil function by this antiprotease. Neutrophils were isolated from healthy controls ($n = 10$), individuals with cystic fibrosis (CF) ($n = 5$) or chronic obstructive pulmonary disease (COPD) ($n = 5$). Recombinant human SLPI significantly inhibited fMet-Leu-Phe (fMLP) and interleukin(IL)-8 induced neutrophil chemotaxis ($P < 0.05$) and decreased degranulation of matrix metalloprotease-9 (MMP-9), hCAP-18, and myeloperoxidase (MPO) ($P < 0.05$). The mechanism of inhibition involved modulation of cytosolic IP_3 production and downstream Ca^{2+} flux. The described attenuation of Ca^{2+} flux was overcome by inclusion of exogenous IP_3 in electropermeabilized cells. Inhibition of IP_3 generation and Ca^{2+} flux by SLPI may represent a novel anti-inflammatory mechanism, thus strengthening the attractiveness of SLPI as a potential therapeutic molecule in inflammatory airway disease associated with excessive neutrophil influx including CF, non-CF bronchiectasis, and COPD.

1. Introduction

The ability of neutrophils to mobilise rapidly to a site of infection is paramount to successful elimination of foreign microbes; however, an excessive infiltrative response can elicit extensive tissue damage. Indeed, neutrophil migration to the airways is a feature of a number of chronic pulmonary disorders including cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), where neutrophil elastase (NE) is largely responsible for protease-mediated damage in the lung. A gradient of attractant molecules, including the bacterial chemotactic peptide fMLP signalling through FPR1 and tissue derived IL-8 signalling via the chemokine receptors CXCR1 and CXCR2, attract neutrophils to migrate towards the site of tissue infection in a directional manner referred to as chemotaxis. Upon cell migration, the normally spherical cell undergoes lateral polarization to form a leading edge and a trailing tail [1]. To achieve this morphological change many processes are initiated including cytoskeletal reorganisation with protein polymers of filamentous actin, microtubules, and intermediate filaments forming at the leading edge and dissolution of polymers occurring at the rear [2].

Molecular regulation of cytoskeletal rearrangements during neutrophil signalling associated with chemotaxis and degranulation of antimicrobial enzymes, requires an increase in cytosolic Ca^{2+} levels [3]. This increase is due to the release of Ca^{2+} from the cell's internal store (the calciosome) or influx of Ca^{2+} from the extracellular environment. Signalling via fMLP or IL-8 GPCRs results in activation of the β isoform of phospholipase C, in turn yielding production of inositol 1,4,5 triphosphate (IP_3) [4]. IP_3 occupancy of receptors on calciosomes induces a rapid release of stored Ca^{2+}, and in a variety of cell types this cascade has been proposed as a potential target for the treatment of a number of diseases including heart failure and arrhythmias [5, 6].

Serine protease inhibitors such as SLPI act locally to maintain a protease/antiprotease balance thereby preventing protease mediated tissue destruction [7]. Disruption of this balance is responsible for much of the extracellular matrix and subsequent lung tissue damage evident in neutrophil driven lung diseases [8, 9]. SLPI is a well-characterised member of the trappin gene family of proteins [10]. It is a highly basic cationic protein that is produced by epithelial cells of the respiratory tract and is also produced by phagocytic neutrophils [11]. SLPI inhibits a broad range of serine proteases including NE, cathepsin G, trypsin, and tryptase. Moreover, SLPI possesses anti-inflammatory characteristics and in monocytes has been shown to inhibit *lipopolysaccharide* (LPS)- and lipoteichoic acid-induced nuclear factor (NF)-κB activation and can compete with p65 for binding to NF-κB binding sites [12, 13]. Moreover, SLPI has been shown to directly bind to bacterial LPS thereby down-regulating production of proinflammatory mediators [14, 15]. However, an area that has received less attention is the role of neutrophil-derived SLPI in modulating inflammatory responses.

Studies have localised SLPI within the cytosol [11] and also in secondary granules of neutrophils, in turn coreleased with lactoferrin [16]. Moreover, the release of SLPI from neutrophils upon phorbol ester (PMA) activation suggests that neutrophil-derived SLPI may regulate the protease antiprotease balance at sites of tissue inflammation [11]. However, the aim of this study was to investigate the intracellular function of SLPI within resting and activated neutrophils. This study has revealed a novel anti-inflammatory role for this antiprotease and demonstrated the ability of SLPI to modulate neutrophil migration and degranulation by inhibiting IP_3 production and Ca^{2+} ion mobilisation.

2. Methods

2.1. Chemicals and Reagents. All chemicals and reagents including inositol 1,4,5-triphosphate were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless indicated otherwise. The concentration of SLPI in neutrophil cytosol was measured using the Quantikine Human SLPI Immunoassay (R & D Systems, Abingdon, Oxon, UK). Recombinant human SLPI (rhSLPI) was obtained from R & D Systems, and recombinant calpain was obtained from Calbiochem (La Jolla, CA, USA). Human NE was purchased from Elastin Products Company, Inc. (Owensville, MO, USA). The IP-One HTRF assay kit was purchased from Cisbio Bioassays (Bedford, MA 01730, USA).

2.2. Study Groups. Control volunteers ($n = 10$, mean age 35.1 ± 1.8) had no underlying medical illnesses and were not receiving any medication. Prior to recruitment patients with CF ($n = 5$) were exacerbation-free over the preceding 6-week period, and patients with COPD were being treated for an acute exacerbation ($n = 5$). There were 2/5 and 3/5 males recruited to the CF and COPD study, respectively. The mean age was 22.5 ± 2.7 and 56.5 ± 2.7 years for the CF and COPD groups, respectively. Informed patient consent was obtained for all procedures, and ethical approval for the use of blood samples from CF and COPD individuals was obtained from the Beaumont Hospital Ethics Review Board.

2.3. Preparation of Human Neutrophils and Cell Fractionation. Blood was obtained from all donors in 7.5 mL heparinised S-monovette tubes (Sarstedt Ltd, Ireland), and neutrophils were purified by dextran sedimentation and Lymphoprep (Axis-Shield PoC AS, Norway) centrifugation as previously described [17]. Purified cells were resuspended in phosphate-buffered saline (PBS) (pH 7.4) containing 5 mM glucose (PBSG) and used immediately. The purity of the neutrophil population was confirmed by flow cytometry measuring the neutrophil membrane marker CD16b and was found to be >99% [18]. All *in vitro* experiments were performed at 37°C. Cells were either left unstimulated (control) or activated with PMA (1.6 μM), fMLP (1 μM), and/or IL-8 (1.2 nM, R & D Systems) for the indicated time points.

For cell fractionation studies, isolated neutrophils were suspended in Lamberts Break Buffer (LBB) (10 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, and 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES); pH 7.2) containing 10% (w/w) sucrose and the following protease inhibitors: 13 μM aprotinin, 5 mM benzamidine, 0.15 mM Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0.5 mM N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 20 mM N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc-AAPV-CMK), 10 μM soybean trypsin inhibitor (SBTI), orthophenanthroline, and 0.2 M pefabloc. Cells were sonicated 3 times for 5 sec on ice and centrifuged at 500 ×g at 4°C for 5 min to generate a post nuclear supernatant (PNS). The PNS was overlaid on discontinuous sucrose gradients of 17.5 and 35% (w/w) dissolved in LBB. This was centrifuged at 137,000 ×g for 45 min at 4°C. The cytosolic fraction was removed from above the 17% (w/w) sucrose layer, and membrane fraction was recovered from the top of the 34% (w/w) sucrose layer. The pellet (containing primary and secondary granules) was resuspended in 10% (w/w) sucrose and overlaid on a discontinuous sucrose gradient of 30, 43, and 55% (w/w). This was centrifuged at 137,000 ×g for 1 h at 4°C. Secondary and primary granules were harvested from above the 43% (w/w) and 55% (w/w) sucrose layers, respectively, as previously described [18]. The concentration of protein present in each purified fraction was quantified by

use of a BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

Quantification of F- and G-actin was carried out as previously described [19]. In brief, neutrophils were either untreated or treated with SLPI (480 nM) or wortmannin (100 nM) for 10 min and then left unactivated or activated with IL-8 (1.2 nM) and fMLP (1 μM) for 10 min. Neutrophils were lysed within an F-actin stabilisation buffer containing ATP (1 mM) and protease inhibitors (as indicated above) for 10 min at 37°C. Clarified cell lysates were centrifuged for 2 h at 100,000 ×g. The supernatant was removed and the pellet was resuspended in an equal volume of buffer containing cytochalasin D (10 mM) and incubated on ice for 1 h. An equal volume of supernatant and pellet was electrophoresed and Western blotted for actin employing a monoclonal anti-actin antibody (1.0 μg/mL; Millipore, Billerica, MA, USA).

2.4. Neutrophil Uptake Assays. Native neutrophil cytosolic SLPI levels were determined by ELISA (SLPI Human ELISA, Cambridge Bioscience, UK). Uptake of exogenous recombinant human SLPI (rhSLPI; 480 nM) by cells (2×10^5/mL in PBSG) was performed at 37°C for 10 min. After incubation, the SLPI loaded cells were washed with ice cold PBSG and neutrophil levels of cytosolic SLPI measured by ELISA. To determine the mechanism of SLPI uptake, cell (2×10^5/mL in PBSG) incubations were performed at 4°C for 10 min or in the presence of the endocytosis inhibitors sodium azide (NaN_3; 15 mM) and sodium fluoride (NaF; 10 mM) as previously described [20].

2.5. SDS-PAGE and Western Blot Analyses. Samples were subjected to SDS-PAGE under denaturing conditions on 4–12% (w/v) NuPAGE gels (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Routinely 25 μg of neutrophil cytosolic protein, 10 μg of membrane protein, 5 μg secondary granule protein, and 10 μg of primary granule protein was loaded on each gel. After electrophoresis gels were stained by Coomassie blue R250 for visualization of proteins or alternatively proteins were transferred onto 0.2 μm nitrocellulose or PVDF membrane by Western blotting using a semidry blotter for 1 h at 100 mA. Membranes were blocked for 1 h in 3% dry milk (w/v) and 1% (w/v) bovine serum albumin (BSA) in PBS containing 0.05% (v/v) Tween 20. Blots were incubated with 1.0 μg/mL polyclonal rabbit (Rb) anti-SLPI specific antibody (Abcam, Cambridge, UK), 1.0 μg/mL polyclonal goat (Gt) anti-SLPI (Synergen Inc., Boulder, Colorado 80301) [21, 22], 1.0 μg/mL polyclonal goat (Gt) anti-SLPI (R&D Systems), 0.2 μg/mL polyclonal rabbit anti-myeloperoxidase (MPO), anti-hCAP-18, or anti-lactoferrin antibody (all purchased from Abcam, Cambridge, UK). Additional primary antibodies included 0.2 μg/mL polyclonal goat antimatrix metalloprotease (MMP)-9 (R & D Systems), 1.0 μg/mL monoclonal anti-talin-1, and 1.0 μg/mL monoclonal anti-vinculin or monoclonal anti-actin antibody (Millipore). The secondary antibodies were HRP-linked anti-rabbit, -goat, or -mouse IgG (Cell Signalling Technology, Danvers, MA, USA). Immunoreactive protein bands were visualized employing SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) after exposure to Kodak X-Omat LS Film.

2.6. Neutrophil Electropermeabilization. Cell permeabilization was performed immediately before use of neutrophils as previously described [23, 24]. In brief, cells (5×10^6) were washed in hypoosmolar buffer (Eppendorf, UK, Ltd.) and then resuspended in 400 μL of ice-cold hypoosmolar buffer in the presence or absence of 1 μM IP_3. The cells were then transferred into an electroporation cuvette (2 mm gap, 400 μL volume purchased from Eppendorf) and subjected to three discharges of 300 V using an Eppendorf Multiporator, with gentle stirring between the three pulses by pipetting [24]. Permeabilized cells were then incubated at room temperature for 5 min to allow incorporation of IP_3 into electroporated neutrophils, immediately washed in PBSG, and then employed in chemotaxis assays or for Ca^{2+} flux measurements. Control experiments indicated that the cell permeabilization procedure did not affect cell viability as determined by trypan blue exclusion assays.

2.7. Neutrophil Chemotaxis Assays. Chemotaxis assays were performed by measuring the percentage of neutrophils (2.5×10^5cells/200 μL) migrating towards IL-8 (1.2 nM) or fMLP (1 μM) by employing a multiwell chemotaxis chamber (Neuro Probe, Inc., USA) and polyvinylprrolidone-free polycarbonate filters (10 μm thick with 5 μm pores). The stimulant was placed in the lower chamber (total volume of 90 μL), and purified neutrophils were placed in the upper chamber (2.5×10^5cells/200 μL) with or without SLPI (0, 120, 240 or 480 nM). Neutrophil chemotaxis was quantified over 30 min at 37°C. The Neuroprobe chamber was then disassembled, and the polycarbonate filter was fixed with methanol, and cells were stained using Speedy-Diff solutions (Clin-tech Ltd, UK). The number of migrated neutrophils was determined microscopically employing a Nikon Eclipse TS100 microscope with 10 standardized × 400-high power fields counted for each well. For comparative analysis neutrophils treated with IL-8 or fMLP were set at a chemotactic index of 1, as indicated. Additional experiments were performed after electropermeabilization with 1 μM IP_3. Cells were then left unstimulated (control) or challenged with fMLP or IL-8. Data was represented as chemotactic index as previously described [25].

2.8. Measurement of Intracellular Ca^{2+} Flux. Measurement of intracellular cytosolic Ca^{2+} was performed employing the Fluo-4 NW Calcium Assay Kit (Invitrogen, Bio Sciences Ltd., Ireland) in the absence of extracellular Ca^{2+} according to the manufactures instructions. In brief, cells (2×10^5/mL) were incubated for 30 min in dye loading solution containing the Ca^{2+} sensitive dye Fluo-4. Subsequently, cells were either left untreated or were exposed to fMLP (1 μM) or IL-8 (1.2 nM) with changes in fluorescence recorded immediately upon addition of the stimulant. Fluorescence was recorded every 10 sec for 5 min employing a Victor X3 (PerkinElmer, Ireland) plate reader with excitation wavelength of 490 nm and

emission at 535 nm. A subset of experiments was performed by incubating cells with either SLPI (480 nM), the PLC-β inhibitor U73122 (5 μM), or the GPCR inhibitor pertussis toxin (500 ng/mL), prior to stimulation with fMLP (1 mM) or IL-8 (1.2 nM). Additionally, experiments were performed by pretreating cells with either SLPI (480 nM) or oxidised SLPI (480 nM), the later oxidised with 20 mM hydrogen peroxide (H_2O_2) as previously described [13].

2.9. Analysis of Neutrophil Degranulation and Secretion of SLPI. Neutrophils (5×10^6/mL per reaction) suspended in PBSG remained untreated or preloaded with rhSLPI (480 nM) for 10 min, followed by fMLP/IL-8 (used in combination at 1 μM and 1.2 nM, respectively, to ensure release of primary, secondary, and tertiary granules) stimulation for 10 or 20 min. Cell free supernatants were harvested following centrifugation at 500 ×g for 5 min at 4°C and analysed for degranulated proteins: MPO as a marker of primary granule release, hCAP-18 as a marker of secondary granule release, and MMP-9 as a marker of tertiary granule release, by Western blotting. In a subset of experiments the extracellular release of native cytosolic SLPI was analysed by Western blotting following cellular activation with PMA (1.6 μM) for 0, 0.5, 1, or 10 min or fMLP (1 μM) and IL-8 (1.2 nM) for 0, 10, 20, 30, 40, or 50 sec. Cell free supernatants were harvested as described above and acetone precipitated prior to SDS-PAGE and Western blot analysis for SLPI.

2.10. Neutrophil Elastase (NE) and Calpain Activity Assays. NE (34 nM) activity assays employing the specific substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as previously described [26]. Liberation of p-nitroaniline was measured at 405 nm at 37°C at 1 min intervals for 5 min. In additional studies the NE inhibitory activity of neutrophil cytosol was assessed before and after immunoprecipitation of SLPI. For immunoprecipitation experiments, neutrophil cytosols were obtained from 1×10^7 cells and precleared by incubation with protein A-Sepharose beads for 1 h as previously described [18]. Goat polyclonal anti-SLPI (5 μg) or isotype control IgG was then added to precleared cytosols for 1 h with rotation at 4°C. SLPI was then removed from the cytosol by incubation with reconstituted protein A-Sepharose beads for 1 h, and the anti-NE capacity of the resulting cytosol (25 μg) was analysed.

Kinetic analysis of calpain activity was determined using the Calpain-Glo protease assay (Promega Corporation, Madison, USA) which detects cleavage of the calpain substrate Suc-LLVY-aminoluciferin with luminescence recorded as per the manufacturer's instructions. The inhibitory effect of rhSLPI and calpastatin was analysed over a range of 0–640 nM and 0–80 nM, respectively.

2.11. Inositol 1,4,5-Triphosphate Measurements. For analysis of IP_3 we employed a recognised protocol and measured levels of IP_1 which accumulates as a stable product of IP_3 [27]. Neutrophils (1×10^5) remained untreated or were preloaded with SLPI (480 nM) as already described. As positive controls the PLC-β inhibitor U73122 (5 μM) and the GPCR inhibitor pertussis toxin (500 ng/mL) were employed prior to stimulation with fMLP (1 μM) or IL-8 (1.2 nM) for 10 sec. Cells were then lysed, and intracellular IP_1 levels were determined by use of an IP-One HTRF assay kit in a final reaction volume of 200 μL as per the manufacturer's instructions.

2.12. Flow Cytometry Analysis. Flow cytometry was carried out to evaluate the membrane expression of CD16b as a measure of cell purity [18] or CD66b (secondary and tertiary granules) and CD63 (primary granule) as a measure of degranulation [24]. Cells remained untreated or were stimulated with fMLP (1 μM) and IL-8 (1.2 nM) for 10 min at 37°C. Neutrophils were then fixed (4% (w/v) paraformaldehyde) and blocked (2% (w/v) BSA) for 30 min at room temperature. After washing (PBS × 2) neutrophils (1×10^6) were incubated with 1 μg/100 μL of mouse monoclonal anti-CD16b (Santa Cruz, Germany), FITC labelled anti-CD66b, or PE labelled anti-CD63 (both from BD Bioscience, UK). Control samples were exposed to relevant nonspecific isotype control IgG or secondary labeled antibody alone (FITC-labelled bovine anti-mouse). In additional experiments the ability of fMLP to interact with its cognate receptors on the neutrophil membrane in the presence of rhSLPI was performed as previously described [28]. In brief, purified neutrophils (1×10^7/mL) were left untreated or exposed to fMLP (10 μM) or rhSLPI (480 nM) for 1 min, followed by incubation with FITC-labeled fMLP (1 μM) for 1 min. Cells were washed in PBS and fluorescence was counted using a BD FACSCalibur flow cytometer (BD Bioscience, Germany) with a total of 10,000 events acquired. The data were analysed and the mean fluorescence intensity (MFI) for each experiment was determined using BD CellQuest Pro software.

2.13. Confocal Immunofluorescence. Adherent neutrophils were produced by placing neutrophils in PBS on Polysine microscope slides (AGB Scientific Ltd., Ireland) after which they were incubated at 37°C for 5 min and then for a further 10 min at 37°C in the presence of fMLP (1 μM) and IL-8 (1.2 nM) or PMA (1.6 μM). Cells were then fixed with 4% (w/v) paraformaldehyde in PBS for 10 min and permeabilised with 0.2% (v/v) Triton X-100 in PBS for 10 min. Subsequently cells were blocked for 1 h with 1% (w/v) BSA in PBS and then incubated with 1 μg/mL FITC-labeled rabbit polyclonal anti-SLPI for 45 min and washed in PBS. Cells were mounted employing VECTASHIELD mounting medium with DAPI for nuclear staining (Vectashield Lab, UK). Control samples were exposed to non-specific FITC-labeled isotype control IgG. All immunofluorescence slides were viewed and images were acquired using a Zeiss LSM710 confocal immunofluorescence microscope.

2.14. Statistical Analysis. The data was analysed with the GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA) and results were expressed as mean ± standard error (S.E.) of the mean. The Mann-Whitney *U*-test or analysis of variance (ANOVA) sample tests followed by Bonferri correction were employed to identify significant

differences. Experiments were performed in triplicate, and a P-value < 0.05 was deemed significantly different.

3. Results and Discussion

3.1. The Immunomodulatory Effects of Cytosolic SLPI on Neutrophil Migration and Degranulation. SLPI has been previously reported to exert an anti-inflammatory effect on immune cell function [20, 29], and in this respect SLPI has been shown to inhibit monocytic CD4 lymphocyte proliferation and Th1 cytokine (INF-γ) release [30]. Indeed, T lymphocytes play a key role in the pathogenesis of CF [31] and COPD [32] lung disease and have been identified as the predominant cell type in subepithelial bronchial tissue of CF patients [31]. Th1, Th2, and Th17 cells may augment the cytokine/chemokine profile in the airways of CF patients thus contributing to the chronic recruitment of neutrophils [31]. Thus the objective of this study was to investigate whether SLPI exerted an anti-inflammatory effect on neutrophil function, and for this reason it was first necessary to confirm its cellular localisation.

Localisation of SLPI within many cell types known to produce this protein is still being elucidated. For example, it has been reported that cellular SLPI is localised in the nuclei of bronchial epithelial cells exposed to 17β-estradiol resulting in NF-κB inhibition and IL-8 gene expression [33]. SLPI has also been detected in the nuclei of alveolar macrophages in patients with pulmonary sepsis, but not in healthy controls [20], indicating that the localisation of SLPI in disease states may differ from healthy control cells. Within the present study by subcellular fractionation of resting neutrophils and Western blot analysis employing both rabbit and goat polyclonal anti-SLPI antibodies, SLPI was identified as a component of the cell cytosol and secondary granule fractions (co-localising with lactoferrin) and was absent from unstimulated membranes and primary granule fractions containing MPO (Figure 1(a)). Localization of SLPI to these compartments is in keeping with previously published data [11, 16]. As the role of cytosolic SLPI was the focus of ensuing experiments, we also confirmed the presence of SLPI in cytosolic fractions of CF and COPD cells (Figure 1(b)).

Having identified the position of SLPI in the neutrophil further experiments were designed to establish whether cytosolic SLPI possessed antiprotease activity. Results of anti-NE kinetic measurements indicated that cytosolic SLPI contained anti-NE capacity (Figure 1(c)) as both the rate and total level of NE catalytic activity (34 nM) were negated by freshly isolated neutrophil cytosol (25 μg/10 μL); an effect significantly reversed in samples in which SLPI was extracted by immunoprecipitation (Figure 1(c)). At the 240 sec time point (4 min), cytosol that had been subjected to immunoprecipitation for SLPI removal was 66.7 ± 2.1% less active in inhibiting NE as compared with complete cytosol ($P < 0.05$) and illustrated no significant difference compared to the NE control reaction (Figure 1(d)).

To begin to understand the role of intracellular SLPI in neutrophil cell function our approach was to increase the level of this anti-protease within the cell cytosol. To achieve this, cells were resuspended in PBSG containing exogenous rhSLPI for 10 min and then washed to remove protein that had not been taken up by the cells. To confirm that rhSLPI was internalized, the concentration of SLPI in neutrophil cytosolic fractions was determined by ELISA. Results revealed that addition of 480 nM rhSLPI significantly raised the concentration of cytosolic SLPI from 96 ± 6.3 ng to 151 ± 4.8 ng per 1×10^5 cells ($P < 0.05$, $n = 6$) indicating permeability of the cell membrane to exogenous rhSLPI (Figure 1(e)). To examine internalization of rhSLPI in further detail experiments were repeated at 4°C, thereby applying a temperature block to inhibit transport processes [20]. Under these reduced temperature conditions results indicated that the uptake of SLPI and localization to the cell cytosol were significantly inhibited by 30.2 ± 3.4% (Figure 1(e)). The role of endocytosis was also assessed by pretreating cells with the endocytosis inhibitors NaN_3 (15 mM) and NaF (10 mM) [20]. These inhibitors had no significant effect on rhSLPI uptake and cytosolic association (Figure 1(e)). Collectively, these results indicate that exogenously added rhSLPI is taken up by the cell and is localized in the cytosol and is not contained within endosomes. These results are in line with previously published data on the internalization of rhSLPI by mononuclear cells [20]. In this later publication exogenously added rhSLPI was localized to the cytosol and nucleus of U937 monocytes. SLPI has been characterised as an arginine rich cationic molecule [34], and it has been reported that the positively charged nature of the protein facilitates its transduction across negatively charged membranes [20, 34]. Moreover, investigation of the anti-HIV-1 inhibitory activity of SLPI has prompted the identification of membrane proteins that are capable of binding to SLPI. In this regard, SLPI has been shown to interact with the phospholipid binding protein annexin II on the surface of human macrophage cell membranes [35] and has also been described as a ligand for phospholipid scramblases 1 and 4 (PLSCR1 and PLSCR4) [36]. This later interaction suggested that SLPI disrupts interplay between PLSCR1 and the CD4 receptor on the surface of $CD4^+$T lymphocytes thus preventing HIV-1 viral infection [36].

Within the present study subsequent experiments investigated the effect of cytosolic SLPI on directional chemotaxis, a fundamental neutrophil cellular response in terms of pulmonary inflammation. Cells (2.5×10^5/200 μL) were first preloaded with exogenous rhSLPI (0, 120, 240 or 480 nM), washed, and then exposed to IL-8 (1.2 nM) or fMLP (1 μM), two stimuli with known effective chemoattractant properties [37, 38]. As shown in Figures 2(a) and 2(b), rhSLPI inhibited both IL-8 and fMLP-induced neutrophil directional chemotaxis in a dose dependent manner with an IC_{50} of approximately 240 nM recorded for both stimuli. Excessive neutrophil infiltration to the lung is a key pathogenic feature for disease progression in individuals with CF and COPD. For this reason we examined the effect of rhSLPI on CF and COPD neutrophil chemotaxis in response to inflammatory stimuli *in vitro*. Results confirmed the inhibitory action on cell migration and demonstrated that cells preloaded with rhSLPI showed a markedly reduced capacity to migrate in

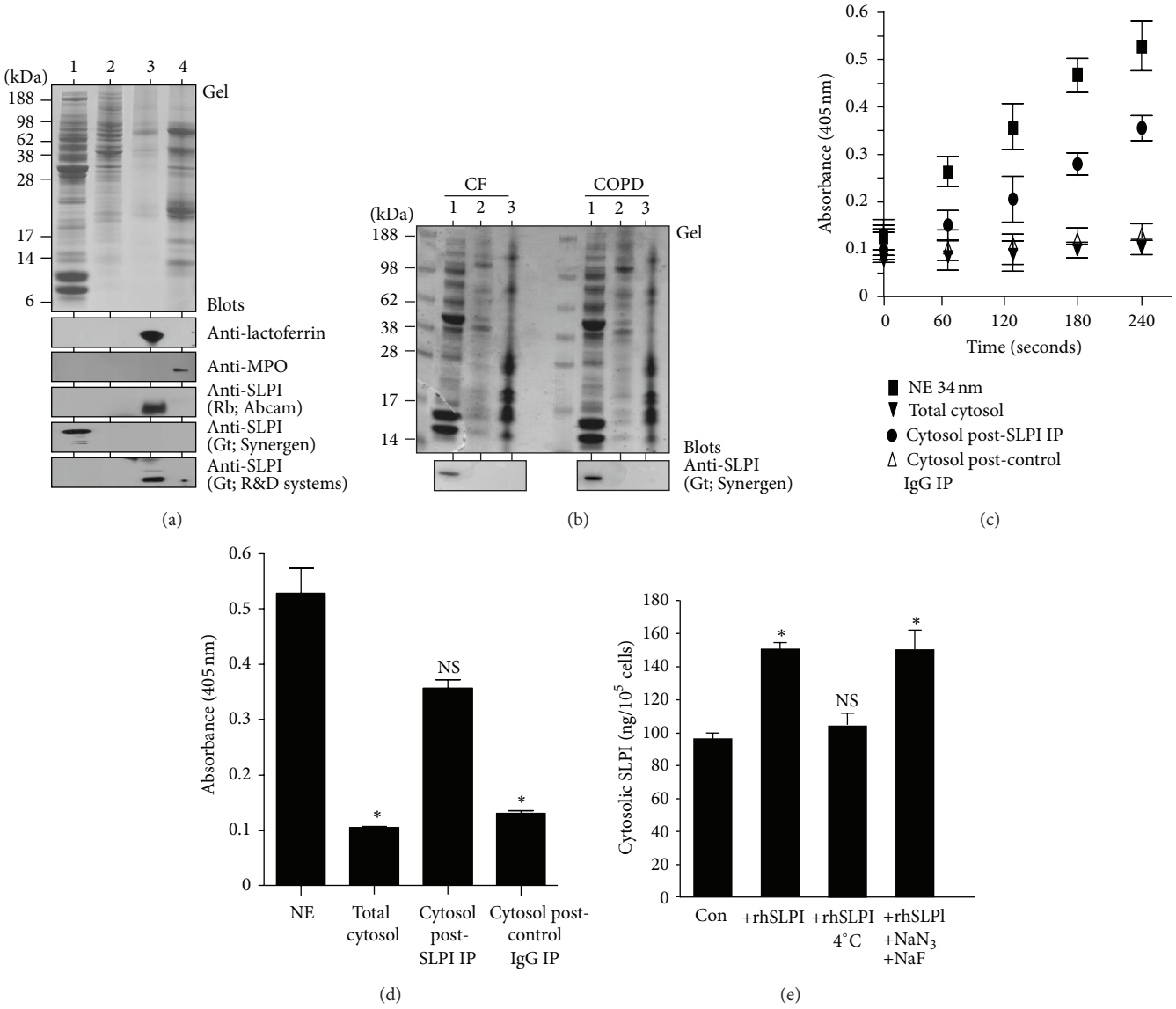

Figure 1: Localization and activity of native SLPI in peripheral blood neutrophils. (a) Coomassie blue stained gel of isolated neutrophils subjected to subcellular fractionation yielding a cytosol fraction (lane 1), membrane fraction (lane 2), and secondary and primary granule fractions, lane 3 and 4 respectively. Western blotting employed polyclonal rabbit (Rb) or goat (Gt) antibody to SLPI, which localized SLPI to both the cytosolic and secondary granule fractions. As controls, MPO and lactoferrin were detected using rabbit polyclonal antibodies as markers for primary and secondary granules, respectively. (b) Coomassie blue stained gel of isolated CF and COPD neutrophils subjected to subcellular fractionation yielding a cytosol fraction (lane 1), membrane fraction (lane 2), and pooled primary and secondary granule fractions (lane 3). Western blotting employed polyclonal Rb antibody confirming cytosolic localization of SLPI in patient samples. (c) The NE inhibitory activity of neutrophil cytosol was assessed before and after immunoprecipitation (IP) of SLPI using Gt polyclonal anti-SLPI antibody (Synergen). The kinetics of inhibition is illustrated in (c), and data of the final time point (240 sec) is plotted in (d). Control experiments included isotype control Gt IgG. $^*P < 0.05$ between NE control. (e) Addition of 480 nM rhSLPI to cells (2×10^5/mL) increased the concentration of cytosolic SLPI by approximately 50% above the untreated cells (Con), as determined by ELISA. Experiments were repeated at 4°C or in the presence of NaN_3 (15 mM) and NaF (10 mM). $^*P < 0.05$ between untreated cells (con). Results illustrated in (a) and (b) are representative gels and blots of 3 separate experiments. Results illustrated in panel (c)–(e) were performed in triplicate and each bar is the mean ± S.E. (NS, no significant difference, $^*P < 0.05$ calculated by Student's t-test).

response to fMLP and IL-8 (Figures 2(d) and 2(c), respectively; $P < 0.05$, $n = 5$). An IC_{50} of 611 nM and 376 nM for CF cells and an IC_{50} of 542 nM and 627 nM for COPD cells were recorded for fMLP and IL-8 stimulation, respectively. Thus a greater level of SLPI was required to inhibit CF and COPD neutrophil chemotaxis compared to control cells. This latter result may be a consequence of neutrophil priming within the circulation of individuals with CF and COPD due to persistent inflammation. Indeed, studies have shown that CF neutrophils are primed and unresponsive to IL-10 induced

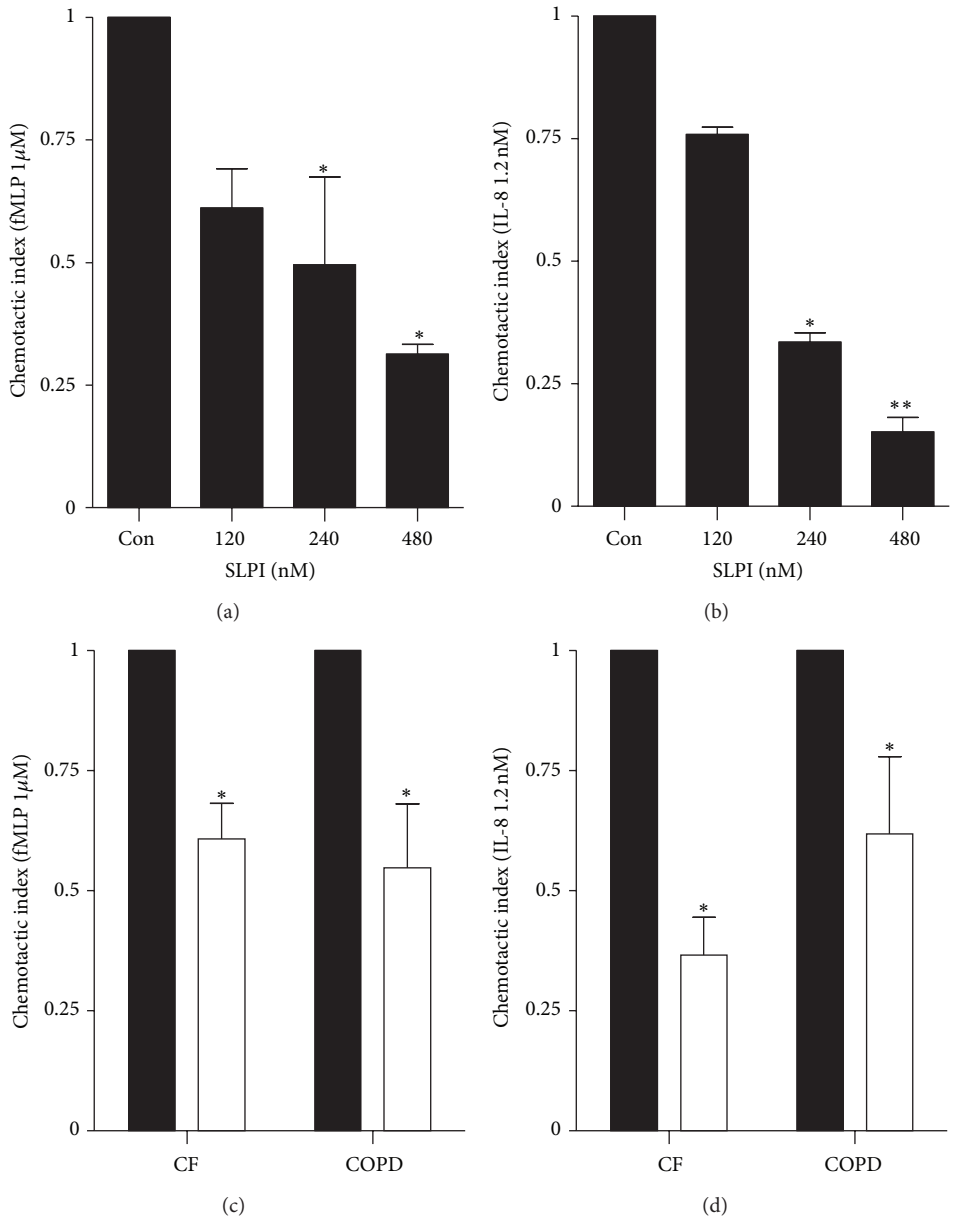

Figure 2: rhSLPI inhibits neutrophil chemotaxis. (a) and (b) An increase in chemotactic inhibition efficiency of increasing concentrations of SLPI (120, 240, and 480 nM). The chemotactic index represents the ratio of neutrophils that migrated towards fMLP (1 μM) or IL-8 (1.2 nM) over 30 min at 37°C. (c) and (d) fMLP and IL-8-induced mean chemotactic index of neutrophils (2.5×10^5 cells/200 μL) isolated from individuals with CF ($n = 5$) or individuals with COPD ($n = 5$), in the presence (□) or absence (■) of 480 nM SLPI. Results illustrated were performed in triplicate and each bar is the mean ± S.E. $^*P < 0.05$ or $^{**}P < 0.01$ versus untreated control cells (Con) with statistical significance calculated by Student's t-test.

anti-inflammatory signals [39]. *Pseudomonas* alginate [40], TNF-α, and IL-8 have been shown to be important priming agents for CF neutrophils, causing greater release of MPO [41] and NE, compared to neutrophils from control subjects and individuals with bronchiectasis [42].

The degranulation of proteolytic enzymes and peptides from the neutrophil upon activation is a tightly regulated process in order to prevent unnecessary damage to tissues [43]. For this reason we evaluated the inhibitory effect of rhSLPI on the kinetics of degranulation. In this experiment we used fMLP (1 μM) and IL-8 (1.2 nM) in combination to ensure exocytosis of all three neutrophil granule types (primary, secondary, and tertiary), and release of granule proteins was quantified in the extracellular supernatant by immunoblotting. The use of equal cell numbers (5×10^6/mL) in each reaction is demonstrated by identical Coomassie blue stained electrophoretic profiles of whole cell lysates prepared form cells employed in each reaction (Figure 3(a)). Levels of cell released MMP-9 from tertiary granules (Figure 3(b)), hCAP-18 from secondary granules (Figure 3(c)),

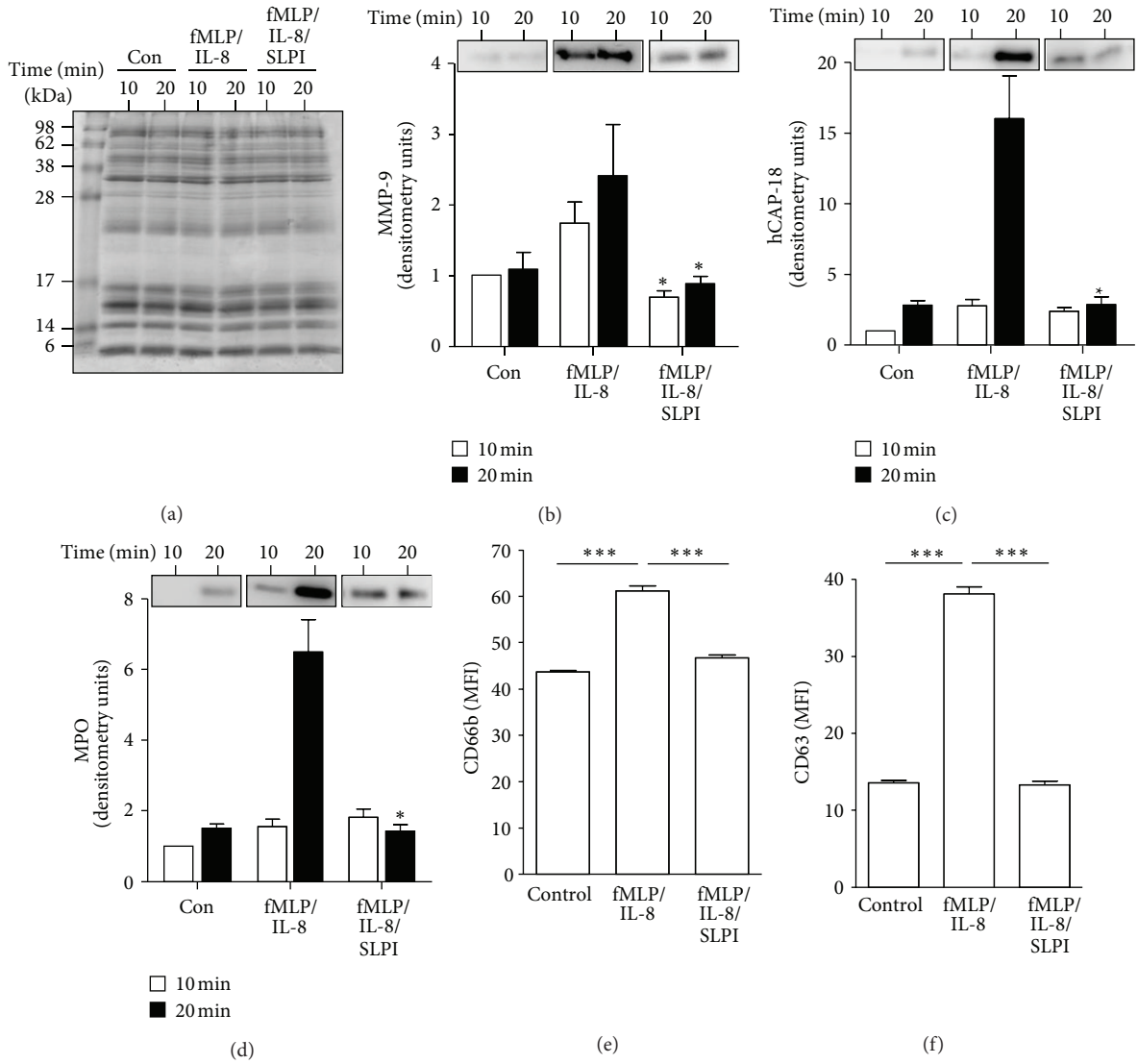

Figure 3: rhSLPI inhibits neutrophil degranulation. Neutrophils (5×10^6/mL) isolated from healthy individuals were either untreated (Con) or exposed to SLPI (480 nM), followed by stimulation with fMLP (1 μM) and IL-8 (1.2 nM) at 37°C. The use of equal cell numbers in each reaction is demonstrated by the identical electrophoretic profile of whole cell lysates in the Coomassie blue stained gel (a). Cell free supernatants were collected at 10 (□) or 20 min (■) and Western blotted for markers of tertiary ((b), MMP-9), secondary ((c), hCAP-18), or primary granule release ((d), MPO). (e) and (f), Healthy control neutrophils were fixed in 4% (w/v) paraformaldehyde following 10 min IL-8/fMLP stimulation in the presence or absence of SLPI (480 nM) and analysed by flow cytometry employing a FITC-labeled CD66b (e) or CD63 antibody (f). Data are represented as mean fluorescent intensity (MFI). All results (expressed as relative densitometry units) were performed in triplicate, and each bar is the mean ± S.E. A representative Western blot is illustrated. $^*P < 0.05$ between SLPI treated and untreated at the respective time points calculated by Student's t-test.

and MPO from primary granules (Figure 3(d)) were significantly reduced post 20 min stimulation in cells preloaded with 480 nM rhSLPI (a 60%, 83%, and 80% reduction respectively, $P < 0.05$). As tertiary granules are more readily discharged, the inhibitory action of rhSLPI was apparent at the 10 min time point (70% reduction, $P < 0.05$). As an alternative approach we investigated the membrane expression of CD66b and CD63. CD66b is a membrane receptor that is exclusively expressed on the membrane of secondary and tertiary granules, and CD63 is present on primary granule membranes [24]. Upon degranulation both CD66b and CD63 become expressed on the cell surface. As illustrated in Figures 3(e) and 3(f), membrane expression of CD66b and CD63 increased by 40% and 180%, respectively, after 10 min fMLP/IL-8 combined stimulation compared to unstimulated cells ($P < 0.001$). In contrast, upregulation of CD66b and CD63 to the plasma membrane was significantly decreased in stimulated neutrophils preloaded with SLPI compared to cells unexposed to SLPI ($P < 0.001$). Collectively, this data successfully demonstrates the inhibitory effect of rhSLPI on neutrophil chemotaxis and degranulation via IL-8 and fMLP GPCR signalling.

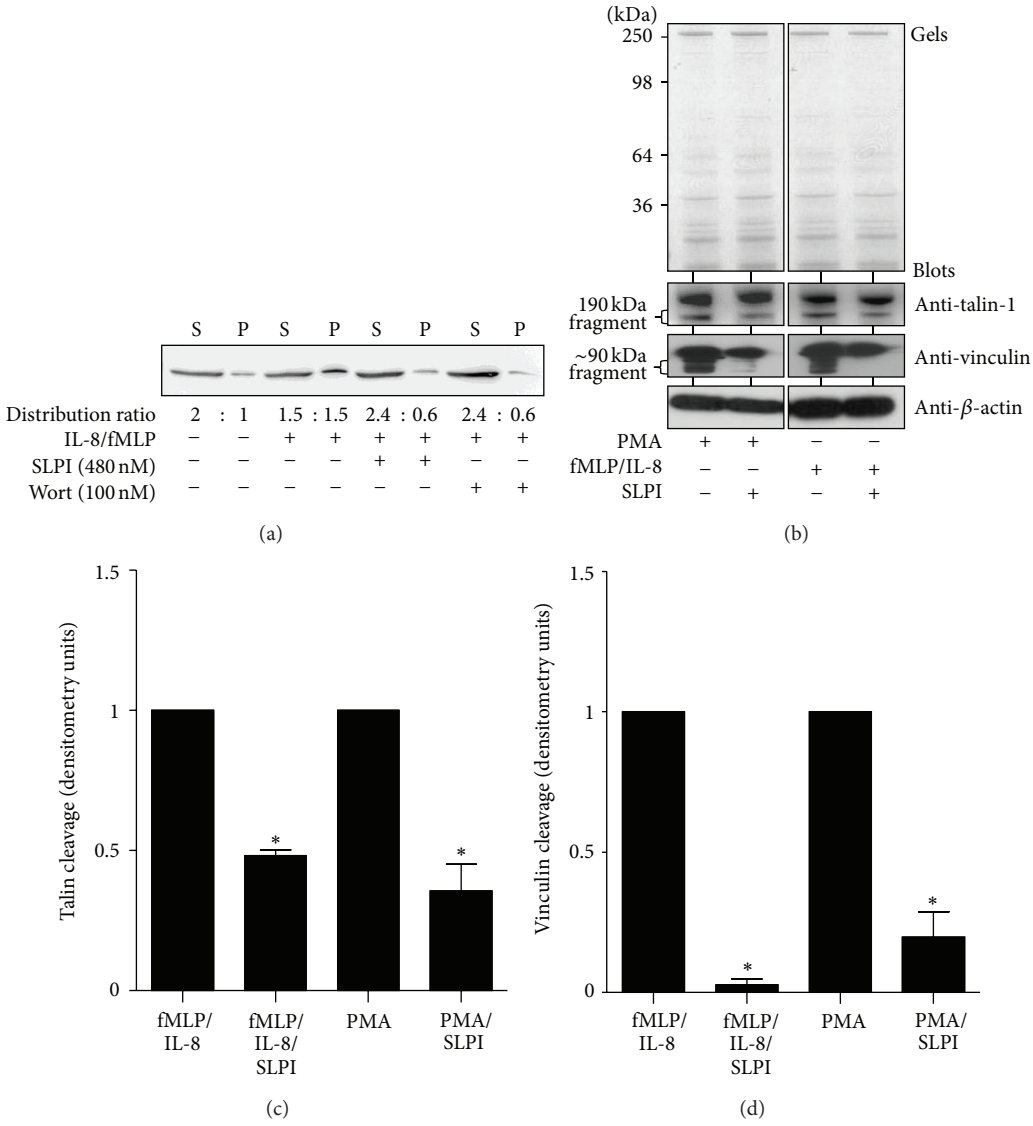

Figure 4: SLPI inhibits neutrophil cytoskeletal rearrangements. (a) Immunoblot with anti-actin antibody for the distribution of G-actin (in the supernatant fraction, S) and F-actin (in the pellet fraction, P) in untreated cells, IL-8/fMLP (1.2 nM/1 μM), SLPI (480 nM), or control wortmannin (Wort; 100 nM) treated cells. The distribution ratios calculated using constants obtained of Western blot densitometry values are illustrated. (b) The effect of SLPI (480 nM) on talin-1 (190 kDa fragment) or vinculin cleavage (90 kDa fragment) after PMA (1.6 μM, positive control), or IL-8/fMLP activation for 10 min was analysed by Western blotting using anti-talin-1 and anti-vinculin monoclonal antibodies. Equal sample loading was confirmed by Coomassie blue stained gels (top panels) and the β-actin immunoblots (lower panels). Results of three separate experiments were expressed as relative densitometry units ((c) and (d)) and each bar is the mean ± S.E. $^*P < 0.05$ between SLPI treated and untreated cells for the respective stimuli calculated by Student's t-test.

3.2. Characterisation of the Mode of Action of SLPI. Remodelling of the actin cytoskeleton is a prerequisite for cell chemotaxis and is a crucial event in the degranulation process. To study the effect of preloading cells with rhSLPI on the redistribution of F-versus G-actin after IL-8/fMLP exposure, a differential centrifugation assay was employed to analyse *in situ* F-actin levels. Preloading of cells with rhSLPI (480 nM) or exposure to wortmannin (100 nM) as a control [18] suppressed the IL-8/fMLP-induced change in the ratio of G-actin (supernatant fraction) versus F-actin (pellet fraction) (Figure 4(a)). In addition, as the C-terminal fragment of talin (190 kDa) is an actin nucleating protein which binds to G-actin and also the fact that cleavage of talin-1 is critical to focal adhesion disassembly [44], we investigated the possibility that SLPI regulates neutrophil chemotaxis by affecting talin cleavage. Neutrophils which were either untreated or preloaded with rhSLPI (480 nM/1 $\times 10^7$ cells) were stimulated with PMA (positive control) or fMLP/IL-8 for 10 min, and the level of talin-1 cleavage was assessed by immunoblotting using a mouse monoclonal anti-talin-1 antibody (Figure 4(b)). Site-specific cleavage of talin-1 yielding a 190 kDa fragment was observed in fMLP/IL-8

and PMA activated whole cell lysates, an effect consistently inhibited by preloading cells with rhSLPI (50% and 64% inhibition, resp.), (Figure 4(c), $P < 0.05$). Moreover, we evaluated the effect of rhSLPI on vinculin cleavage, a protein involved in neutrophil adhesion and pseudopod formation [45]. Results revealed that vinculin cleavage in response to fMLP/IL-8 and PMA was significantly reduced in cells preloaded with rhSLPI, as demonstrated by Western blot analysis (Figure 4(b)) and densitometric quantification (96% and 84% inhibition, respectively, Figure 4(d)).

The observation that rhSLPI could inhibit talin and vinculin cleavage prompted us to initially investigate the hypothesis that SLPI inhibited the Ca^{2+} dependent neutral cysteine protease activity of calpain-1 and calpain-2, which play an important role in cell migration by cleaving talin and vinculin [46]. SLPI has been shown to inhibit a broad range of serine proteases [47], but, unlike the antiprotease alpha-1 antitrypsin (AAT), SLPI has not been shown to inhibit other protease classes. For example, AAT has been shown to inhibit caspase-3 activity [48] and to modulate metalloprotease ADAM-17 activity, thereby regulating neutrophil chemotaxis in response to soluble immune complexes [18]. However, unlike AAT or the natural inhibitor calpastatin, which have been shown to inhibit neutrophil calpain [49], the results of the present study demonstrate that SLPI had no direct effect on calpain-1 or calpain-2 activity (see Supplementary Figure 1 in Supplementary Material available online on http://dx.doi.org/10.1155/2013/560141).

Upon activation of neutrophils in the absence of extracellular Ca^{2+}, the rise in cytosolic Ca^{2+} levels occurs by release from intracellular sites. As a result of the rise in Ca^{2+}, the neutrophil initiates cytoskeletal rearrangements required for degranulation and chemotaxis [50]. Therefore, we next explored the possibility that SLPI inhibits activation of neutrophils by regulating Ca^{2+} mobilisation from intracellular stores. Preloading neutrophils (2×10^5/mL) isolated from healthy individuals with rhSLPI (480 nM) in a Ca^{2+} free buffer significantly inhibited the rise in cytosolic Ca^{2+} levels from intracellular stores triggered by fMLP (Figure 5(a), $P < 0.01$ at 10 sec and $P < 0.05$ at 20 sec) and IL-8 (Figure 5(b), $P < 0.05$ at 20 and 30 sec) compared to untreated control cells. Of note, the observed spike in Ca^{2+} flux in healthy control cells not treated with SLPI is consistent with previously published data [18]. Furthermore, neutrophils isolated from CF stable individuals preloaded with rhSLPI also failed to initiate a Ca^{2+} spike upon stimulation with fMLP (Figure 5(c), $P < 0.05$ at 10 and 20 sec) or IL-8 (Figure 5(d), $P < 0.01$ and $P < 0.05$ at 10 and 20 sec, resp.). Additionally, when Ca^{2+} levels were analysed in neutrophils isolated from individuals during an acute exacerbation of COPD, there was a statistically significant decrease in the cytosolic Ca^{2+} levels of fMLP and IL-8 stimulated cells preloaded with rhSLPI (480 nM) compared to untreated cells ($P < 0.05$ at 10 and 20 sec, Figures 5(e) and 5(f)). Taken together, these results indicate that cytosolic SLPI inhibits Ca^{2+} flux in cells from patients or healthy donors when exposed to proinflammatory stimuli.

To rule out the possibility that SLPI inhibits Ca^{2+} flux in cells by preventing IL-8 and fMLP interacting with their respective receptors, studies on agonist-receptor interactions were performed. Whilst exposure to FITC-labeled fMLP resulted in specific binding of FITC-fMLP on neutrophil membranes by flow cytometry (Figures 6(a) and 6(b), pre-exposure of cells (1×10^7/mL) to unlabeled fMLP prevented FITC-fMLP binding thereby reducing the mean florescence intensity reading by approximately 75% (40.05 ± 4.7 to 11.04 ± 1.45 MFI, Figures 6(a) and 6(b)). In contrast, pre-loading of neutrophils with rhSLPI (480 nM) had no effect on the FITC-fMLP fluorescence reading (42.67 ± 4.164 compared to 40.05 ± 4.471 in the presence of SLPI), indicating that the immuno-regulator activity of SLPI was not a result of inhibiting fMLP binding to the cell membrane. Additionally, results indicated that preloading cells with rhSLPI did not impair IL-8 membrane binding (results not shown). Moreover, reaction with H_2O_2, a major oxidant generated by activated neutrophils, oxidizes all four methionine residues in SLPI, resulting in substantial diminution of its NE inhibitory activity [13]. In the present study, oxidised rhSLPI possessed decreased inhibitory capacity over intracellular Ca^{2+} flux upon activation with fMLP (1 μM) or IL-8 (1.2 nM, Figures 6(c), 6(b), and 6(d)) suggesting the requirement of the active site of SLPI for the observed inhibitory effect on Ca^{2+} flux.

In response to either fMLP or IL-8 the initial rise in intracellular cytosolic Ca^{2+} requires IP_3 production, and subsequent IP_3 occupancy of receptors on calciosomes induces a rapid release of Ca^{2+} [51, 52]. Therefore, we next explored the possibility that cytosolic rhSLPI inhibits Ca^{2+} cytosolic flux by preventing upstream IP_3 production. For this analysis we measured levels of IP_1 which accumulates as a stable product of IP_3 [27]. Pre-loading of neutrophils (1×10^5/mL) with rhSLPI (480 nM) significantly reduced cumulative levels of IP_1 upon fMLP or IL-8 activation (an approximate 75% reduction for both stimuli, $P < 0.05$), with similar levels to unstimulated cells observed (Figure 7(a)). Positive controls for this experiment included addition of the PLC-β inhibitor U73122 (5 μM) or the GPCR inhibitor pertussis toxin (PTX, 500 ng/mL), with significant inhibition of IP_1 accumulation observed for both ($P < 0.05$). Substantiating this result, it was observed that the inhibitory effect of rhSLPI (480 nM) on Ca^{2+} flux induced by fMLP and IL-8 was overcome by augmenting cytosolic levels of IP_3 (1 μM) (Figure 7(b)). For this latter experiment IP_3 was incorporated into electroporated neutrophils and the cells immediately employed in Ca^{2+} flux measurements. Moreover, results revealed that rhSLPI employed at a concentration of either 240 or 480 nM was unable to reduce the chemotactic index of cells loaded with IP_3 in response to either fMLP (Figure 7(c)) or IL-8 (Figure 7(d)).

Collectively, these results confirm that cytosolic SLPI did not impede agonist receptor interaction but successfully modulated IP_3 production and subsequent release of Ca^{2+} from IP_3-regulated internal stores. However, as the production of IP_3 is a direct outcome of PLC-β activation, further experiments are required to fully understand how SLPI modulates PLC-β activation. Moreover, it is possible

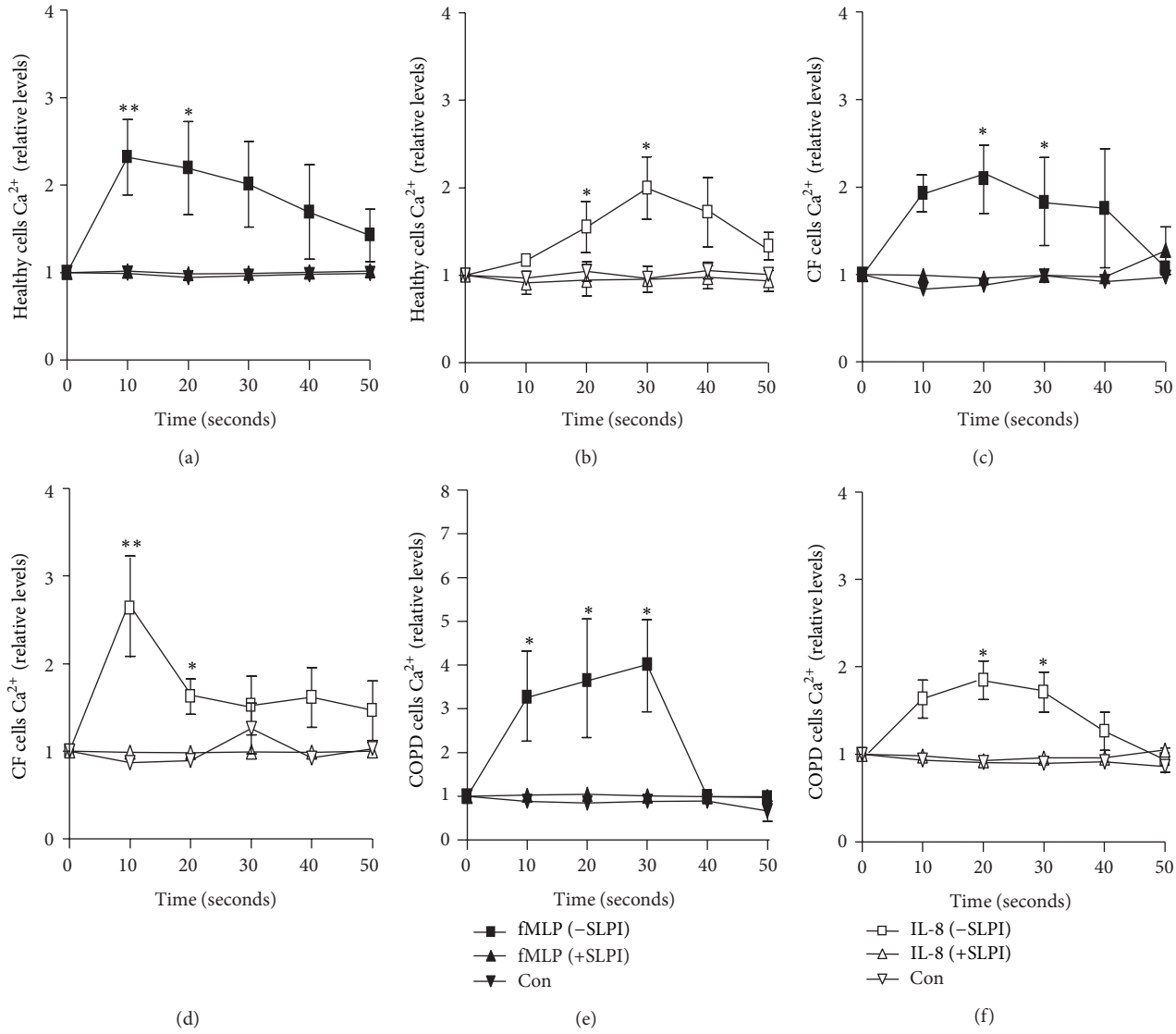

FIGURE 5: SLPI inhibits intracellular Ca^{2+} flux. Neutrophils (2×10^5/mL) were isolated from healthy donors ((a) and (b)), individuals with CF ((c) and (d)) or COPD ((e) and (f)) ($n = 5$ for each cohort). Cells remained untreated (control, con) or treated with 480 nM SLPI for 5 min prior to stimulation with fMLP (1 μM) or IL-8 (1.2 nM) for 10, 20, 30, 40, or 50 sec. Intracellular cytosolic Ca^{2+} was analysed using the Invitrogen Fluo-4NW Calcium Assay kit. Each point is the mean ± S.E. ($n = 5$, $^*P < 0.05$ and $^{**}P < 0.001$ between untreated and SLPI treated cells calculated by 2 way ANOVA followed by Bonferroni test).

that SLPI affects Ca^{2+} flux via modulation of other immune signalling pathways including the mitogen-activated protein kinase (MAPK) cascade, ERK1/2 and p38 MAPK [53–55], modulation of the increase in intracellular Ca^{2+} associated with activation of the transcription factor NF-κB [56], or by regulation of cAMP-dependent protein kinase A [3]. Nevertheless, the maintenance of intracellular Ca^{2+} levels via IP_3 modulation represents a novel therapeutic strategy in inflammatory conditions such as COPD and CF, where neutrophil infiltration to the airways causes excessive tissue damage. In line with this theory, U73122, a membrane permeable aminosteroid PLC inhibitor, which we have been used as a positive control in this study, has previously been shown in an *in vivo* animal model to significantly inhibit neutrophil infiltration to the peritoneal cavity upon LPS injection, in addition to reducing IL-8 and leukotriene B_4 induced Ca^{2+} flux in neutrophils [57]. The findings of the present study are highly relevant in the consideration of rhSLPI as a modulator of Ca^{2+} flux and therapeutic modality in chronic neutrophilic airway inflammatory disorders. In support of this concept applications of aerosolized anti-proteases such as SLPI have been investigated as potential therapeutics for people with CF and COPD [58, 59]. Aerosolized rhSLPI (100 mg, twice daily for 1 week) has previously been administered to individuals with CF [59] with results revealing increased epithelial lining fluid levels of SLPI and significantly reduced levels of active NE (pretreatment 11.1 ± 1.8 μM NE to posttreatment 6.1 ± 1.2 μM NE). A further *in vivo* study carried out in rats

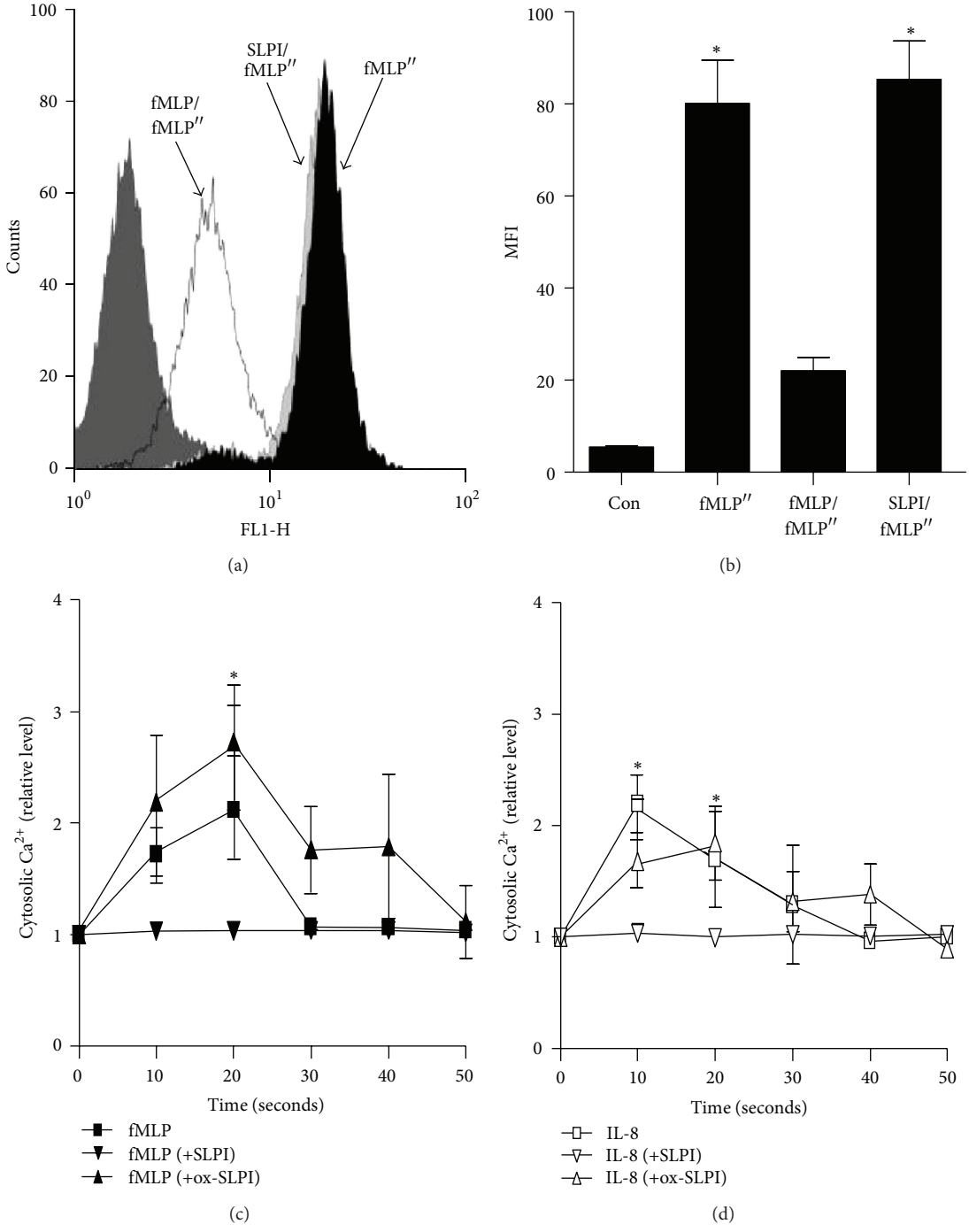

FIGURE 6: Intracellular nonoxidised SLPI exerts immunomodulatory activity. (a) Neutrophils (1×10^7/mL) were exposed to unlabeled fMLP (10 μM) or SLPI (480 nM) for 1 min, followed by FITC-labeled fMLP (fMLP″) (1 μM), and the level of bound fMLP″, quantified by FACS. The negative control (unlabeled cells) is illustrated in dark grey and a total of 10,000 events were collected. (b) Results in mean fluorescence intensity units (MFI) demonstrate that pre-incubation with unlabeled fMLP, but not rhSLPI, prevented binding of fMLP″ to the cell membrane ($^*P < 0.05$ compared to fMLP/fMLP″ cells). (c) and (d); Neutrophils (1×10^7/mL) remained untreated or treated with 480 nM SLPI or 480 nM oxidised SLPI (ox-SLPI) for 5 min, prior to stimulation with fMLP ((c), 1 μM) or IL-8 ((d), 1.2 nM). Ox-SLPI did not inhibit the fMLP or IL-8 induced Ca^{2+} flux ($^*P < 0.05$ between SLPI and ox-SLPI). Each experiment was performed in triplicate, each point is the mean ± S.E. and statistical significance was calculated by 2-way ANOVA followed by Bonferroni test or Student's t-test.

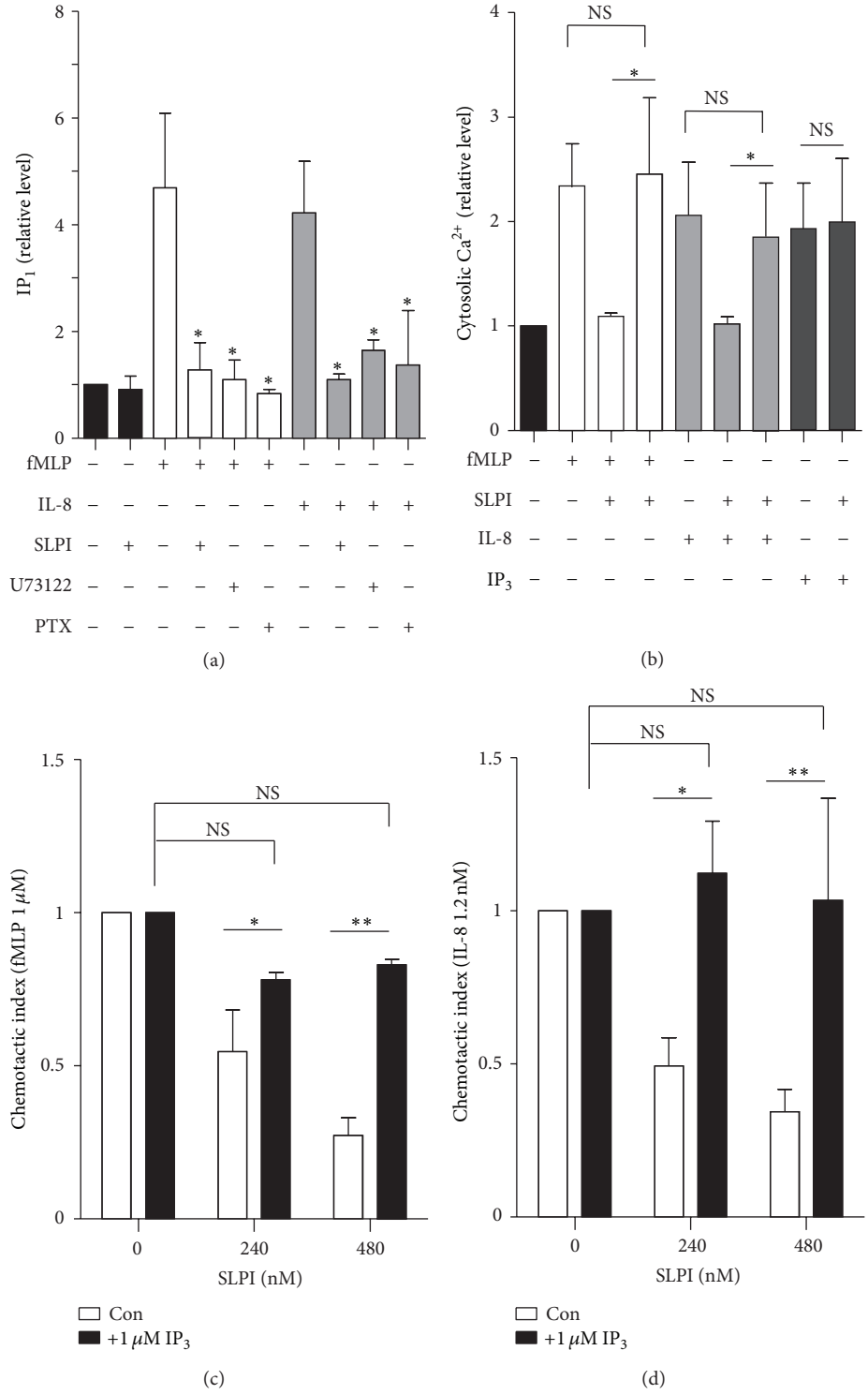

FIGURE 7: SLPI inhibits production of IP_3. (a) Preloading neutrophils (1×10^5/mL) with SLPI (480 nM) significantly reduced the accumulative levels of IP_1 induced by fMLP (1 μM) or IL-8 (1.2 nM) activation (final reaction volume of 200 μL). Positive controls included U73122 (5 μM) and pertussis toxin (PTX, 500 ng/mL). (b) Cells remained untreated or preloaded with SLPI (480 nM), electropermeabilized in the presence or absence of 1 μM IP_3 and then stimulated with fMLP (1 μM) or IL-8 (1.2 nM) for 10 sec. (c) and (d) Neutrophils were isolated from healthy individuals and the effect of SLPI (240 or 480 nM) on the cell chemotactic index induced by fMLP (1 μM) or IL-8 (1.2 nM) was determined in the presence (■) or absence (con, □) of 1 μM IP_3. SLPI had no significant inhibitory effect on neutrophil chemotaxis in the presence of augmented cytosolic IP_3. Each point is the mean ± S.E. ($n = 5$, NS: no significant difference, and $^*P < 0.05$ and $^{**}P < 0.001$ calculated by Student's t-test).

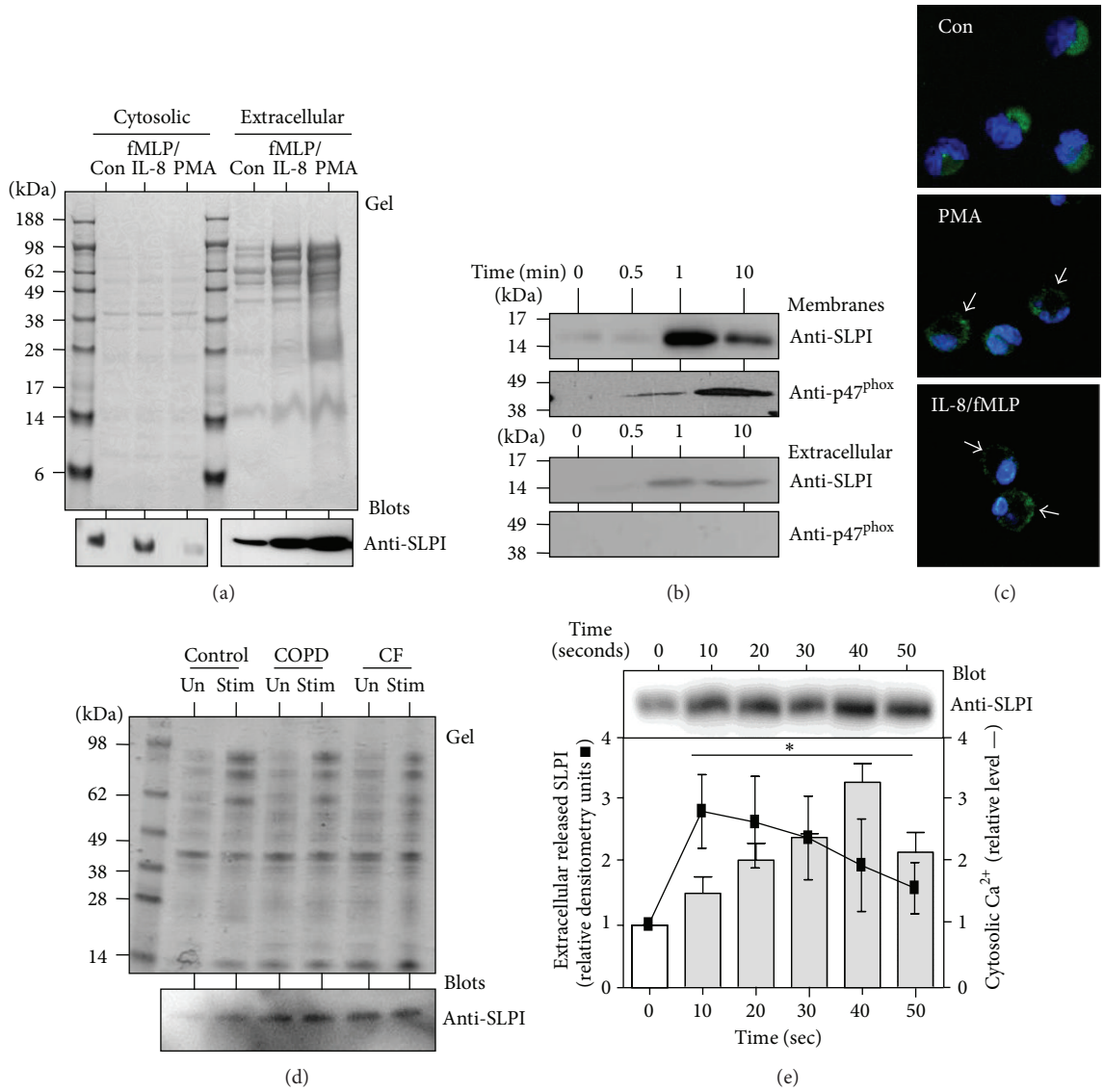

FIGURE 8: SLPI is secreted from the cell upon activation. (a) Coomassie Blue stained gel (top panel) and immunoblots (bottom 2 panels) employing anti-SLPI antibody (Gt, Synergen) for its respective distribution (intracellular or extracellular) in control unstimulated cells (Con) and after IL-8 (1.2 nM) and fMLP (1 μM) or PMA (1.6 μM) activation for 10 min. (b) Donor neutrophils (5×10^6/mL) were stimulated with PMA (1.6 μM) for 0, 0.5, 1, or 10 min before sonication and preparation of membrane (top panels) and extracellular released protein fractions (bottom panels). SLPI and $p47^{phox}$ translocation was analysed by Western blotting employing polyclonal rabbit antibodies. (c) The distribution of SLPI in resting unstimulated cells (Con) and after PMA or fMLP (1 μM) and IL-8 (1.2 nM) activation for 10 min was detected using an FITC labeled rabbit polyclonal anti-SLPI antibody (green fluorescence). The distribution of SLPI was predominantly cytosolic in unstimulated cells, and after stimulation condensed around the margin of the cell (indicated by white arrow). DAPI stained nuclei are represented in blue (×40 magnification, ×10 zoom). (d) Coomassie blue stained gel (top panel) and immunoblot (bottom panel) employing rabbit anti-SLPI antibody for detection of released SLPI in the extracellular supernatants of unstimulated (Un) or fMLP/IL-8 stimulated (Stim) healthy control (Con), COPD or CF cells (5×10^6/mL). (e) Neutrophils isolated from healthy donors were incubated at 37°C and were treated with fMLP/IL-8. Cell free supernatants were collected at 0, 10, 20, 30, 40 or 50 sec and Western blotted for cell secreted SLPI (top panel) and intracellular cytosolic Ca^{2+} was analysed using the Invitrogen Fluo-4 Calcium Assay kit. Results of three separate experiments were expressed as relative densitometry units (bar graph), and each bar is the mean ± S.E. $^*P < 0.05$ is between 0 time point and statistical significance was calculated by Student's t-test.

reported that rhSLPI could remain biologically active in the lungs for at least 8 h [60]. When administered intratracheally, (8.6 mg/kg) the reported half-life of SLPI was 4 to 5 h, indicating minimal metabolism in the airways. rhSLPI has also been administered intravenously to sheep [21]. In this latter study, following an infusion of rhSLPI (1 g) over 10 min the half-life of rhSLPI was reported as 1.8 h; however if the rate of infusion was slowed, SLPI excretion was significantly

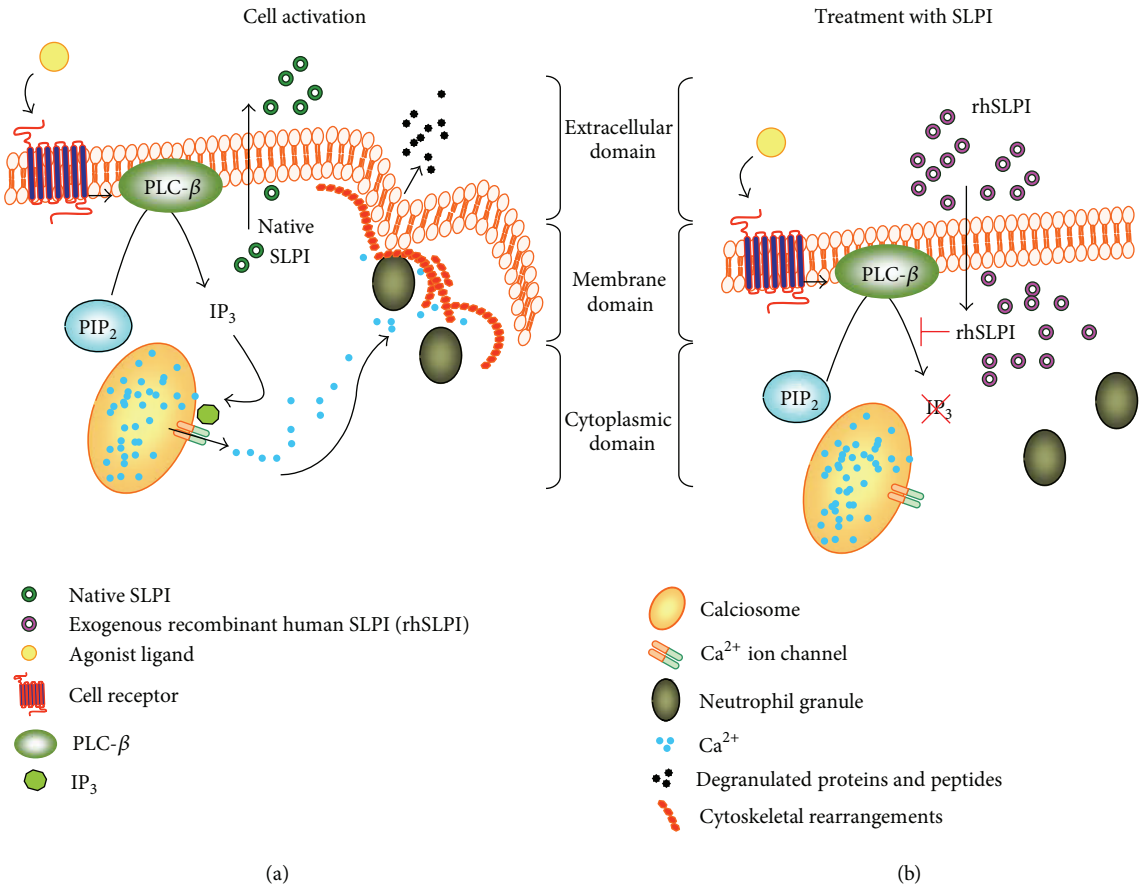

FIGURE 9: SLPI regulates neutrophil activity by modulating IP_3 production. (a); Upon neutrophil stimulation SLPI is secreted from the cell to the extracellular environment and IP_3 is formed during PLC-β activation. IP_3 binds to Ca^{2+} voltage-gated channels on calciosomes (or endoplasmic reticulum) and allows Ca^{2+} ions to flow from the lumen of the calciosome toward the cell interior. The presence of Ca^{2+} ions within the cytosol allows cytoskeletal rearrangements such as actin polymerization and talin cleavage facilitating the process of cell degranulation and chemotaxis. (b); Exogenously added rhSLPI effectively accumulates within the cell and reduces IP_3 production thereby inhibiting degranulation of antimicrobial peptides and proteins and cell migration.

decreased with a 3 h infusion associated with 9% excretion [21]. Intact SLPI was detected in lymph and epithelial lining fluid, which displayed anti-NE capacity in line with the levels of SLPI [21].

3.3. Secretion of SLPI from the Neutrophil Coincides with Cell Activation. The question next investigated was how cells overcome the inhibitory effect of native cytosolic SLPI thereby leading to Ca^{2+} flux in neutrophils. Indeed, neutrophils have previously been shown to secrete SLPI [11], and for this reason ensuing experiments were designed to determine whether the kinetics of SLPI release upon cell activation corresponded to the spike of Ca^{2+} flux. By Western blotting, it was found that neutrophils (5×10^6/mL) secrete cytosolic SLPI to the outside of the cell in response to both IL-8 (1.2 nM) and fMLP (1 μM) or PMA (1.6 μM) activation for 10 min (Figure 8(a)). A reduction in the level of cytosolic SLPI was observed, most prominently after PMA activation, with a concomitant increase in the level of extracellular SLPI detected. These results are in keeping with a previous study that recorded significant amounts of SLPI secreted from activated neutrophils (3 μg/10^6 cells/24 h) as compared with an epithelial and type II pneumocyte cell line [11]. This set of experiments were expanded and included subcellular fractionation and isolation of membranes following cell (5×10^6/mL) activation with PMA (1.6 μM) at 0, 0.5, 1, and 10 min. Results revealed that over the timecourse explored, progressively higher quantities of SLPI became associated with the plasma membrane (Figure 8(b) upper panel) followed by sequential secretion to the outside of the cell (Figure 8(b) lower panel). Translocation of the NADPH oxidase component $p47^{phox}$ from the cytosol to the membrane upon cell activation was used as a positive control [61]; however $p47^{phox}$ was not released from the cell. To corroborate results, confocal microscopy was employed and revealed that SLPI was localized diffusely and uniformly throughout the cytosol of control resting cells (green). In contrast, after fMLP/IL-8 or PMA stimulation for 10 min the majority of SLPI translocated to the periphery of the cell (Figure 8(c)). Moreover, a greater level of SLPI secretion from CF and COPD neutrophils

(5×10^6/mL) was detected in extracellular supernatants by Western blot analysis (Figure 8(d)). This latter result is in agreement with data indicating a requirement for increased SLPI for modulation of COPD and CF neutrophil activity (Figures 2(c) and 2(d)) possibly due to the occurrence of cell priming. On the assumption that extracellular release of SLPI must follow the same time course of seconds as Ca^{2+} flux, we monitored release of SLPI from the cell after 10, 20, 30, 40, and 50 sec fMLP/IL-8 stimulation. Release of SLPI was quantified in the extracellular supernatant by immunoblotting and results demonstrate that levels of extracellular SLPI were significantly increased after just 10 sec stimulation (Figure 8(e), $P < 0.05$). Although the time course of Ca^{2+} flux peaked at 10 sec, a lag period of 30 sec for the maximum level of detectable cell free SLPI was evident. This may in part be explained by the time required for immunodetection of measurable amounts of the protein on the outside of the cell.

SLPI release from the cell following activation has been comprehensively studied [11]; however the signalling events including possible phosphorylation of SLPI and necessary membrane receptor interactions required for its release, have yet to be evaluated. Nevertheless, collectively these results indicate that upon exposure to proinflammatory stimuli cytosolic SLPI is actively secreted from the cell, and the time of elimination of SLPI coincides with the time of Ca^{2+} flux.

4. Conclusion

This study presents evidence that SLPI is present in the cytosol and also the secondary granules of resting neutrophils and is secreted from the cell upon exposure to the phagocyte activator PMA or the chemokines IL-8 and fMLP. We show that neutrophil cytosolic SLPI is effective as a serine protease inhibitor with potent activity against NE. Moreover, within the neutrophil SLPI maintains the ability to modulate cellular processes involving cytoskeletal restructuring including directional chemotaxis and degranulation of antimicrobial peptides and proteases. This investigation elucidates a hitherto undescribed function of intracellular neutrophil cytosolic SLPI and indicates that the described anti-inflammatory effects of SLPI may be orchestrated through inhibition of Ca^{2+} flux by modulating IP_3 production (Figure 9). Due to the correlative relationship between neutrophil activation and cytosolic Ca^{2+} levels, inhibition of Ca^{2+} flux by SLPI may pose as a potential anti-inflammatory therapy during acute exacerbation of severe chronic obstructive lung disease, thereby reducing neutrophil influx into the airways.

SLPI, elafin, and AAT have been a focus of interest from a therapeutic viewpoint for a number of years. Modifying excessive neutrophil activation and an overexuberant inflammatory response would be a relevant treatment objective in a wide variety of conditions including COPD and CF and also in the modulation of periodontal disease, sepsis syndromes, asthma, bronchiectasis, and in transplant rejection. Recent studies have employed aerosolized liposomal [62] and hydrogel [63] formulations for successful delivery of SLPI. Our finding demonstrating the inhibitory capacity of SLPI on IP_3 production and Ca^{2+} flux strengthens its attractiveness as a potential therapeutic, and the finding that SLPI has an anti-inflammatory role via inhibition of neutrophil chemotaxis and degranulation is a novel concept that opens up a new field of investigation.

Abbreviations

SLPI: Secretory leukoprotease inhibitor
COPD: Chronic obstructive pulmonary disease
CF: Cystic fibrosis
IP_3: Inositol 1,4,5-triphosphate
NE: Neutrophil elastase.

Authors' Contributions

Emer P. Reeves and Nessa Banville share joint first authorship.

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Research Article

The SDF-1α 3′A Genetic Variation Is Correlated with Susceptibility of Asthma in Iranian Patients

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Background and Aim. Chemokine/receptor axis is a predominant actor of clinical disorders. They are key factors of pathogenesis of almost all clinical situations including asthma. Correspondingly, CXCL12 is involved in the immune responses. Therefore, this study was designed to explore the association between gene polymorphism at position +801 of CXCL12, known as SDF-1α 3′A, and susceptibility to asthma in Iranian patients. *Material and Methods.* In this experimental study, samples were taken from 162 asthma patients and 189 healthy controls on EDTA. DNA was extracted and analyzed for CXCL12 polymorphisms using PCR-RLFP. The demographic information was also collected in parallel with the experimental part of the study by a questionnaire which was designed specifically for this study. *Findings.* Our results indicated a significant difference ($P < 0.0001$) between the *A/A*, *A/G*, and *G/G* genotypes and *A* and *G* alleles of polymorphisms at position +801 of CXCL12. We also showed an elevated level of CXCL12 circulating level in Iranian asthma patients. *Conclusion.* Our findings suggest that SDF-1α 3′A (CXCL12) polymorphism plays a role in pathogenesis of asthma. It can also be concluded that circulatory level of CXCL12 presumably can be used as one of the pivotal biological markers in diagnosis of asthma.

1. Introduction

Asthma is characterized as the airways chronic inflammatory disease with mostly airway obstruction, which is accompanied by increased bronchial high responsiveness to various external and internal stimuli. Asthmatic patients suffer from varying symptoms from breathlessness, wheezing, and cough to chest tightness. It is sometimes coupled with allergy, an acquired potency for progression of unwanted reactions that are controlled via immunological mediators [1]. Approximately more than 22 million people are suffering from asthma. Asthma and allergic diseases are believed to follow the pattern of genetic diseases that are caused by the interaction of multiple genes and also environmental stimuli [2].

Although several lines of evidence have indicated the central role of chemokines in regulating multiple aspects of the asthmatic response, the full picture of chemokines' involvement in allergic airway inflammation, the exact role of specific chemokines at various stages in the evolution of allergic lung inflammation and their regulatory pathways in vivo are only partially identified.

In the process of allergic airway disease, at least one part of diagnosis is the infiltration of blood leukocytes, including

eosinophils, neutrophils, lymphocytes, and macrophages in the lung [3]. The CD4+ Th2-type lymphocytes (Th2 cells) and other inflammatory leukocytes in airways mucosa secret a wide range of inflammatory mediators that participate both directly and indirectly in remodeling of the airway wall, excessive mucus secretion, airway obstruction, and finally airway hyperreactivity [4–6].

CXCL12 as a homeostatic CXC chemokine is widely expressed with a broad range of functions (from recruitment of mature B and T cells to migration of haematopoietic progenitor cells from the bone marrow). It is well documented that CXCL12 plays a wide variety of roles in two unrelated set of diseases such as human immunodeficiency virus (HIV) and cancer, because its known receptor (CXCR4) is a coreceptor used by HIV T-tropic strains, and it is also the most widely expressed chemokine receptor in many different types of cancers and asthma [7, 8]. "The SDF-1 gene is located on human chromosome 10q11.1 and SDF1-3′A polymorphism is a single nucleotide polymorphism (SNP) at position 801 relative to the start codon in the 3′ untranslated region involving a G > A transition in SDF-1. The A allele is probably a target of cis-acting factors, and it is assumed to upregulate the expression of SDF-1" [9]. This polymorphism is the best-studied polymorphism in CXCL12 gene [1].

The association between this polymorphism and a variety of diseases from lung and breast cancer, acute myeloid lymphoma, leukemia, and chronic myeloproliferative disease to viral infections such as acute hepatitis is well documented [1].

There are no reports describing any association between SDF-1α 3′A polymorphism and pathogenesis of asthma. Therefore, the authors of this study hypothesized that this polymorphism may play a critical role in development of asthma in Iranian population; thus, this study investigated the predicted role of the +801G/A polymorphism in pathogenesis and progression of asthma in Iranian population.

2. Material and Methods

2.1. Subjects. In this study, we recruited 162 unrelated asthmatic patients with a mean age of 51 ± 11 years (ranged between 15 and 79 years) using easy convenience method. Women being pregnant, patients having a history of inflammatory and infectious diseases rather than asthma, and patients with a history of cigarette smoking were excluded from the study. The occurrence of asthma was diagnosed by an expert infectious diseases specialist according to the American Thoracic Society (ATS) criteria. According to the clinical findings and history, patients were classified into 2 groups, that is, allergic and nonallergic groups. We also enrolled 189 genetically unrelated subjects having normal spirometric values and no respiratory symptoms which were matched for sex and similar ethnicity origin with the patients. The study protocol was approved by the ethical committee at our institution (Rafsanjan University of Medical Sciences), and written informed consent was obtained from all participants, either patients or controls, prior to sample collection. Some characteristics of the subjects are summarized in Table 1.

2.2. DNA Extraction. Asthmatic and control subjects' peripheral blood samples were collected on Ethylenediaminetetraacetic acid (EDTA) precoated tubes and subjected to genomic DNA extraction using a commercial kit (Bioneer, South Korea). The extracted DNA samples were then stored at −20°C for further use.

2.3. Polymorphism Detection. The CXCL12 gene polymorphism at the position +801 was analyzed by polymerase chain reaction-restriction length polymorphism (PCR-RFLP) method. As previously described by Hassanshahi et al., Azin et al., and Ryabov et al. [10–12], a PCR reaction mixture was made up by addition of the following reagents to a 0.2 mL microcentrifuge tube on ice:

2.5 μL of *taq* DNA polymerase buffer (10X), 0.5 μL of MgCl2 (stock concentration 1.5 mM), 0.5 μL of each dNTP (dATP, dCTP, dGTP, and dTTP) stock concentration of 10 mM), 1 μL of each primer (forward: CAGTCAACCTGGGCAAAGCC and reverse: AGCTTTGGTCCTGAGAGTCC), stock concentration of 25 ng/μL, 1 μL of prepared DNA, and sterile double-distilled water to a final volume of 25 μL. The following program was used for fragment amplification: one cycle of 93°C for 2 min, 93°C for 1 min (denaturation), 1 min at 57°C for annealing of CXCL12, 72°C for 40 sec (elongation) followed by 30 cycles of 93°C for 20 sec, 55°C for 20 sec, and 72°C for 40 sec. Within the last 45 sec of the first stage, approximately 0.3 μL of Taq DNA polymerase was added to the mixture. *The final volume was 20 μL which contains 2 μL of primer mix of forward and reverse, 10 μL of Master mix (dNTP, $MgCl_2$,* and *Taq enzyme), 3 μL of DNA, and 5 μL of DNase-free water.* The amplified PCR product of CXCL12 gene covers +801 regions with a molecular size of 302 bp. The Sac-1 (Fermentas, Lithuania) restriction enzyme has only one restriction site on this region, and is capable to digest the fragment and produce two fragments of 202 and 100bp following digestion. In fact, in case of heterozygotic form (A/G), 3 different fragments with 302, 202, and 100 bp are then visible, while in homozygotic form, a 302 bp fragment (without any digestion (A/A)) or two 202 and 100 bp (digesting both alleles (G/G)) was then observed (Table 2 and Figure 2). The digested products were electrophoresed on a 2.5% agarose gel following addition of 4 μL of loading buffer (Cinnagen, Iran) and studied on ChemiDoc model XRS (Bio-Rad, USA) subsequent to ethidium bromide staining.

2.4. Chemokine Assay. The serum level of CXCL12 was measured by ELISA (R&D systems, UK) in patients and healthy controls immediately after blood collection. Assays were performed as per manufacturer's guidelines. The sensitivity of kits was 2 pg/mL, and inter- and intra-assay assessments of reliability of the kit were conducted.

2.5. Statistical Analysis. Hardy-Weinberg equilibrium was assessed using genotype data. Allele and genotype frequencies were calculated in patients and healthy controls by direct

Table 1: Subjects characteristics.

Variant	Controls	Asthma patients
Age	48 ± 12 years	51 ± 11 years
Gender: M/F	88/101 (189)	99/129 (228)
Frequent symptoms (cough, wheeze, and shortness of breath)	−	+
History of allergy	−	70.3%
Familial history of asthma	−	43.2%
FEV1 (predicted %)	87.3 ± 14	79.2 ± 21
FEV1/FVC	88.1 ± 9	82.3 ± 9
PEF (L/predicted %)	91.6 ± 21	74.6 ± 19
FEF.sub.25–75>	84.7 ± 17	62.9 ± 34
FVC (L/predicted %)	93.4 ± 8	79.9 ± 19
Eosinophils (106/L)	117 ± 96	612 ± 837
IgE (IU/mL)	24 ± 9	1,112 ± 1,082

FEV1: forced expiratory volume in 1 second, FVC: forced vital capacity, PEF: peak expiratory flow, and FEF.sub.25–75>: forced expiratory flow 25–75% or 25–50% (mean ± SD).

Table 2: Frequency of polymorphisms of SDF-1α (CXCL12) gene in asthma patients and controls.

	Patient n (%)	Control n (%)	OR	95% CI	P value
Genotype					
AA	18 (4.3%)	27 (6.5%)	2.541	1.17–5.51	*0.018
AG	190 (45.6%)	60 (14.4%)	0.144	0.77–0.268	* <0.0001
GG	20 (4.8%)	102 (24.5%)	4.021	2.21–7.29	* <0.0001
Alleles					
G	230 (27.6%)	264 (31.7%)	1		
A	226 (27.1%)	114 (13.7%)	2.276	1.70–3.03	* <0.0001

*Significant difference was observed.

gene counting. Statistical analysis of the differences between groups was determined by χ^2, t-test, and ANOVA using SPSS software version 17 which power of test was %90. P value of less than 0.05 was considered significant.

3. Results

Statistical analysis of demographic parameters indicated that the mean age, gender, and socioeconomical status of the participants had no markedly differences, which were as follows: the mean age of patients was 51 ± 11 years and of control group was 48 ± 12 years ($P = 0.85$), and the gender variation of patients was 129 (56.58%) female and 99 (43.42%) male and for control group was 101 (61%) female and 88 (38.6%) male ($P = 0.9$) (Table 1).

Analysis of the polymorphisms in +801 of CXCL12 by *Sac*-1 restriction enzyme showed that the frequency of *A/A* genotype was 18 (4.3%) in patients and 27 (6.5%) in controls (OR = 2.541, 95% CI = 1.17−5.51, $P < 0.018$). Our results also revealed that the frequency of *A/G* genotype was 190 (45.6%) and 60 (14.4%) in patients and controls, respectively (OR = 0.144, 95% CI = 0.77 − 0.268, $P < 0.0001$).

The frequency of the *G/G* genotype in patients was 20 (4.8%) and in controls was 102 (24.5%) (Table 2), where statistically analyzed data indicated a significant difference between the two groups ($P < 0.0001$) (OR = 4.021, 95% CI = 2.21–7.29, $P < 0.0001$). The frequency of *A* allele was 226 (27.1%) and 114 (13.7%) in patients and controls, respectively. In case of *G* allele, 230 (27.6%) were observed in patients, while the frequency of this allele was 264 (31.7%) in controls (Table 2). Statistical analysis of alleles exhibited a significant difference between patients and controls ($P < 0.0001$). The results of this study also showed that the plasma level of CXCL12 was 312.53 ± 28.75 and 83.57 ± 6.74 pg/mL in asthma patients and healthy controls, respectively (Figure 1). Statistical analysis showed that the difference was significant ($P < 0.0001$). Our results indicated that the protein levels of SDF-1 in AG genotype in asthma and control were 448.75 ± 281 and 171.81 ± 54.11, respectively, which the difference was significant ($P < 0.0001$). Analysis of our data showed that the protein levels of SDF-1 in AA genotype in asthma and control were 22.84 ± 8.41 and 21.62 ± 17.61, respectively, which the difference was not significant ($P < 0.822$). Analysis of data by Eta test did not show significant difference between genotype and protein level of SDF-1 in patient group (approx. sig = 0.654, OR = 11.89/13.61); it also showed that there is no significant difference between FEV1 percent and genotype in patient group (Eta Test, approx. sig = 0.654, OR = 58.40/59.58) and that there is no significant difference between genotype and FEV1/FVC percent in patient group (Eta Test, approx. sig = 0.654, OR = 64.60/65.60). Analysis data by Spearman's test (Spearman's correlation coefficient) showed significant difference between protein level of SDF-1 and FEV1 percent and between protein level of SDF-1 and FEV1/FVC percent in patient group ($P < 0.0001$); this is an inverse relationship between protein level of SDF-1 and FEV1 percent and between protein level of SDF-1 and FEV/FVC percent (Table 3).

Analysis data with Kruskal-Wallis Test (because this kind of data does not have normal distribution, we had to use this test to obtain mean rank of three genotype polymorphisms for protein level of SDF-1, FEV1 percent, and FEV1/FVC percent) showed that AA, AG, and GG genotypes have the mean rank of 71.38, 46.81, and 56.79 for the protein level of SDF-1, 32.63, 57.19, and 47.21 for the FEV1 percent and 32.63, 57.19, and 47.21 for the FEV1/FVC percent, respectively, in patient group. For all of them, = 0.021, which showed significant difference ($P < 0.05$) for protein level of SDF-1, FEV1 percent, and FEV1/FVC percent (Table 4).

4. Discussion

The current study was undertaken to investigate the role of CXCL12 + 801 G/A SNP polymorphism in susceptibility to asthma in Iranian asthma patients. All of study groups (patients and controls) had same ethnic background origin and shared a common geographic origin in Southeast part of Iran. We demonstrated a closed association between this polymorphism and asthma in Iranian asthma patients. To our best knowledge, this is the first study to report an association between this polymorphism and asthma. The paramount

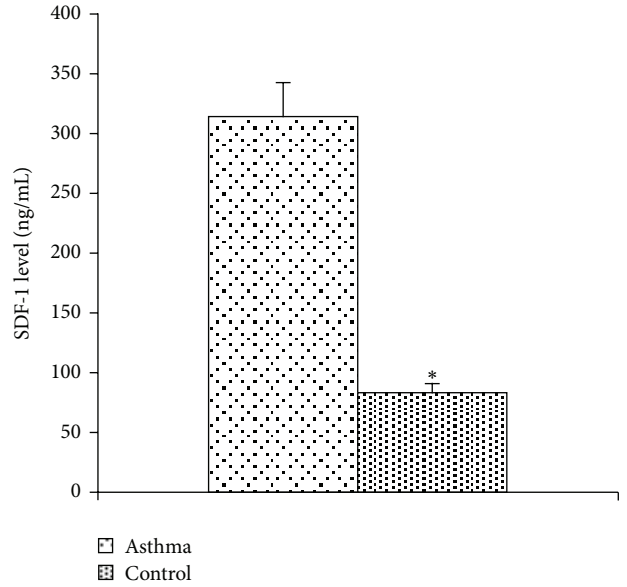

Figure 1: SDF-1α (CXCL12) circulating level in asthma patients and controls. $*$= significant difference was observed in SDF-1α (CXCL12) serum level ($P < 0.0001$). Data are shown as mean ± SEM.

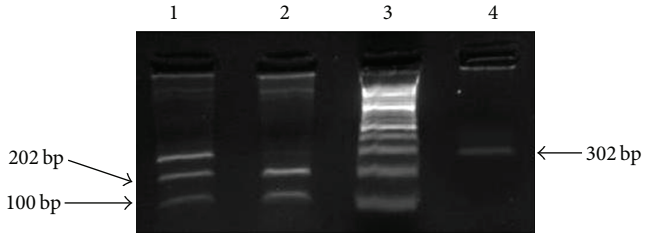

Figure 2: *Sac*-1 digestion of PCR product of SDF-1 gene. Lane 1: heterozygotic digestion of PCR product of SDF-1 (A/G). Lane 2: homozygotic digestion (G/G). Lane 3: 100 bp ladder marker. Lane 4: homozygotic nondigested PCR product (A/A). bp = base pair.

Table 3: The results of analyzing data by Spearman's correlation coefficient test for relationship between protein level of SDF-1 and FEV1 percent (%) and between protein level of SDF-1 (pg/mL) and FEV/FVC (%) in patient group; significant difference ($P < 0.0001$) and inverse relationship (correlation coefficient: –1.00) were observed.

	Protein level of SDF-1			Correlation coefficient
	N	Mean ± SD	P value	
FEV1	103	79.2 ± 21	<0.0001	–1.00
FEV/FVC	103	82.3 ± 9	<0.0001	–1.00

Table 4: The mean rank of AA, AG, and GG genotypes for the protein level SDF-1, FEV1 percent, and FEV1/FVC percent in patient group. These data were produced by the Kruskal-Wallis test. There is a significant difference in all of them ($P < 0.05$ in all).

	Genotype	N	Mean rank	The Kruskal-Wallis test result
SDF-1	AA	12	71.38	$P = 0.021$
	AG	67	46.61	
	GG	24	56.79	
FEV1	AA	12	32.63	$P = 0.021$
	AG	67	57.19	
	GG	24	47.21	
FEV1/FVC	AA	12	32.63	$P = 0.021$
	AG	67	57.19	
	GG	24	47.21	

importance of chemokines and their receptors, as the central actors in the initiation and development of asthma, has been evidenced [13, 14]. Results of our study may confirm the role played by SDF-1α (CXCL12) angiogenic alterations associated with asthma which was reported by Hoshino et al., 2003 [15].

Hoshino et al. showed that immunoreactivity of CXCL12 is increased within the airways of asthmatic patients and reported that expression of CXCL12 plays an important role in angiogenesis in the airways of asthmatic patients [15].

The expanded vascularity that has been reported in the asthmatic mucosa might possibly confirm that angiogenesis is a component of the chronic inflammatory response in asthmatic patients, and increased CXCL12 circulatory level in asthmatics may confirm that angiogenesis is a specific characteristic of asthma, which is probably at least partially mediated by CXCL12 [16–18].

However, angiogenesis function of chemokines is currently the focus of intense investigations; little information is known on the roles of chemokines in pathological remodeling of the asthmatic airway. Mice lacking the CXCL12 gene have defective vascular development, suggesting that CXCL12 plays an important role in organ vascularisation [19]. Recent studies have demonstrated that endothelial cells are strongly chemoattracted to CXCL12 [20–22]. Cytokine-mediated regulation of CXCL12 has been demonstrated in eosinophils and lung epithelial cells [23, 24]; the effects of CXCL12/CXCR4 axis showed a contribution to the inflammatory response in a murine model of asthma [25]. Because CXCL12 is a potent chemotactic factor for T and pre-B lymphocytes [26, 27], plasma cells [28], and dendritic cells (DCs) [29] and expression of SDF-1 (CXCL12) receptor (CXCR4) is also documented on eosinophils [24, 30] and mast cells [31–33] - the two critical components in allergic conjunctivitis [33]. It may facilitate infiltration of these cell types to the lung vascular system. CXCL12 may also recruit a wide variety of mature and immature CXCR4 positive leukocytes to the lungs and stimulate neovascularization [34]. CXCR4 and CXCL12 are considered to be essential factors of allergic airway disease in the mouse [25, 35] and humans [36]. Thus, the increased circulating level of these chemokines that we have shown in our study may contribute to the process of recruitment of these cell types to the asthmatics' lungs. On the other side, the defective myelopoiesis and lymphopoiesis in parallel with lack of cardiovascular development in CXCL12 or CXCR4 (its receptor) may prove its role in homeostasis roles rather than inflammatory properties [19, 37, 38]; thus,

increased circulatory level of CXCL12 in our asthma patients may be related to its role in remodeling of airways as an homeostatic chemokine rather than its other roles. Although previous reports indicated that the A allele is associated with higher CXCL12 expression [39], further studies pointed to the opposite side [40], and few studies claimed that there were no differences in the CXCL12 production by the A or G alleles [41, 42]. A more recent haplotype-based investigation [43] demonstrated that other polymorphisms in LD with the CXCL12 + 801G/A SNP, rather than the CXCL12 + 801G/A, are involved in control of the different transcription levels of CXCL12. Although the role of autoimmunity is not that much clear in the pathogenesis of asthma, SDF1-3′ G801A polymorphisms in Polish patients with systemic lupus erythematous which follow an autoimmune pattern are documented by Warchoł et al. [44]. As far as we sought to obtain more related studies in relation of asthma with this polymorphism we were unable but our studies on multiple sclerosis (Azin et al., [11]) and also unpublished data in type 1 diabetes as the two important diseases which similarly as asthma follow the pattern of autoimmunity revealed a close relation between autoimmunity and this polymorphism. Hence, there may possibly be an association between autoimmunity and the SDF-1α 3′A polymorphism that presents in autoimmune disorders.

Nevertheless, our study was affected by some limitations. It should be considered that all of our study population (both control and asthma patients) were within Iranian population, and thus, the possibility of ethnicity as a confining factor could be excluded. However, it should be noted that different geographical and ethnic backgrounds of the individuals can influence the consequences of the association studies. Therefore, findings of the current study need to be examined and confirmed in other populations and ethnic groups. Moreover, further replication studies using a wider sample size and different population would be essential for further evaluation of the relationship between the CXCL12 genetic variations and the risk of asthma.

Conflict of Interests

All of authors of this paper declare that there are no competing financial interests in relation to this work. Furthermore, none of the authors have a relationship with any factory or company for financial support.

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Research Article

Carbon Nanofibers Have IgE Adjuvant Capacity but Are Less Potent Than Nanotubes in Promoting Allergic Airway Responses

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There is a growing concern for the possible health impact of nanoparticles. The main objective of this study was to investigate the allergy-promoting capacity of four different carbon nanofiber (CNF) samples in an injection and an airway mouse model of allergy. Secondly, the potency of the CNF was compared to the previously reported allergy-promoting capacity of carbon nanotubes (CNT) in the airway model. Ultrafine carbon black particles (ufCBP) were used as a positive control. Particles were given together with the allergen ovalbumin (OVA) either by subcutaneous injection into the footpad or intranasally to BALB/cA mice. After allergen booster, OVA-specific IgE, IgG1, and IgG2a in serum were measured. In the airway model, inflammation was determined as influx of inflammatory cells (eosinophils, neutrophils, lymphocytes, and macrophages) and by mediators (MCP-1 and TNF-α present in bronchoalveolar fluid (BALF)). CNF and CNT both increased OVA-specific IgE levels in the two models, but in the airway model, the CNT gave a significantly stronger IgE response than the CNF. Furthermore, the CNT and not the CNF promoted eosinophil lung inflammation. Our data therefore suggest that nanotube-associated properties are particularly potent in promoting allergic responses.

1. Introduction

Allergic airway diseases are characterized by eosinophil and lymphocyte lung inflammation, as well as allergen-specific IgE in serum. Ultrafine particles present in ambient air have, in animal models and in humans, been demonstrated to modulate airway inflammations and promote allergic responses in the lung [1–4]. Carbon nanotubes (CNT) are particles applied in an increasing number of consumer products and are also incidental components in indoor air pollution [5, 6]. Carbon nanofibers (CNF) are useful in many of the same applications as CNT, such as Li-ion batteries and polymer nanocomposites [7]. In parallel to the increasing manufacture of CNT and CNF, there is a growing concern for the health impact of these nanoparticles in occupational workers and consumers in general, also in relation to allergy. Inhalation studies in rodents suggest that CNT may induce toxic effects like transient inflammation, fibrosis, and granuloma formation [8], and mice with a preexisting allergic inflammation have been reported to be particularly susceptible [9]. Further, single-walled (sw) and multiwalled (mw) CNT have been reported to increase allergen-specific IgE levels, eosinophil airway inflammation, and Th2-associated cytokine responses in mice models [2, 10, 11]. In support, Park and coworkers demonstrated increased total IgE, Th2-associated cytokine, and B cell levels in mice after a single intratracheal exposure to mwCNT [12]. However, little is known about the characteristics of CNT responsible for the allergy-promoting effect. The possible allergy-promoting properties of CNF have not been investigated.

The main objective of this study was to investigate the allergy-promoting capacity of four different CNF samples in an injection and an airway mouse model of allergy. Secondly, the potency of the CNF was compared to the

previously reported allergy-promoting capacity of CNT and ultrafine carbon black particles (ufCBP) in the airway model. Knowledge on which physicochemical characteristics of the particles are important for their biological effects is essential to enable manufacturing of particles with low toxicity. We, therefore, explored the importance of major physicochemical properties of carbon nanoparticles on their capacity to modulate allergy, by comparing the allergy-promoting capacity of samples having different physicochemical properties. Four samples of well-characterized, qualitatively different CNF produced in the same pilot plant were tested in both mouse models (CNF A, B, C, and D [7]). By deliberate use of different conditions and purification steps during the CNF manufacturing process, four samples with different particle properties such as fiber width and length, open versus closed channels, metal impurity content, relative surface area, presence of structural defects, and relative amount of fibers to other graphitic material were produced. Samples of swCNT, mwCNT, and "spherical" ufCBP (Printex90), previously shown to promote allergic responses in the two mouse models used [2], were also included in the airway model. The allergy parameters allergen-specific IgE levels in serum and eosinophil lung inflammation were measured. Allergen-specific IgG2a antibodies and the presence of inflammatory cells, MCP-1, and TNF-α in bronchoalveolar lavage fluid (BALF) were also determined, to reflect Th1- and general inflammatory responses.

2. Material and Methods

2.1. Animals. Female inbred BALB/cAnNCrl mice (Charles River, Sulzfeld, Germany) were 6-7 weeks old upon arrival and were acclimatised for one week. Four animals were housed per cage in Innorack IVC (Innovive Inc., CA, USA), containing Nestpaks filled with aspen 4HK bedding (Datesand Ltd, Manchester, UK). The mice were exposed to a 12 hr/12 hr light/dark cycle (30–60 lux in cages), regulated room temperature (20 ± 2°C), and 40–60% relative humidity. Pelleted food (RM1, SDS, Essex, UK) and tap water were provided *ad libitum*. The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway and were approved by the Norwegian Experimental Animal Board under the Ministry of Agriculture (FOTS ID numbers 678 and 1005).

2.2. Particle Source and Characteristics. Four qualitatively different batches of carbon nanofibers (CNF A, B, C and D) were kindly provided by Statoil and Elkem Carbon AS (Kristiansand, Norway). The particles were produced from natural gas in a catalytic chemical vapor deposition (CVD) process and processed as reported [11]. The swCNT and mwCNT, also produced by CVD method, were obtained from Sigma-Aldrich (St. Louis, MO, USA, Cat. numbers 636797 and 636487, resp.). Ultrafine carbon black particles (ufCBP, Printex90), kindly provided by Degussa (Köln, Germany), were included as a positive reference particle for adjuvant activity in mice [1, 2]. All particle samples were characterized by transmission electron microscopy (TEM), and the specific surface area was determined by nitrogen adsorption using the Brunauer, Emmet, and Teller method (BET). The endotoxin levels were determined in particle supernatants by the limulus amebocyte lysate (LAL) assay as previously described [2, 7], and the pH of the particle supernatants was measured (Mettler Toledo AS, NY, USA).

2.3. Particle and Allergen Preparations. Ovalbumin (OVA, Gal d1; chicken egg albumin, grade VII, Sigma, St. Louis, MO, USA) was used as allergen after removal of endotoxin by Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL, USA). The final endotoxin level measured less than 0.025 ng/mg OVA, as determined by the LAL assay. Particle suspensions were prepared in Hank's balanced salt solution (HBSS), with BALB/cA mouse serum to facilitate suspension of particles, and microtip probe sonicated as described in Nygaard et al. [2]. The CNT and CNF were even then mainly observed as agglomerates in TEM.

2.4. Injection Model. The IgE adjuvant capacity of the CNF particles was initially investigated in a footpad injection model suitable for studying respiratory allergy adjuvants (method reviewed in [13], performed as described in [2]). In short, groups of eight mice were given a single dose of 200 μg of CNF A, B, C, D, or ufCBP together with 10 μg OVA, OVA boosted (10 μg) after 21 days and terminated on day 26. Sera were collected and stored at −20°C until quantification of OVA-specific IgE, IgG1, and IgG2a by ELISA (see below). A particle dose of 200 μg was chosen, a dose previously shown to give a pronounced adjuvant effect of CNT in this injection model [2].

2.5. Intranasal Model. The adjuvant capacity of the particles after airway exposure was examined in an intranasal mouse model. Exposures were performed as described in [2], and a particle dose previously giving pronounced effects of CNT in the intranasal model was chosen [2]. In short, 133.3 μg CNF A, B, C, D, swCNT, mwCNT, or ufCBP together with 10 μg OVA was given intranasally to groups of ten mice on three consecutive days, giving a total dose of 400 μg particles and 30 μg OVA during sensitization. All mice were OVA-boosted intranasally (10 μg per day) on days 21, 22, and 23. On day 26, the animals were deeply anesthetized by a 0.3 mL intraperitoneal injection of a mixture of Zoletil forte (17 mg/kg tiletamine and 17 mg/kg zolazepam; Virbac International, Carros Cedex, France) and Narcoxyl (13.6 mg/kg xylazine; Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands). Sera were stored at −20°C until quantification of OVA-specific IgE, IgG1, and IgG2a by ELISA (see below). Lung inflammation was determined by total and differential cell counts and cytokine levels in BALF (see below). Based on previous findings with sw and mw CNT [2], the cytokines TNF-α and MCP-1 in BALF were selected.

2.6. Detection of IgE, IgG1, and IgG2a Anti-OVA Antibodies. Detection of OVA-specific IgE, IgG1, and IgG2a antibodies was performed by ELISAs as described in Nygaard et al. [2]. The sera to be tested were diluted 1 : 10 for IgE and IgG2a, and for IgG1 diluted 1 : 4 000 and 1 : 20 000 for the

injection and intranasal models, respectively. Values outside the dynamic range of the standard curve were set to a value just below/above the detection limit.

2.7. BALF Collection, Preparation, and Analyses. BALF was collected and prepared, and total and differential cell counts were determined as previously described [2, 14]. In short, the supernatant from a first lavage was stored at −80°C until cytokine measurements, while the cells from all three lavages were pooled, counted, and stained, and 400 cells per slide were differentiated into neutrophils, eosinophils, epithelial cells, lymphocytes, and macrophages. A number of macrophages appeared to contain particles, but since we could not determine whether the particles were taken up or were adsorbed to the cell membranes, they were counted as particle-associated macrophages. In BALF supernatants, the levels of tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) were determined using BD Cytometric Bead Array (CBA) Mouse Soluble Protein Flex Sets, as previously described [2].

2.8. Data Analysis. The data were log10-transformed to meet the assumptions for running one-way ANOVAs. In order to determine which groups differed significantly from the others when the ANOVAs were positive ($P \leq 0.05$), pairwise comparisons were performed by Tukey's post hoc test. Group differences were considered statistically significant if $P \leq 0.05$. All statistical analyses were performed with SigmaStat Statistical Analysis System for Windows Version 2.03 (Jandel Scientific, Erkrath, Germany).

3. Results

3.1. Particle Characterization. Particle sample properties are shown in Table 1, while the methods used for particle characterization and more comprehensive characterization of the samples are previously reported [2, 7]. TEM micrographs at similar magnifications from CNF A, B, C, D, swCNT, and mwCNT samples are presented in Figures 1(a)–1(f), respectively. Systematic inspection of the TEM micrographs revealed that the majority of the CNF agglomerates were within the size range 0.6 μm to 2.6 μm. The CNF were wider than the CNT, and the mwCNT were wider than the swCNT, as illustrated in the micrographs and given by the mean fiber width presented in Table 1. Most CNF are fractured or broken and therefore, on an average, are shorter than the CNT (as illustrated by dotted arrows in Figure 1, Table 1). Inspection of the TEM micrographs also showed that CNF samples and the swCNT sample contain disordered graphitic material, while the mwCNT sample is more homogeneous. High numbers of metal particles were observed in CNF A, CNF C, and swCNT, mainly embedded in the carbon material (indicated by arrows in Figures 1(c), 1(e), and 1(f)). CNF A and CNF C have a higher surface area, a lower mean fiber diameter, and a higher presence of structural defects than CNF B and CNF D samples (Table 1).

Figure 2 shows the typical internal structure of CNF and CNT in high-resolution TEM images. The CNT consist of single or multiple concentric graphene sheets rolled into form of cylinders (Figures 2(c) and 2(d)). In contrast, the CNF were classified into two main types of stacked arrangements of graphite cones: with channels that are mostly open (Figure 2(a)) or with periodically closed channels (Figure 2(b)). The latter are also characterized by closed layers on the CNF surface, as indicated by arrows. Although the open channel type was found in all CNF samples, only the CNF B and CNF D samples contained a high fraction of the periodically closed channel type. As previously reported [2], only half of the tubes in the swCNT sample were identified as single-walled, the other half being multiwalled. Most tubes in the mwCNT sample were defective, as exemplified in Figure 2(d) (arrowhead).

The particles were also studied after preparation in the medium used for the *in vivo* studies. As judged by TEM analyses, the sonication process did not affect the length of the CNT, whereas some breakage of the CNF was observed (data not shown). This was most probably due to a lower mechanical strength of the fibers compared to the tubes, caused by the different stacking of the graphite layers. The acidity (pH) of the particle supernatants did not differ markedly between the particles, and the endotoxin levels were below detection limit for all particles, except swCNT which had a detectable but low level (Table 1).

3.2. OVA-Specific IgE, IgG1, and IgG2a Levels in Serum after Coinjection of Particles and OVA in the Footpad. In the footpad injection model, OVA-specific IgE levels were statistically significantly higher in mice treated with OVA and CNF A, CNF C, or ufCBP than with OVA alone (Figure 3(a)). ufCBP elicited significantly higher IgE levels than CNF B and CNF D, and CNF A elicited significantly higher levels than CNF D. OVA-specific IgG1 levels were significantly elevated by all particles, but CNF A and CNF C had higher IgG1-adjuvant activity than CNF B and D, but statistically significant only for CNF D (Figure 3(b)). CNF A, CNF C, and ufCBP gave weakly but significantly elevated levels of OVA-specific IgG2a levels (Figure 3(c)).

3.3. OVA-Specific IgE, IgG1, and IgG2a Levels in Serum after Intranasal Coexposure to Particles and OVA. In the intranasal model, all particles significantly increased OVA-specific IgE levels compared to the OVA control group (Figure 4(a)). The four CNF samples and ufCBP elicited similar levels of IgE, while in comparison, the sw and mw CNT elicited very high IgE levels. Also the OVA-specific IgG1 levels were significantly increased by all particles, and mwCNT elicited significantly higher levels than CNF A and CNF B (Figure 4(b)). As compared to the OVA control group, OVA-specific IgG2a levels were significantly increased after exposure to CNF C and mwCNT and also tended to be increased by swCNT ($P = 0.079$) and ufCBP exposure ($P = 0.088$; Figure 4(c)).

3.4. Airway Inflammation after Intranasal Coexposure to Particles and OVA. In the intranasally exposed mice, CNF B, CNF C, sw and mw CNT, and ufCBP with OVA significantly increased the total number of BALF cells, compared to the

Table 1: Selected characteristics for CNF A, B, C, and D, swCNT, mwCNT, and ufCBP. The CNF have been thoroughly characterized, reported in Jensen et al. [7]. Data for the swCNT, mwCNT, and ufCBP were previously reported in Nygaard et al. [2].

	CNF A	CNF B	CNF C	CNF D	swCNT	mwCNT	ufCBP, Printex90
				Description of morphology (SEM, TEM)[a]			
	Aggregated particles. Medium fraction of fibres, mainly open channel fibres	Aggregated particles. High fraction of fibres, mainly periodically closed channel fibres	Aggregated particles. Low fraction of fibres, mainly open channel fibres	Aggregated particles. Medium fraction of fibres, mainly periodically-closed channel fibres	Aggregated particles. ~50% graphitic material of arbitrary shape ~25% swCNT ~25% mwCNT Open channels	Aggregated particles. High fraction of tubes (>90%), but most are fibres with CNT aspect (the graphite layers make low angles with the channel direction). The fibres contain many defects. Open channels	Aggregated particles. Near spherical particles whose structures are low-graphitized
				Percent fibers/tubes versus other carbonous material[a]			
	>50%	>80%	<50%	>50%	~50%	>90%	Not relevant
				Fibre/tube widths ± standard error [range] (nm)			
	37.01 ± 1.57 [11.25–108.69][a]	83.14 ± 4.18 [18.90–302.21][a]	35.82 ± 2.13 [14.46–185.75][a]	70.57 ± 2.68 [18.52–286.85][a]	4.05 ± 0.23 nm [1.41–10.91 nm][a]	15.04 ± 0.47 nm [7.62–29.01 nm][a]	
					1.1 nm[b]	20–30 nm[b]	14.3 nm[c]
				Fibre lengths			
	Up to 10 μm[a]	Up to 10 μm[a]	Up to 5 μm[a]	Up to 5 μm[a]	0.5–100 μm[b]	0.5–200 μm[b]	Not relevant
				Surface area (BET, m^2/g)			
	103[d]	61[d]	124[d]	56[d]	543[e]	140[e]	321[d]
				Impurities			
	>95% carbon[a]	>95% carbon[a]	>95% carbon[a]	>95% carbon[a]	>95% carbon[b]	>95% carbon[b]	>95% carbon[a]
ADF-STEM[a]	High number of Ni [5–500 nm] particles, some large Ti-Fe [>1 μm] particles	Traces of Ni [3–50 nm] and Ni-Fe [50–1000 nm] particles	High number of Ni [10–1000 nm] particles, traces of Cr, Fe and Ti	Traces of Ni [10–50 nm] particles	High number of impurities, mainly Co [1–20 nm], but some Fe [50–500 nm]	Lower number of impurities, mainly Ni-(Fe) [5–40 nm]	Traces
Ni[d] (AAS, %)	1.29	0.070	4.97	0.036	n.a.	n.a.	<0.0003
Fe[d] (AAS, %)	0.008	0.023	0.148	0.002	n.a.	n.a.	0.003
				Structural defects ($R = I_D/I_G$ as determined by Raman spectroscopy)[d]			
	1.7	0.7	0.9	0.6	n.a.	n.a.	1.2
				Endotoxin (ng/mg)[f]			
	<0.0066	<0.0066	<0.0066	<0.0066	0.0079	<0.0066	<0.0066
				Acidity (pH)[f]			
	7.34	7.36	7.38	7.35	7.28	7.31	7.32

Abbreviations: ADF-STEM: annular dark field scanning transmission electron microscopy. AAS: atomic absorption spectrometer. BET: Braunauer, Emmet, and Teller method. Fe: iron. Ni: nickel. n.a.: not analyzed. SEM: scanning electron microscopy. TEM: transmission electron microscopy. $R = I_D/I_G$: intensity ratio of the first order bands G and D.

[a]Authors observation by electron microscopy. [b]Supplied by the manufacturer. [c]According to Renwick et al. [15]. [d]Jensen et al. [7]. [e]Nygaard et al. [2]. [f]Levels in supernatants after ultracentrifugation of particle suspensions. Endotoxin detection limit = 0.0066 ng/mg (0,25 EU/mL).

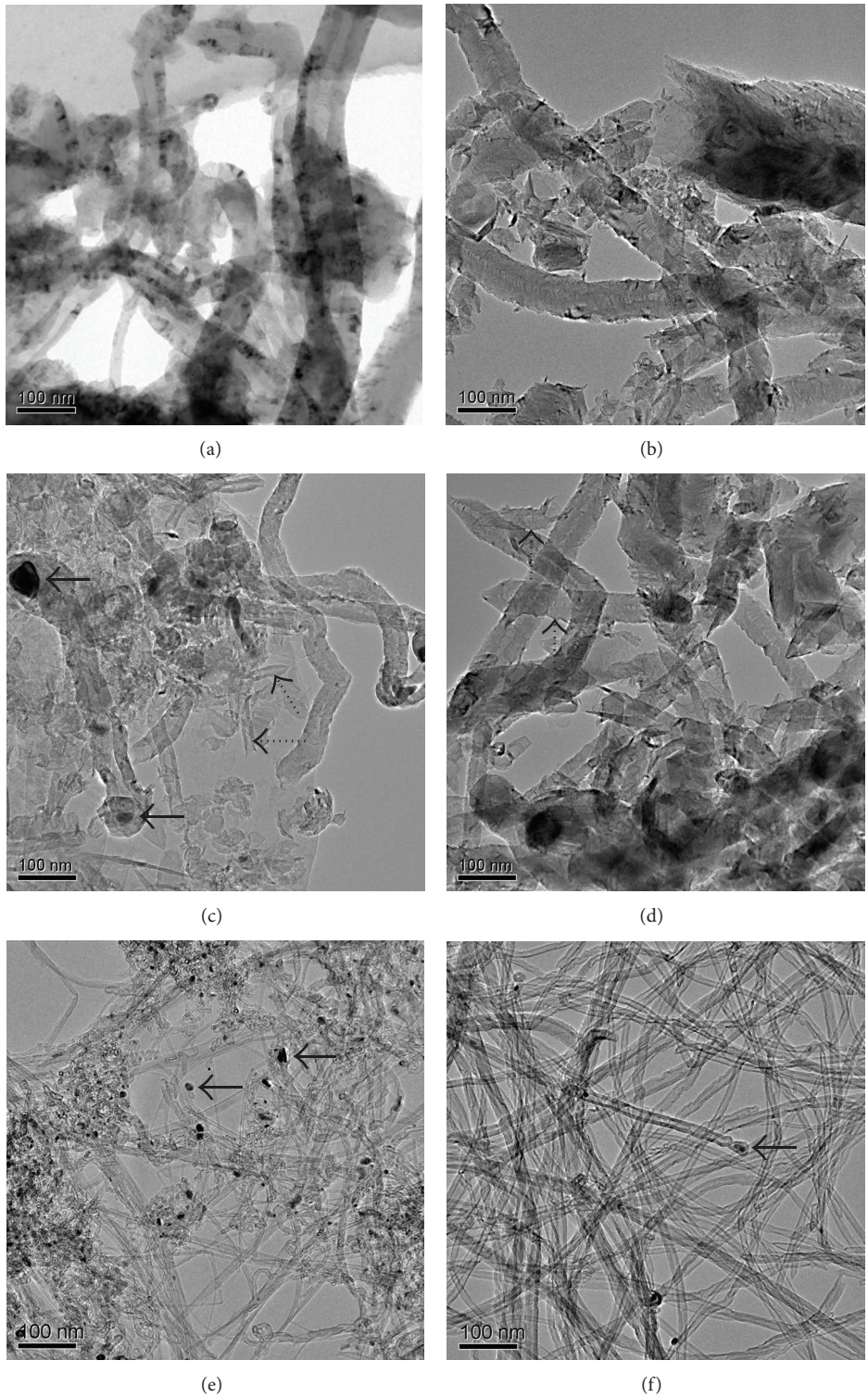
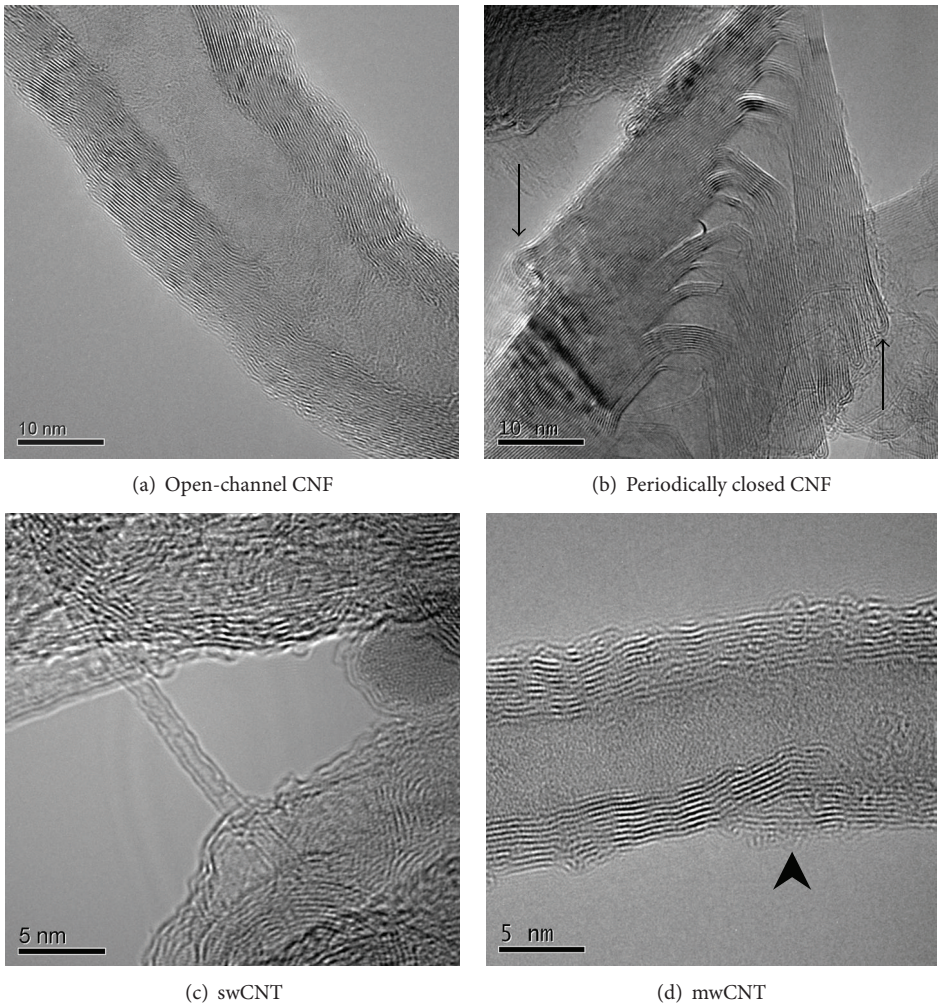


Figure 1: TEM micrographs of the particle samples. Micrographs of CNF A (a), CNF B (b), CNF C (c), CNF D (d), swCNT (e), and mwCNT (f) illustrate the difference in width and length between the CNF and CNT. Examples of metallic particle presence are indicated by dense arrows. Most metallic particles are embedded in the carbon material. Shorter fragments of fibers are indicated by dotted arrows.



(a) Open-channel CNF

(b) Periodically closed CNF

(c) swCNT

(d) mwCNT

FIGURE 2: High-resolution images of particles, illustrating their internal structure. The herringbone CNFs consisted of graphite layers arranged at an angle to the axis of the filament structure ((a), (b)). The micrographs show the structure of a typical open-channel fiber found in all CNF samples (a) and a periodically closed fiber present in large amounts only in CNF B and CNF D (b). Many graphite layers in the periodically-closed type of fiber have closed ends at the surface, indicated by arrows in (b). A typical example of a swCNT, together with disordered graphitic material, is shown in (c). An example of a typical defect mwCNT is shown in (d); a defect in the wall is indicated by an arrow head.

group given OVA alone (Figure 5(a)). The total cell numbers were significantly higher in the groups given swCNT, mwCNT, and ufCBP than in the CNF groups, with the exception of CNF C which elicited significantly higher cell numbers than CNF A and CNF D. The particles that increased the total cell numbers also significantly increased the neutrophil cell numbers (Figure 5(b)). The eosinophil numbers were low in the groups treated with OVA alone or together with the CNF samples. Exposure to OVA with swCNT or mwCNT, however, induced significantly higher eosinophil numbers than OVA alone or OVA with any of the CNF samples (Figure 5(c)). ufCBP with OVA also increased the eosinophil numbers, although the numbers were only significantly higher than in mice treated with OVA alone or together with CNF A and CNF B. The number of lymphocytes was slightly, but statistically significantly, increased in the swCNT group only (Figure 5(d)), and the epithelial cell numbers were only marginally affected (data not shown). swCNT, mwCNT, and ufCBP with OVA significantly increased the macrophage numbers compared to OVA alone (Figure 5(e)), while CNF C was the only CNF sample that significantly increased the macrophage numbers. The percentage of macrophages associated with particles did not differ markedly between the different particle types (Figure 5(f)). However, the CNF D, swCNT, mwCNT, and ufCBP tended to be associated with a lower percentage of macrophages than CNF A, B, and C and were significantly different for swCNT and ufCBP only, as compared to CNF B.

Due to accidental thawing, only half of the BALF samples were analyzed for cytokines, reducing the ability to detect statistically significant differences. The levels of MCP-1 after OVA booster apparently were increased by all particles with OVA except CNF B (Figure 5(g)). The tendencies were most pronounced for CNF A, CNF C, mwCNT, and ufCBP, although statistical significance was reached only for the latter. Although the levels of TNF-α were low at this time

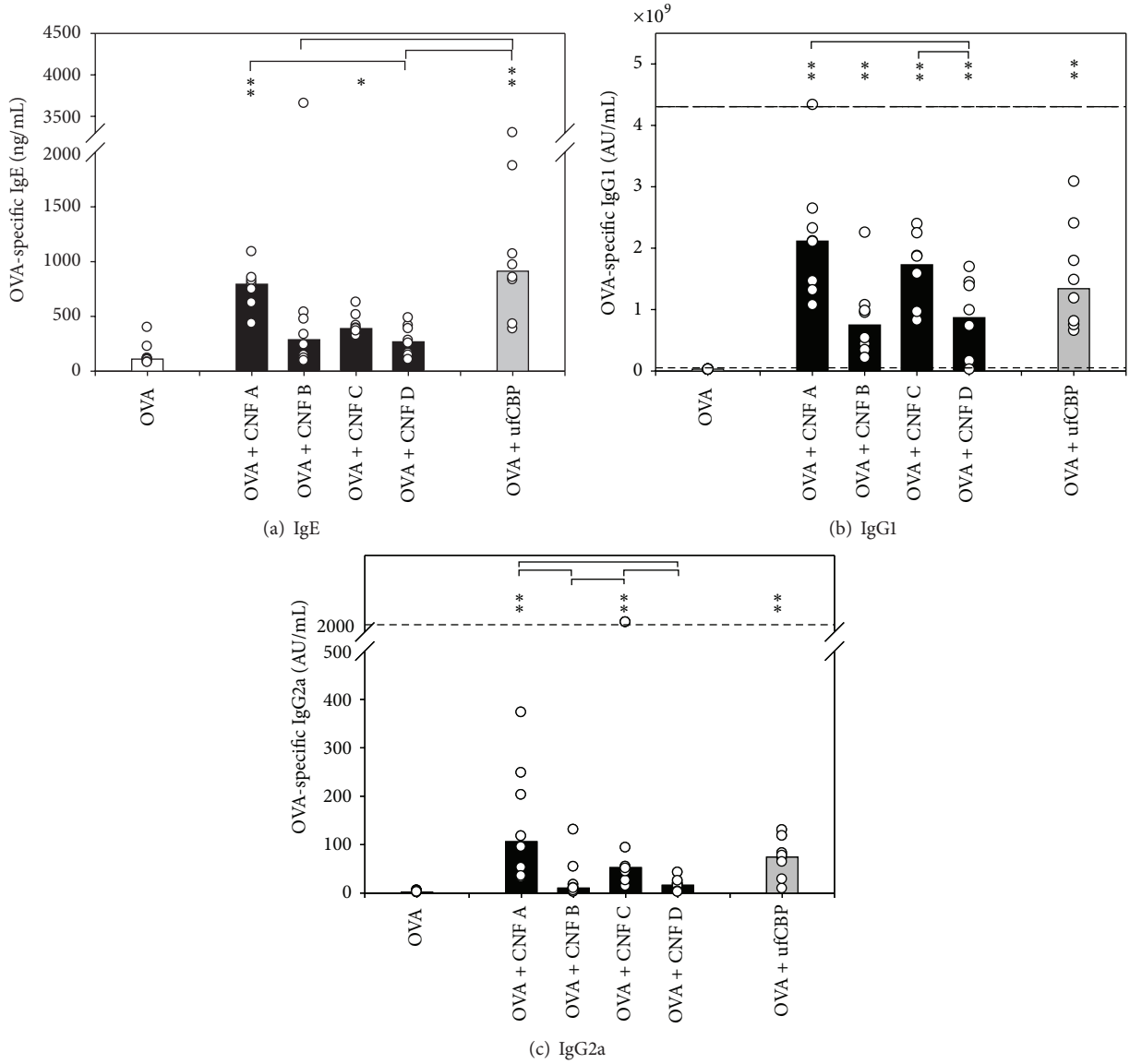


Figure 3: Allergen-specific antibodies after subcutaneous coinjection of allergen and particles. Serum levels of OVA-specific IgE (a), IgG1 (b), and IgG2a (c) 26 days after subcutaneous injection into one hind footpad of 10 μg OVA alone (white bars) or together with 200 μg CNF (samples A, B, C, and D; black bars) or ufCBP (light grey bars). On day 21, all mice were boosted with 10 μg OVA in the footpad. Values (ng or arbitrary units (AU) per mL) for individual mice (circles) and median values (columns) for groups of eight mice are shown. If values were outside the dynamic range of the ELISA assays, the dotted lines indicate the lower or upper quantitative detection limits for the assays. Asterisk denotes significant differences compared to the OVA group, $^{**}P < 0.001$, $^{*}P < 0.05$. Brackets denote statistically significant differences between the particle groups, $P < 0.05$.

point (23 days after particle exposure), CNF A and ufCBP with OVA tended to induce higher levels than the other groups (Figure 5(h)).

4. Discussion

Studies of airway effects of CNF are scarce in the literature. In the present study, we demonstrate that CNF can promote production of allergen-specific IgE after injection and intranasal exposure to mice. While CNF A and CNF C, and not CNF B and CNF D, demonstrated IgE adjuvant capacity in the injection model, all four CNF samples elicited similar levels of IgE in the airway model. The increase in IgE levels was not associated with a clear allergic airway inflammation in the intranasal model used, since no significant eosinophil influx was observed after allergen booster. Our observation that CNF promote antibody production is in agreement with previous studies reporting allergy-promoting activity of nanosized particles like ultrafine polystyrene particles, ufCBP and CNT [1, 2, 10, 11, 16].

In the intranasal model, the sw and mw CNT samples elicited markedly higher IgE levels than the CNF samples, accompanied with a clear eosinophil airway inflammation.

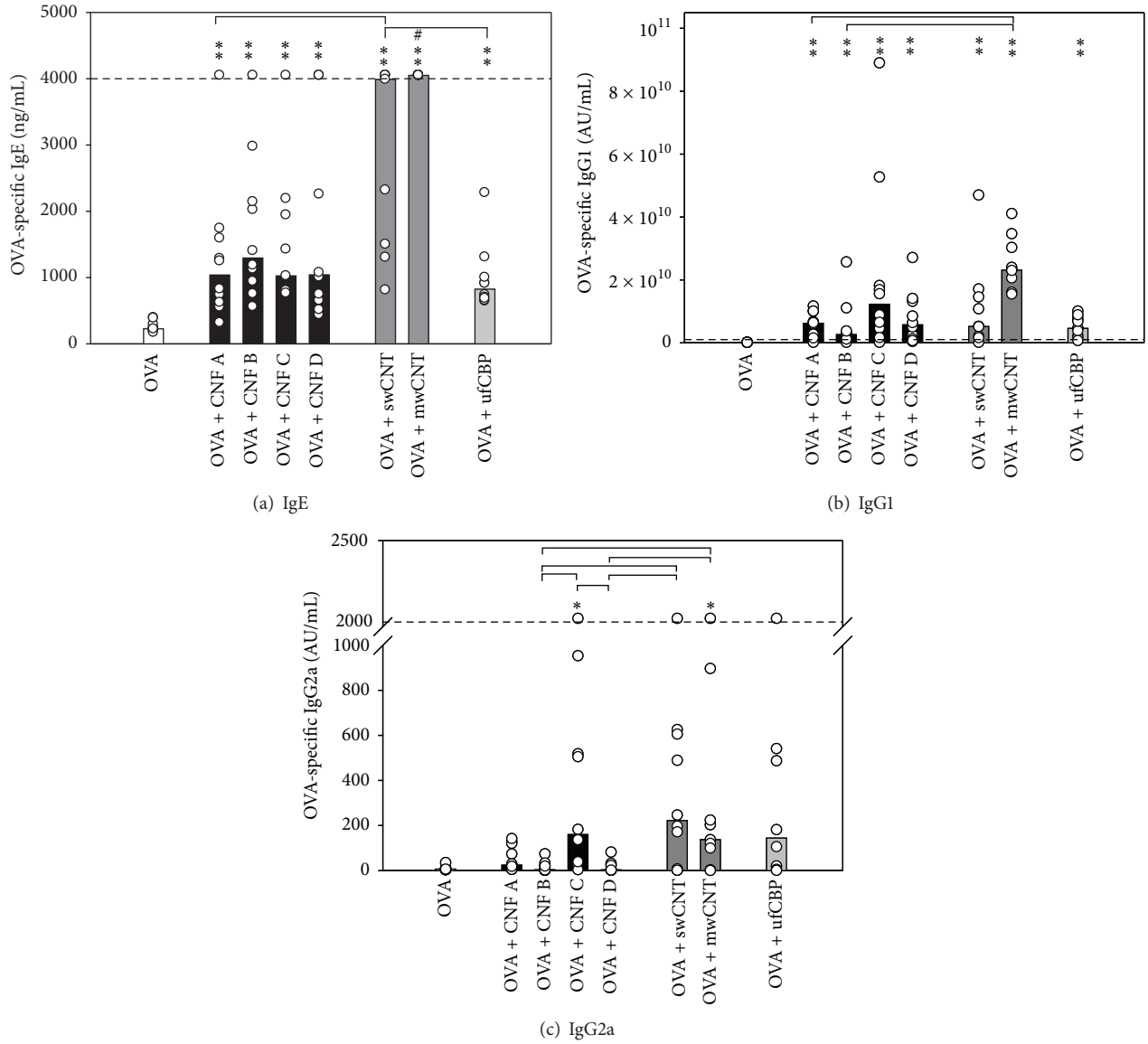


Figure 4: Allergen-specific antibodies after intranasal coexposure to allergen and particles. Serum levels of OVA-specific IgE (a), IgG1 (b), and IgG2a (c) 26 days after intranasal exposure to 10 μg OVA alone (white bars) or together with 133 μg particles on days 0, 1 and 2 (total dose of 30 μg OVA and 400 μg particles). The four samples of CNF (black bars), swCNT and mwCNT (dark grey bars), and ufCBP (light grey bars) were used. On days 21, 22, and 23, all mice were boosted intranasally with 10 μg OVA (per day). Values (ng or arbitrary units (AU) per mL) for individual mice (circles) and median values (columns) for groups of ten mice are shown. If values were outside the dynamic range of the ELISA assays, the dotted lines indicate the lower or upper quantitative detection limits for the assays. Asterisk denotes significant differences compared to the OVA group, $^{**}P < 0.001$,$^{*}P < 0.05$. # Denotes significant differences compared to all other particle groups, $P < 0.05$. Brackets denote statistically significant differences between particle groups, $P < 0.05$.

The features of the two CNT samples, making them different from the CNF samples, are the presence of long, thin, and hollow structures (tubes), as opposed to the wider, hat-stacked, more compact structures of the CNF (Figure 1, Table 2). In addition, the present CNT are longer and have 1/2–1/20 of the diameter as compared to the CNF, giving them a higher aspect ratio (i.e., the length/width ratio). Particles with high aspect ratios, such as asbestos fibers, have demonstrated high toxicity, and CNT have been reported to have pathogenic features similar to asbestos fibers [17]. The main characteristic distinguishing nanofibers from nanotubes is the stacking of graphene sheets of varying shapes [18]. While sw and mw CNT consist of single or multiple graphene sheets rolled into concentric cylinders, herringbone (hat-stacked) CNF consist of graphite layers arranged at an angle to the axis of the filament structure, forming a stacked arrangement of cones (Figure 2). This intrinsic difference results in less mechanical strength of CNF compared to CNT [18], as also observed in our study, since the CNF were more susceptible to breaking during the sonication process. In contrast to what has been

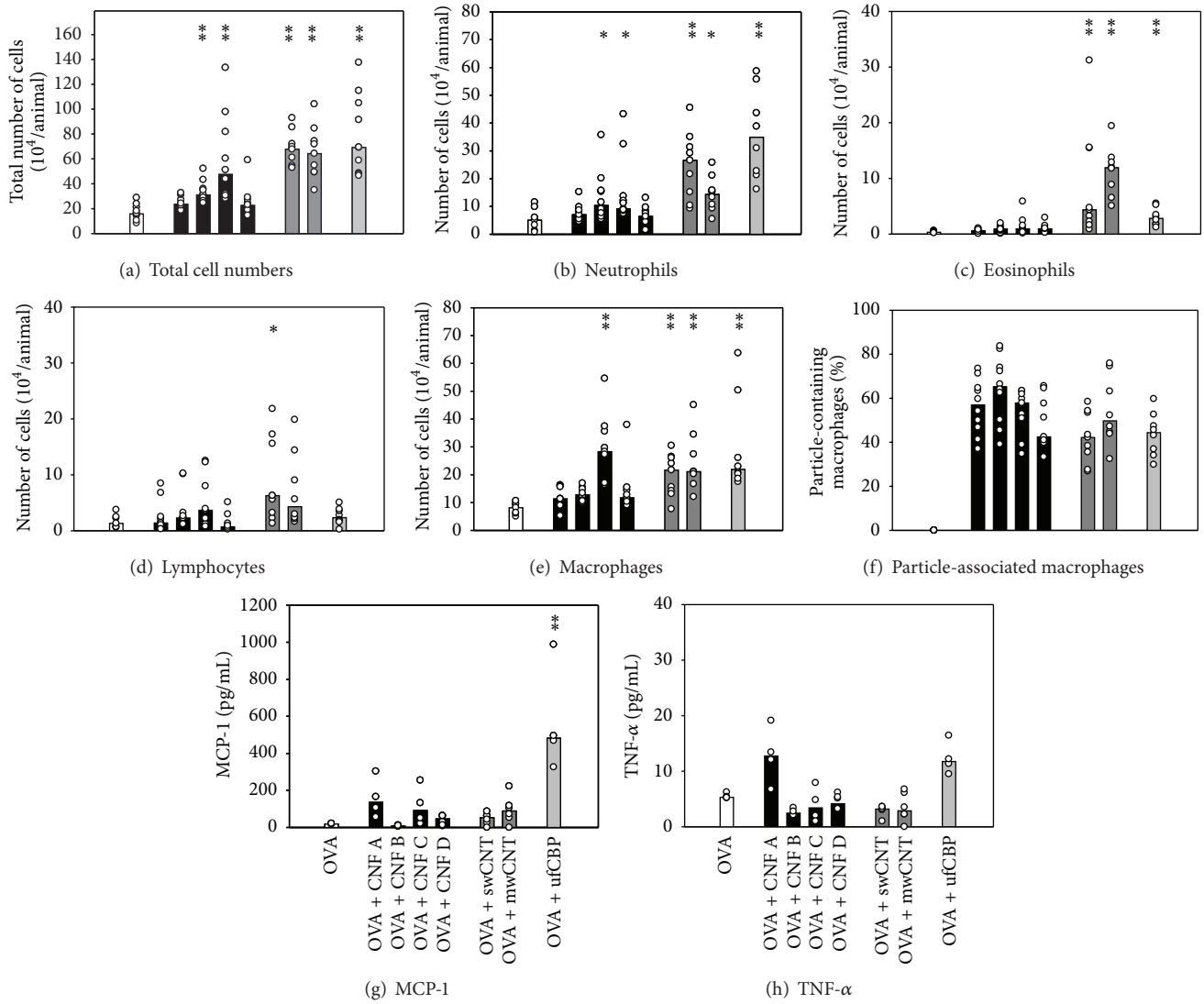


FIGURE 5: Airway inflammation measured after intranasal coexposure to allergen and particles. Cell numbers and cytokines in BALF collected following OVA booster, 26 days after intranasal exposure of mice to allergen alone or together with particles (as described in legend of Figure 4). The total cell numbers (a) were determined on a Coulter cell counter, whereas the number of neutrophils (b), eosinophils (c), lymphocytes (d), and macrophages (e) and the percent of particle-associated macrophages (f) were determined by counting 400 cells per stained cytoslide. The amount of MCP-1 (g) and TNF-γ (h) in BALF supernatants was determined by CBA assay. Values for individual mice (circles) and group medians (columns) for groups of ten mice are shown, except MCP-1 and TNF-α where only half of the samples could be measured. Asterisk denotes significant differences compared to the OVA group, $^{**}P < 0.001$, $^{*}P < 0.05$. Statistically significant differences between particle groups are given in the results section.

observed for mwCNT [19], hat-stacked CNF implanted in subcutaneous tissue of rats appeared to become shorter and display decreased degree of aggregation over time [20]. The authors suggested that delamination of graphene layers by hydrophilic substances or energy from cytoplasmic motion may be involved in CNF shortening. Thus, although studies have demonstrated some biodegradation *in vitro* [21], the present and previous data suggest that the CNF samples have lower biopersistence than the CNT samples.

Further, the open, hollow structure of the CNT may be of importance for the allergy-promoting capacity, for instance, as a depot of allergens. Carbon particles have previously been shown to act as allergen carriers [22, 23], and a depot effect has been suggested as one mechanism for particle adjuvant effects [24]. The above mentioned reports support the present finding that tube-associated characteristics, such as a thin, hollow tube structure with assumed high biopersistence, appeared to be particularly important features for the allergy-promoting effect of particles in the airways. CNT also generate reactive oxygen species [25], which probably plays an additional role in their induction of airway allergic inflammation. The capacity of CNF to form ROS is unknown.

Knowledge on which particle characteristics are important for the different biological effects is a prerequisite to enable production of less toxic particles [26]. An advantage of this study was the availability of four CNF batches

Table 2: Distribution of selected particle characteristics and the allergy-promoting capacity for all particles tested (as measured by high levels of OVA-specific IgE and eosinophil airway inflammation). To simplify the identification of particle characteristics of importance for the modulated allergy responses after airway exposure, the levels of each particle property or allergic response are subjectively categorized into three levels, illustrated by roman, bold italic, or bold font.

	Main carbon structure	Fraction of fibers/tubes versus disordered material	Fiber/tube width (nm)	Surface area (m^2/g)	Structural defects ($R = I_D/I_G$)	Metallic contaminants	Allergy-promoting capacity
CNF A	***Fibers, open channels***	***Medium***	***37.01 ± 1.57 [11.25–108.69]***	***103***	**1.7**	**High, mainly Ni**	***Medium***
CNF B	**Fibers, periodically closed channels**	**High**	83.14 ± 4.18 [18.90–302.21]	61	0.7	Traces	***Medium***
CNF C	***Fibers, open channels***	Low	***35.82 ± 2.13 [14.46–185.75]***	***124***	***0.9***	**High, mainly Ni**	***Medium***
CNF D	Fibers, periodically closed channels	***Medium***	70.57 ± 2.68 [18.52–286.85]	56	0.6	Traces	***Medium***
swCNT	**Tubes, open channels**	***Medium***	**4.05 ± 0.23 [1.41–10.91 nm]**	**543**	n.a.	**High, mainly Co**	**High**
mwCNT	**Tubes, open channels**	**High**	**15.04 ± 0.47 [7.62–29.01 nm]**	***140***	n.a.	***Less, mainly Ni(Fe)***	**High**
ufCBP	Spherical	Not relevant	Not relevant	**321**	***1.2***	Traces	***Medium***

manufactured in the same facility but with varying particle characteristics deliberately introduced by changing manufacturing conditions [7]. Despite their qualitative differences, the allergy-promoting capacity of the four CNF samples did not differ in the intranasal model, neither with regard to IgE levels nor eosinophil influx. In the footpad injection model, on the other hand, only the CNF A and CNF C increased OVA-specific IgE levels. CNF A and CNF C also tended to differ from the other CNF samples with regard to other endpoints, such as higher levels of IgG1 and IgG2a antibodies in both models and some of the inflammatory markers in the BALF. Although the biological implications of these observations are unclear, they indicate that the CNF A and CNF C samples differ from the CNF B and CNF D samples with regard to induction of biological responses. Common for CNF A and CNF C is a higher metal content, about twice as large relative surface area, a lower mean fiber width, a higher proportion of open channel fibers, and a tendency towards a higher number of defective sites compared to CNF B and CNF D (Table 2). These are all parameters affected by the high temperature treatment performed to reduce the metal content in CNF B and CNF D (described in [7]), and our results therefore suggest that heat-treated CNF may be less toxic with regard to some biological responses. Unfortunately, the association between these heat-treatment sensitive parameters makes it hard to identify single key properties responsible for the higher responses to the CNF A and CNF C. Both metal content, surface area, and structural defects have been suggested to play a role in particle-induced lung inflammation and allergy-promoting effects in mice [16, 27–32]. However, we cannot exclude that the lower fiber mean width and the higher proportion of open channeled fibers in the CNF A and CNF C samples may have contributed to the stronger responses to these particles. Indeed, this would be in agreement with the apparent importance of a thin, hollow structure on the allergic adjuvant effect, as discussed previously.

5. Conclusion

The present data demonstrate that CNF and CNT modulate airway responses to allergens, resulting in allergic airway inflammation and production of allergen-specific IgE in mice. When different CNF samples were compared with swCNT and mwCNT, however, the CNT samples appeared to be especially potent in promoting allergic responses, possibly due to their thin, hollow tube structure and assumed high biopersistence. This study provides a basis for studies aiming to further identify the particle properties of importance for airway effects and studies aiming to better understand which airway mechanisms are underlying the allergy-promoting effect of nanoparticles such as CNF and CNT.

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present study design, interpretation of data, or the resulting publication.

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Review Article

The Immune Interplay between the Host and the Pathogen in *Aspergillus fumigatus* Lung Infection

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The interplay between *Aspergillus fumigatus* and the host immune response in lung infection has been subject of studies over the last years due to its importance in immunocompromised patients. The multifactorial virulence factors of *A. fumigatus* are related to the fungus biological characteristics, for example, structure, ability to grow and adapt to high temperatures and stress conditions, besides capability of evading the immune system and causing damage to the host. In this context, the fungus recognition by the host innate immunity occurs when the pathogen disrupts the natural and chemical barriers followed by the activation of acquired immunity. It seems clear that a Th1 response has a protective role, whereas Th2 reactions are often associated with higher fungal burden, and Th17 response is still controversial. Furthermore, a fine regulation of the effector immunity is required to avoid excessive tissue damage associated with fungal clearance, and this role could be attributed to regulatory T cells. Finally, in this work we reviewed the aspects involved in the complex interplay between the host immune response and the pathogen virulence factors, highlighting the immunological issues and the importance of its better understanding to the development of novel therapeutic approaches for invasive lung aspergillosis.

1. Introduction

An infection due to an *Aspergilli* was first described in animals in 1815 when its presence was observed in the air sacs and lungs of a crown [1]. However, the first human case was only described almost 30 years later in Scotland, when the sputum of a patient was microscopically analyzed by Benett [2]. Those findings were followed by the description of bronchial and pulmonary aspergillosis in humans by Virchow [3] and the remark that this infection may occur coupled to other lung diseases such as tuberculosis [4]. Although the disease was already known by that time, the fungus *Aspergillus fumigatus* was only described by Fresenius in 1850 when air sacs and bronchi of a great bustard were analyzed [5]. Therefore, aspergillosis was firstly described as an opportunistic infection when an immunocompromised patient had a disseminated mycosis with the presence of *A. fumigatus* in lung and kidney, besides *Candida* in liver and spleen, or the concomitant presence of both fungi in other tissues [6].

Ubiquitous in nature and without geographic predilection, *Aspergillus* species are found in the air, food, water, soil, and decomposing vegetation where they play an essential role in recycling environment carbon and nitrogen. Inhalation of *Aspergillus* spp. conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are efficiently eliminated by immune mechanisms. Thus, the isolation of *A. fumigatus* from respiratory secretions in normal hosts generally reflects colonization rather than infection [7–9]. In the last decades, with the advent of solid organ and bone marrow transplantation, the increased use of immunosuppressive drugs, and the epidemic infection with the human immunodeficiency virus (HIV), the disease caused by *A. fumigatus* has emerged as a severe infection in immunocompromised patients. Established infection in these patient groups has proven difficult to eradicate, and

despite significant advances in antifungal therapy in recent years, overall mortality with invasive disease remains high [10]. The most frequent pathogenic agent of aspergillosis is *A. fumigatus,* followed by *A. flavus* and *A. niger* [11]. Among them, the first has some characteristics which may allow a greater adhesion to the airways before invasion such as its ability to bind to laminin [12], thus conferring an evolutionary advantage to *A. fumigatus* during infection over the others.

The incidence of invasive aspergillosis is increasing [13–16], and *A. fumigatus* causes approximately 90% of this disease [8]. Thus, the development of pulmonary aspergillosis, which involves severe pulmonary manifestations, relies on a series of predisposing aspects and on a complex interplay between both the host immune competence and the pathogen virulence factors.

2. *Aspergillus fumigatus* Virulence Factors

The virulence of *Aspergillus fumigatus* is multifactorial and is combined with both the immune status of the patient and the biological characteristics of the fungus. There is a high connection between them, as demonstrated by the differences in the activation of the innate immune system which depend on the *Aspergillus* spp. morphology, growth stage, environment sensing and species, representing a key factor in fungal pathogenicity [17]. Once the fungus conidia reached the host environment, which is a different condition found in their normal environment niche, it must continually adapt to survive. These adjustments are classified according to the process they are involved in, for example, thermotolerance, toxins, cell wall composition and conservation, resistance to the immune response, nutrient uptake, signaling, metabolism, and response to stress condition.

The decaying organic matter is the normal environment for *A. fumigatus*, which is subjected to a wide temperature variation as a consequence of intense microorganism activity. The ability to grow at 37°C is shared by all successful human pathogens and is a feature that has been shown to correlate with virulence potential. *A. fumigatus* can grow between 37 and 50°C and is therefore more resistant and has better thermotolerance than other *Aspergillus* species [18, 19]. The ribosomal biogenesis proteins encoded by *crgA* [20], α-mannosyltransferase (*kre2/mnt1*) [21], and the endoplasmic reticulum-transmembrane sensor encoded by *ireA* [22] are to date the proteins associated with thermotolerance growth and hypovirulence, when the respective genes were deleted from *A. fumigatus* genome.

The fungal cell wall of *A. fumigatus* represents the first point of contact with the hosts and plays an important role in the infection process. It is a polysaccharide-based three-dimensional network which is a physical protection and provides a dynamic structure that is continuously changing as a result of the modification of the culture conditions and environment stress. The cell wall is composed by β(1,3) and β(1,4)glucans, α(1,3)glucans, chitins, and galactomannans [23]. A melanin layer and sialic acid are found in the conidia surface, and a hydrophobic component layer is present on both conidia and hyphae [24]. The skeleton pattern arrangement of the cell wall is composed by the β(1,3)glucan branched with β(1,6)glucan that is covalently bound to chitin and β(1,3/1,4)glucan. Dectin-1, an innate immune receptor of the mammalian cell, recognizes the fungal cell wall pathogen-associated molecular patterns [25] and may initiate immune response against the fungus. Additionally, β-glucan is present in almost all fungi and has been used for the diagnosis of invasive mycosis [26].

The most abundant polysaccharide in the *A. fumigatus* cell wall is α(1,3)glucan and among the three α(1,3)glucan synthases identified in the fungus genome, only Δ*ags3* mutant has shown virulence changes (hypervirulence) in an experimental mouse model of aspergillosis [27]. Seven chitin synthases encoding genes have been identified in *A. fumigatus* genome, among them four genes were assessed for virulence profile and only the *chsG* null mutant strain displayed hypovirulence phenotype [28]. The main exoantigens released by the fungus during tissue invasion are the galactomannans [9], which may activate the innate immune response away from the focus of the infection. In the cell wall the galactomannans are composed by a linear α-mannan backbone and short chains of β(1,5)galactofuranose residues [29]. Galactofuranose biosynthesis starts with the isomerization of UDP galactopyranose to UDP galactofuranose by UDP galactomutase encoded by the *glfA* gene. The absence of UDP galactomutase in *A. fumigatus* led to attenuated virulence in a mouse model of invasive aspergillosis [30].

Glycosyl phosphatidyl inositol (GPI) motif proteins anchored to the plasma membrane play important roles in the biosynthesis and organization of the fungal cell wall. Many proteins such as cell surface enzymes, receptors, and adhesion molecules are anchored to the cell membrane by the GPI anchor which in turn may transfer the cell-wall-related information across the cell membrane. In this context, the absence of glucanosyltransferase encoded by *gel2* gene has been related to hypovirulence of *A. fumigatus* [31]. Another gene related to the virulence attenuation in *A. fumigatus* is *Afpig-a,* which encodes the catalytic subunit of a complex that catalyzes GPI anchor biosynthesis [32]. On the other hand, *ecm33* gene, which encodes a GPI-anchored protein, plays an important role in maintaining fungal cell wall integrity, and the absence of this enzyme enhances the virulence of the fungus [33].

Signal transduction pathways play a critical role in the biology of all living cells contributing to the integration of environmental cues into appropriate cellular activities. Proteins that participate in the signal transduction such as the G-proteins, MAP kinases, adenylate cyclases, and protein kinases (PKA) have been associated with virulence control and development of fungal pathogens [34]. The cyclic adenosine monophosphate (cAMP) PKA dependent is the central component of the cAMP signaling cascade. PKA is a serine/threonine kinase composed by the catalytic subunit *pkaC1* and the regulatory subunit *pkaR*. The central messenger of the signal transduction pathway is cAMP, produced by the adenylate cyclase, which is regulated by GpaB, a G-protein-α subunit. The malfunction of the PKA pathway by the disruption of *gpaB*, *pkaC1* [35], or *pkaR* [36] in *A. fumigatus* leads to virulence attenuation in mice. cAMP,

the second messenger molecule, is produced after perception of an extracellular signal by the G-protein-coupled receptor (GPCR). GprC and GprD in *A. fumigatus* are GPCRs and important for fungal metabolism regulation, conidia germination, and hyphae elongation and branching. Furthermore, these receptors regulate resistance towards environmental stress caused by reactive oxygen intermediates and elevated temperatures. They also play a role during the infection process, as the mutant strains were attenuated in virulence. Additionally a connection of the receptors with calcineurin-mediated signal transduction [37] is proposed.

Calcium-dependent signaling mechanisms in fungi have been associated with the regulation of wide variety responses to stress including survival in the host environment and resistance to antifungal drugs [38]. Calcium enters the cell via plasma membrane channels in response to external stress and activates the calcium-binding protein calmodulin that in turn activates calcineurin, a protein phosphatase responsible for the stimulation of downstream target genes. In fungi calcineurin regulates localization and activity of Crz1p-like transcription factor [39, 40]. The deletion of the catalytic subunit of calcineurin, *calA*/*cnaA* [41, 42] and *crzA* [43, 44] in *A. fumigatus*, led to significant defects in conidial germination, polarized hypha growth, cell wall structure, and attenuation of mortality rate in a neutropenic murine model of invasive pulmonary aspergillosis. Calcineurin and Ras proteins have been implicated in parallel activity in the regulation of hyphal and cell wall formation [45]. Ras are small monomeric GTPases proteins that act as molecular switcher which transduce signals from outside of the cell to the signal pathways inside the cell. Three Ras proteins have been studied in *A. fumigatus*, RasA, RasB, and RhbA. RasA is critical for polarized morphogenesis and cell wall stability [46] and RasB have been implicated in the control signaling modules important to the directional growth of fungal hyphae [47]. On the other hand, RhbA is implicated on nitrogen-dependent nutrient sensing and acquisition [48]. The deletion of Ras proteins encoding genes, Δ*rasA,* Δ*rasB*, and Δ*rhbA* leads to *A. fumigatus* virulence decrease in murine model of invasive pulmonary aspergillosis [46–48].

The genome of *A. fumigatus* conidia is protected from enzymatic lysis, ultraviolet light and oxidation by a gray-green melanin layer adhered to the cell wall [49]. The transcription of the essential genes for the biosynthesis of both types of melanins, pyomelanin and dihydroxinaphthalene (DHN)-melanin, are detected during infection and also protect *A. fumigatus* against reactive oxygen species (ROS), which are important compounds from the host innate immunity against pathogens [50, 51]. Pyomelanin production is associated with the tyrosine degradation pathway by the oxidative polymerization of an intermediate of the pathway, the homogentisic acid (HGA) [50]. In response to human neutrophils [52] and dendritic cells [53], four genes of the tyrosine degradation pathway (*hppD, hmgX, hmgA,* and *fahA*) showed increased transcription, leading to believe that pyomelanin is involved in fungal survival by escaping from the host immune system. DHN-melanin production starts by the polyketide synthase Alb1/PksP. The presence of a functional *alb1*/*pksP* gene in *A. fumigatus* conidia is associated with an inhibition of phagolysosome fusion in human monocyte-derived macrophages [54], which can justify the virulence attenuation of the pigmentless *alb1*/*pksP* null mutant in murine infection model [55–57].

Macrophages and polymorphonuclear cells such as neutrophils produce reactive oxygen species (ROS) as defense mechanism against the fungal conidia and hyphae, respectively. On the other side, the fungus produces specific enzymes for ROS detoxification. One category of ROS detoxification proteins is the catalases peroxidases, Cat1/CatB and Cat2/KatG, produced by the fungus mycelia. The null mutant of each gene in *A. fumigatus* led to mycelial hydrogen peroxide sensitivity and virulence reduction in the lungs of immunosuppressed rats [58]. Additionally, the cyclic AMP-dependent protein kinase (PKA), a well-known regulator of stress response in eukaryotes, contributes to the growth, germination, ROS response, and the virulence response of *A. fumigatus* [36]. Another group of genes related to oxidative stress response are the fatty acid oxygenases *ppoA, ppoB*, and *ppoC*, which are similar in sequence to specific mammalian prostaglandin synthases, the cyclooxygenases. The fatty acid oxygenase encoding genes in *A. fumigatus* were tested for virulence, and the triple mutant strain was found to be hypervirulent in an invasive murine model, showing increased tolerance to hydrogen peroxide [59]. Finally, the null mutant of a nonribosomal peptide synthetase gene, *pes1* showed a significant reduction in virulence in the *Galleria mellonella* model system and an increased sensitivity to oxidative stress in culture and during neutrophil-mediated phagocytosis [60].

In fungi, such as *A. fumigatus*, transcription factors represent an important genetic component for the establishment of an infection by the activation or repression of different mechanisms that regulate virulence and pathogenicity. The putative C_2H_2 transcription factor DvrA was identified in *A. fumigatus* as a negative regulator of host cell damage and stimulation as well as virulence during invasive pulmonary disease. The deletion of this gene led to the stimulation of CCL20, interleukin-8, and the tumor necrosis factor mRNA expression in a pulmonary epithelial cell line. Additionally, there were increased virulence and pulmonary inflammatory response in neutropenic mouse model of invasive pulmonary aspergillosis [61]. In a similar way, the deletion of the transcription factor Ace2 in *A. fumigatus* induced accelerated mortality, greater pulmonary fungal burden, and increased pulmonary inflammatory responses in nonneutropenic mice immunosuppressed with cortisone acetate. The phenotype of Δ*ace2* mutant in *A. fumigatus* was characterized by dysmorphic conidiophores, reduced conidia production, and abnormal conidial cell wall architecture, besides the reduced mRNA expression of *ppoC*, *ecm33,* and *ags3*. The null mutants of these genes have shown increased virulence in mice, as well as other phenotypic similarities to the Δ*ace2* mutant [62].

The filamentous fungi are well known microorganisms producers of secondary metabolites [63]. It is believed that the production of these secondary metabolites is linked to the protection of the fungus against the host [64]. In fungi, the genes required for the biosynthesis of secondary metabolites are clustered [65]. The gene *laeA* encodes the transcription

factor methyltransferase which is the global regulator of the secondary metabolite clusters in *A. fumigatus* [66, 67]. The deletion of *laeA* in this fungus reduces the secondary metabolite production, including gliotoxin and reduces the virulence in a murine neutropenic model [68]. Gliotoxin is a member of the epipolythiodioxopiperazine class of toxins and is both the major and the most potent toxin produced by *A. fumigatus*. This fungus maintains its normal virulence after deletion of the gene that encodes a nonribosomal peptide synthase of the gliotoxin biosynthetic cluster, *gliP*, in neutropenic mice immunosuppressed with a combination of cyclophosphamide and corticosteroids. However, the fungus becomes hypovirulent when the mice are immunosuppressed with corticosteroids alone [69]. In the same way, the deletion of the gene *pld* which encodes Phospholipase D attenuates the virulence in mice immunosupressed with hydrocortisone acetate but not with cyclophosphamide [70]. Phospholipase D modulates the internalization of *A. fumigatus* conidia into host epithelial cells. Phospholipases cleave host phospholipids, resulting in membrane destabilization and host cell penetration [71].

Fungal amino acid biosynthesis is vital to the pathogen metabolism and a conserved transduction cascade which links the environmental stimuli to amino acid homeostasis is the cross-pathway control (CPC) system. *cpcA* gene in *A. fumigatus* encodes the transcriptional activator of the CPC system of amino acid biosynthesis and the *cpcA* null mutant displayed attenuated virulence in a murine model of pulmonary aspergillosis [72].

The transcription factor SebA was demonstrated in *A. fumigatus* to be involved in the response to poor nutritional condition, osmotic, oxidative, and heat shock stress. Additionally, in the absence of the gene *sebA,* there is attenuated virulence of *A. fumigatus* in the neutropenic murine model of invasive pulmonary aspergillosis and decreased viability of the fungus during alveolar macrophages phagocytosis [73].

The fungal virulence traits are determined by regulatory elements which control their development and asexual reproduction. MedA is a developmentally regulated protein initially identified in the related model organism *A. nidulans* [74]. The *A. fumigatus medA* null mutant produced conidiophores with impaired phialide and conidia formation, impaired biofilm formation on inorganic substrate, and pulmonary epithelial cell interaction abnormalities such as decreased adherence, damage, and stimulation of cytokine production. MedA is required for normal virulence in an invertebrate and in a murine model of invasive aspergillosis [75].

An essential precondition for the beginning and manifestation of an infection is the nutrient uptake availability at the site of the infection. The pathogenic fungi are well adapted to deal with competitors for nutrients and are adapted to the fast environmental shift [76]. *A. fumigatus* is able to uptake nutrients from destruction of the host tissue by its secreted proteases. The main *A. fumigatus* secreted protease is the alkaline serine protease Alp1/asp f 13, which had been shown to be abundant in infected lung tissue and able to degrade some complement components (C3b, C4b, and C5) [77].

In living cells tissue, the iron and zinc availability are in low levels, enough to restrict the growth of pathogens. The transcriptional activator ZafA from *A. fumigatus* regulates the zinc homeostasis and is essential for the pathogenicity and virulence of the fungi, once the *zafA* null mutant is avirulent in a cortisone acetate-immunosuppressed mice [78].

Among micronutrients of the environment, iron is an essential nutrient for *Aspergillus* sp. and a key component of several enzymes that participates in a variety of cellular processes [79]. The iron sequestration is an important factor in host defense against invading fungi, since it prevents *in vivo* fungal development [80]. Low-molecular mass iron-specific chelators called siderophores are employed by the fungus to regulate iron load, which are of great importance in fungal virulence [81]. *A. fumigatus* can acquire iron by two different ways, by reductive iron assimilation and by siderophore-assisted iron uptake. The protein FtrA is a high affinity iron permease which belongs to the reductive mechanism for iron assimilation. The inactivation of *ftrA* gene in *A. fumigatus* does not change the virulence in a murine infection model. By contrast, the low virulence after the inactivation of siderophore-assisted iron uptake components, *sidC*, *sidD*, *sidF,* and *sidG* indicates that siderophore biosynthesis but not reductive iron assimilation mechanism is essential for *A. fumigatus* virulence [82–84]. Among the *Aspergillus* species, the acquisition of iron under depleted condition of this compound is controlled by the proteins SreA and HapX [85, 86]. AcuM is the transcription factor involved in the suppression of *sreA* and induction of *hapX* to stimulate expression of genes involved in both reductive iron assimilation and siderophore-mediated iron uptake, besides gluconeogenesis regulation. *A. fumigatus* Δ*acuM* mutant had reduced iron incorporation, decreased extracellular siderophore production, impaired capacity to grow under iron-limited conditions, and decreased virulence in *Galleria melanogaster* larvae model, as well as in murine models of hematogenously disseminated and invasive pulmonary aspergillosis [87]. Curiously, *sreA* null mutant is as virulent as an *A. fumigatus* wild-type strain, but HapX-deficiency causes significant attenuation of virulence in a murine model of aspergillosis [86].

Iron also influences processes such as ergosterol biosynthesis, azole drug resistance, hypoxia adaptation, and the interaction with the host immune cells [88–90]. The iron availability in eukaryotes and cellular response to low oxygen are intimately related [91].

In an infection, *A. fumigatus* is exposed to active changes in the oxygen concentration, once the quantity of oxygen at the site of infection is low due to the inflammatory response. SrbA, related to the sterol regulatory element-binding protein, is critical for coordinating genes involved in iron acquisition and ergosterol biosynthesis under hypoxia and low iron conditions found at the site of the human infection [88]. *A. fumigatus srbA* null mutant is incapable of growth in a hypoxic environment and consequently is ineffective in causing disease in a murine model of invasive pulmonary aspergillosis [92]. Mitochondrial respiration is active in hypoxia and critical for fungal pathogenesis. The deletion of the cytochrome C (*cycA*) which is involved in

mitochondrial respiration in *A. fumigatus* led to significant impaired conidia germination, growth in normoxia/hypoxia, and the fungus displayed attenuated virulence in murine model of invasive pulmonary aspergillosis [93].

In summary, fungi are versatile organisms able to adapt to diverse environmental conditions in order to grow and survive. In a hostile environment like the human body, *A. fumigatus* must evade intricate host defenses with the help of a growing number of virulence factors that are continuously being described. These molecules, which are expressed and secreted by fungus, enable host colonization, and promote immunoevasion, immunosuppression, and pathogen nutrition. On the other hand, the host immune response status is the key point that defines the progression of a disease, like the pulmonary invasive aspergillosis.

3. Host Response to *Aspergillus fumigatus*

Clinical manifestations of aspergillosis are determined by the host's immune response against the fungi and have been classically divided into allergic, saprophytic, and invasive forms [94]. In this context, in atopic individuals, the fungus triggers immune-mediated phenomena such as allergic rhinitis, asthma, and allergic bronchopulmonary aspergillosis (ABPA) [95], involving the upper and lower respiratory tracts. Allergic bronchopulmonary aspergillosis affects 1-2% of asthmatic subjects and 7–9% of cystic fibrosis patients [96, 97]. This pathology is characterized by a T helper 2 (Th2) lymphocyte response, eosinophilia, and increased serum IgE levels [98, 99]. However, in patients with preexisting cavitary lesions as a result of diseases such as tuberculosis, sarcoidosis, bronchiectasis, or cavitary neoplasia [100], growth of the fungus leads to saprophytic aspergilloma [101], which is a saprophytic form of aspergillosis [102]. In immunocompromised individuals, some conidia germinate in the lungs as hyphae, the invasive form of the fungus, causing serious angioinvasive infection, often fatal, known as invasive pulmonary aspergillosis [103, 104].

3.1. Innate Immune Response. *Aspergillus* species are moulds widespread in the world, and the most common source of infection is the inhalation of spores (conidia) into the lungs and sinuses [105]. Due to the small size of *A. fumigatus* conidia (3–5 μm) and the presence of a hydrophobic protein-coat layer which underlies immunologically active polysaccharides and consequently protect them from host defense, the spores may penetrate deeply into the respiratory tract where they find appropriate conditions to develop [11, 106, 107]. Although *A. fumigatus* has not been described as an intracellular pathogen, the *in vitro* ability of its conidia to bind to respiratory epithelium cells, to be phagocytosed, and to survive into acid organelles of these cells was already shown [97, 108] suggesting that *A. fumigatus* may use epithelium cells to escape from phagocytes. Besides, the ability of this fungus to cause damage to epithelial cells by reducing the beating of cilia from ciliated cells and thus impairing an important physical barrier which protects host against fungal invasion was already shown in an *ex vivo* model using human cells. This detrimental effect was probably due to the production of mycotoxins such as gliotoxin, fumagillin, and helvolic acid [109]. Other findings showed that respiratory cells may be activated by double-stranded RNA from *A. fumigatus* conidia and initiate an immune response via IFN-β production [110].

The recognition of *A. fumigatus* constituents by innate immunity and subsequent signaling is related to pattern recognition receptors (PRRs), which include C-type lectin and toll-like receptor (TLR) families that recognize pathogen-associated molecular patterns (PAMPs) like fungal wall components [111, 112]. As described before, C-type lectin, such as dectin-1, is a receptor for β-glucans, one of the fungus wall compounds and one of the major innate receptors for protection against *A. fumigatus.* Dectin-1 deficient mice are more susceptible to fungal infection when compared to control group, as demonstrated in a study where the deficient mice showed an impaired production of inflammatory cytokines and chemokines such as IL-1β, TNF-α, CCL3, CCL4, and CXCL1, leading to insufficient lung neutrophils recruitment and reactive oxygen species (ROS) production besides uncontrolled growth of *A. fumigatus* in lungs. In this study it was also observed an impaired capability of macrophages to produce proinflammatory mediators in response to fungus infection in the absence of dectin-1 [112]. This receptor, which is also a structural component of the corneal epithelial cells, has its expression increased early after *Aspergillus fumigatus* infection of the cornea of rats [113].

The role of TLR family in the host defense against *A. fumigatus* infection was already described too. These receptors are able to activate the antifungal properties of many innate leukocytes such as macrophages and polymorphonuclear (PMN) cells. Netea et al. demonstrated that TLR2 is related to the recognition of yet unidentified ligands of conidia and hyphae, whereas TLR4 was only associated with the recognition of conidia ligands [114]. The activation of TLR2 by *A. fumigatus* was observed with the formation of the heterodimers TLR2/6 in mice and TLR2/1 both in human and mice cells *in vitro* [115]. The activation of TLR4 led to the oxidative pathways with azurophil and myeloperoxidase granules release [116]. Rubino et al. using an *A. fumigatus* strain (Δ*rodA* CBS 144-89) deficient in a protein (Rod A), which prevents innate immune recognition, demonstrated both in human and murine cells the role of TLR2 and TLR4 in the recognition of this fungus by innate immune cells beyond the dependence of TLR1 (in human and mice cells) and TLR6 (in murine cells) for recognition [115]. Therefore, to evaluate the role of TLR in the response against *A. fumigatus,* bone marrow-derived macrophages from wild-type, TLR1, TLR2, TLR3, TLR4, and TLR6 knockout mice, were coincubated with the fungus. A reduced production of proinflammatory cytokines and chemokines such as IL-12p40, CXCL2, IL-6, and TNF-α from TLR1 deficient macrophages was observed, and this production was almost abolished in TLR2, TLR4, and TLR6 knockout cells but not in wild-type or TLR3 deficient macrophages, showing the importance of TLR1, TLR2, TLR4 and TLR6 in the fungal recognition and clearance. Moreover, to evaluate the role of TLR in human cells, Rubino et al. also used HEK293T cells transiently transfected with vectors of human TLR1,

TLR2, or TLR6 and demonstrated that only in the presence of human TLR1 and TLR2 *A. fumigatus* was recognized, and there was activation of the inflammatory transcription factor NF-κB. Furthermore, the importance of TLR6 was reinforced in a study conducted with mice deficient in this receptor, in a model of *A. fumigatus* allergic induced asthma. In this work it was observed that in the absence of TLR6, there was a lower production of IL-23 and Th17 response, which resulted in exacerbated asthma response [117]. Regarding TLR4, when this receptor (and coreceptor CD14) was blocked with specific monoclonal antibodies in human monocytes *in vitro* (using the concentrations of 1, 3, or 10 μg/mL), an inhibition between 35–70% of TNF-α releasing was observed especially when the highest concentration was used, thus highlighting its relevance on innate immune recognition of *A. fumigatus* [118]. On the other hand, triggering TLR2 induced oxidative pathways in PMN with the release of gelatinases and inflammatory cytokines [116]. To note, gelatinases that are extracellular matrix destructive enzymes [119] are associated with PMN migration during inflammation and are mobilized more readily than other molecules such as lactoferrin and azurophil granules [120]. In ABPA, caused by *A. fumigatus*, an increased inflammatory infiltrate containing neutrophils is found as well as elevated levels of the gelatinase MMP-9 and the cytokine IL-8, compared to asthma patients and controls. Moreover, IL-8 correlated with neutrophils and MMP-9 in the sputum, suggesting that the influx of neutrophils modulated by this cytokine may lead to a putative MMP-9-mediated tissue damage in the lungs [121]. Moreover, triggering of TLR3 were attributed to *A. fumigatus* conidia double-stranded RNA [110, 122], leading to contrasting results when compared to the study of Rubino et al. [115]. The opposite responses generated by TLR3 activation throughout the different studies were probably due to the strains used in them, besides different cellular and activation contexts. Therefore, such divergent results may be the consequence of diverse experimental conditions used by the authors of the studies. In addition, TLR9 activation was related to unmethylated CpG motifs present on the fungus DNA [123]. Thus, the importance of TLR seems clear, especially, TLR1, TLR2, TLR3, TLR4, TLR6, TLR9, and C-type lectin such as dectin-1 on *A. fumigatus* recognition by innate immune cells. Furthermore, MyD88, which is a molecule that mediates signaling downstream activation of most of TLRs mediates fungal clearance, inflammation, and tissue injury early after pulmonary infection with *A. fumigatus* [124].

Nucleotide-binding oligomerization domain (NOD) proteins (NOD1 e NOD2) are a subset of PRRs that recognize intracellular pathogens containing molecular patterns such as peptidoglycan. Therefore, NOD2 recognizes bacterial muramyl dipeptide (MDP) both from gram-positive and gram-negative bacteria [125], and its importance on immune response in different organs such as intestine [126] and lungs [127] was also observed. After the peptidoglycan detection the NOD proteins recruit the downstream adaptor molecule RIP2 hence activating proinflammatory pathways such as NF-κB and mitogen-activated protein (MAP) kinases such as p38, ERK, and JNK [128]. NOD2 is also expressed by a human corneal epithelial cell line, especially after activation with *A. fumigatus* conidia, which then triggers the production of proinflammatory cytokines such as TNF-α and IL-8 through the NF-κB pathway [129]. Similarly, the stimulation of a macrophage cell line with *A. fumigatus* conidia resulted in significantly increased expression of NOD2, RIP2 and NF-κB with the production of proinflammatory cytokines in a NOD2-dependent manner [130]. Therefore, this initial innate detection of *A. fumigatus* triggers important events for fungal control and clearance such as the production of inflammatory mediators, recruitment, and activation of immune cells.

The recognition of fungal cell wall compounds through PRRs is the beginning of the immune response, and the first components of defense against *Aspergillus* spp. in the alveoli are the alveolar macrophages (Figure 1), which phagocyte dormant or swollen spores, killing only swollen spores, especially due to the capability of lung resident macrophages to produce ROS [131]. Accordingly, Ibrahim-Granet et al., using murine and human alveolar macrophages, demonstrated that the activity of phosphatidylinositol (PI) 3-kinase during the initial steps of phagocytosis is required to properly kill spores in addition to the acidification of phagolysosome [132].

Although macrophages were described for many years as the main responsible for *A. fumigatus* clearance [133], human and murine neutrophils (Figure 1) also seem to be essential to a successful response against this fungus, due to their ability to form NETs (neutrophil extracellular traps) or inhibit the growth and formation of both spores and hyphae [134, 135]. This fungal control by NETs occurs by the ability of neutrophils to reduce the polar germ tube growth coupled to the presence of calprotectin which may mediate the chelation of Zn^{+2} ions. Moreover, the importance of formation of NETs to control fungal infection was reinforced in different studies using cells from patients with chronic granulomatous disease, which is a rare inherited disorder where neutrophils have a defect on ROS production, thus leading to increased susceptibility to bacterial or fungal infections [136, 137]. To note, the formation of NETs was first described in bacterial infection, as a property of innate immune cells to prevent microorganisms spreading due to a high local concentration of DNA coupled to a set of cytoplasmatic and granular proteins such as histones, elastase, and calprotectin, thus enhancing the capability of immune system to control infections [138–140]. To reinforce the importance of neutrophils in the control of *A. fumigatus,* Mircescu et al. observed, after depletion of alveolar macrophages or neutrophils, that mice infected with *A. fumigatus* had a greater presence of invasive fungus hyphae only when neutrophils were depleted when compared to the macrophages depleted group [141]. Despite the formation of NETs, neutrophils may also act against *A. fumigatus* infection through the production of ROS, as described by Sugui et al. who observed an up-regulation of catalases, superoxide dismutase, and thioredoxin reductase in the phagolysosome in an *ex vivo* model using human neutrophils [142]. Moreover, fungus phagocytosis by neutrophils led to a shift on fungus metabolism resembling a state observed under glucose limitation, suggesting that neutrophils may create nutrient limiting conditions to facilitate fungal killing [142].

Despite the importance of macrophages and neutrophils as the first barrier to control *A. fumigatus* infection, the role of

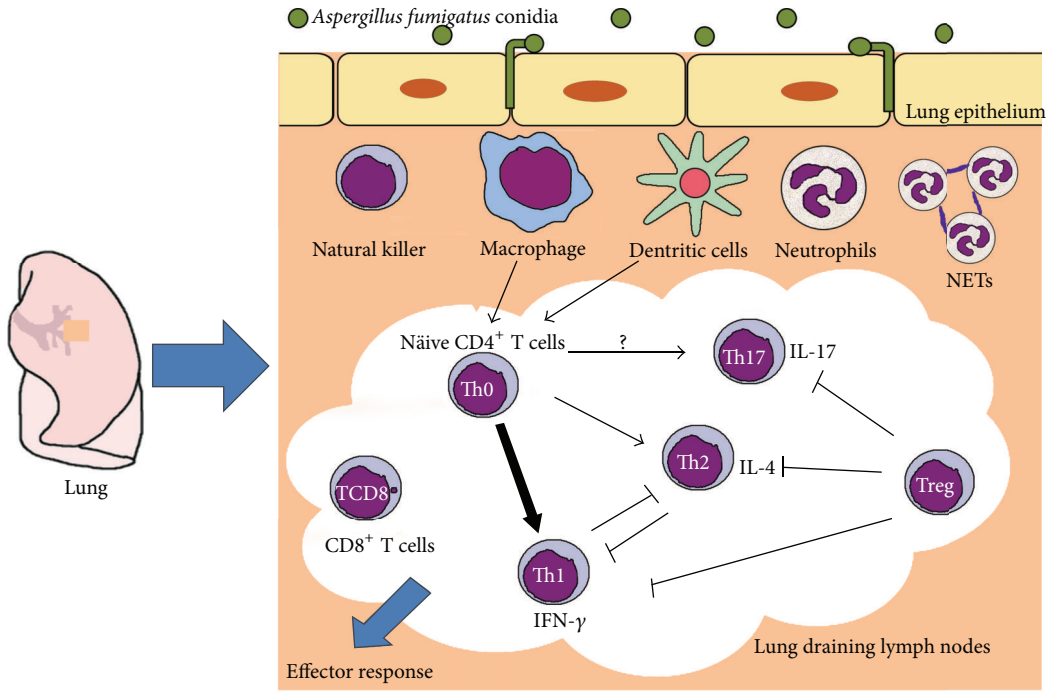

FIGURE 1: Summary of *Aspergillus fumigatus* and host immune system interplay. The lung pulmonary epithelium is magnified, and the immune response against fungal invasion is demonstrated. First, fungal components are recognized through pathogen recognition receptors (PRRs) which led to the activation of the innate immune response, depicted by macrophages, dendritic cells, natural killer cells, and neutrophils. The role of neutrophils during fungal infection is also represented by formation of neutrophils extracellular traps (NETs). The innate immunity triggers the development of an acquired immune response in the lung draining lymph nodes, which may induce the differentiation of Th1, Th2, or Th17 cell phenotypes depending on the specific stimuli and cytokine milieu, which also accounts with the presence of cytotoxic CD8 T cells against the fungus. These effector antifungal responses may also be modulated by the action of regulatory T cells (Tregs) to avoid excessive tissue damage.

other innate immune cells cannot be discarded. The ability of monocytes to phagocyte and inhibit the spores germination was already showed using human cells. The different monocyte subsets $CD14^+CD16^-$ and $CD14^+CD16^+$ react distinctly to *A. fumigatus* conidia either by controlling germination and secreting low levels of TNF or by producing high levels of inflammatory cytokines while not being able to suppress germination of conidia, respectively. These data indicate that the monocyte subsets may differ in their response to *A. fumigatus* infection depending on the cell phenotype that encounters the pathogen [143]. During experimental lung infection with *A. fumigatus*, inflammatory monocytes can differentiate into monocyte-derived dendritic cells and transport spores to lung-draining lymph nodes, thus making a link to acquired immunity and expansion of CD4-specific T cells [144]. Moreover, a differential regulation of expression of 1.827 genes was observed *in vitro* in human monocytes after their coincubation with *A. fumigatus* conidia [145]. Among these genes those related to host defense against fungal infection were upregulated between 2 and 6 hours of exposure to the fungus, such as IL-1β, IL-8, CXCL2, CCL4, CCL3, and CCL20, coinciding with an increase in phagocytosis. Another study, using the same model of coincubation, showed a differential regulation of 602 genes with an upregulated expression of IL-8, CCL20, and CCL2 on monocytes after 3 hours of coincubation with *A. fumigatus* hyphae, in contrast to only 206 genes in response to resting conidia [146].

The importance of natural killer (NK) cells to control *A. fumigatus* infection was first described in neutropenic mice that received adoptive transfer of NK cells [147]. The authors observed a reduction in CCL2 expression in the lungs early during fungal infection, and when this protein was neutralized a greater mortality and fungal burden in lungs were observed. This neutralization was also accompanied by a reduction in NK cells recruitment into the lungs, suggesting an important role of these cells in the control of *A. fumigatus* infection. Similarly, early during *A. fumigatus* infection in neutropenic mice, NK cells were the main source of IFN-γ production, and when these cells were depleted a reduction in the level of this cytokine was observed, coupled to a higher fungal load in the lungs [148]. In addition, when the authors used adoptive transfer of NK cells from wild-type, but not from IFN-γ deficient mice, there was a greater fungal clearance from the lungs, indicating that NK cells function as a relevant source of IFN-γ for this fungal control (Figure 1). Furthermore, the importance of NK cells was also related to its *in vitro* capability to damage *A. fumigatus* hyphae, but not resting spores, especially when incubated with alveolar macrophages, resulting in 2–8-fold greater killing and a marked induction of CXCL9, CXCL10, and CXCL11 when

compared to macrophages alone or macrophages incubated with IFN-γ-deficient NK cells [148]. NK cells may also damage *A. fumigatus* by directly releasing soluble factors such as IFN-γ [149]. Taken together, these findings indicate that complex and multifactorial innate processes may act as the initial defense mechanisms to avoid *A. fumigatus* lung infection and disease establishment.

3.2. Acquired Immune Response. Innate immunity alone is able to control fungal infections especially at low doses; however, when the airway infections become frequent or high fungal burdens occur, acquired immunity is necessary to host protection [150]. Accordingly, the role of CD4 (including Th1, Th2, Th17 and regulatory) or CD8 T-cell responses during *A. fumigatus* spore exposure was already showed in experimental (mice) and *in vitro* assays using human cells. In these studies different T helper cell responses were observed depending on the fungal specific components to which T lymphocytes were exposed, thus inducing protective or harmful reactions; that is, fungal secreted proteins activated nonprotective (Th2) IL-4 secreting clones, glycolipids led to nonprotective (Th17) IL-17 activated response, and polysaccharides induced protective IFNγ, IL-17, or IL-10-clones besides GPI-anchored proteins (Gel1p and Crf1p) and proteases (Pep1p) leading to Th1/Treg protective reactions [151]. Therefore, Th1/Treg response plays a protective/regulatory role, whereas Th17 could have a protective or a harmful role depending on which fungal specific component this T helper cell subtype interacts with. However, Th2 cells seem to play a nonprotective role during *A. fumigatus* infection and are especially activated after interaction with fungal secreted proteins.

The predominance and contribution of Th2 cells (Figure 1) to disease progression were also observed in cystic fibrosis patients who develop ABPA [152, 153]. To note, individuals with cystic fibrosis present an overall risk of 4–15% to develop ABPA [153]. In fact, the dendritic cells of cystic fibrosis patients with ABPA present elevated expression of the costimulatory molecule OX40 ligand coupled to a lower secretion of TGF-β, a profile linked to a Th2-biased cell response which is not observed in cystic fibrosis patients without ABPA [152]. In an experimental murine model with repeated challenges using *A. fumigatus* conidia, especially after four exposures, there was a predominance of IgE, eosinophils, and IL-4, all are hallmarks of Th2 response [154], thus suggesting that the Th2 response predisposes to *A. fumigatus* infection. Moreover, Shreiner et al. demonstrated the importance of IL-4 and especially IL-10 in the generation of a Th2 response during an 8-week experimental chronic infection with this fungus, rather than acting as anti-inflammatory cytokine [155].

On the other hand, the protective role of Th1 response (Figure 1) during *A. fumigatus* infection was reinforced both in immunocompromised patients [156, 157] and in neutropenic mice due to its capability to neutralize Th2 cytokines [158]. In this context, Cenci et al. showed that when CD4 T cells produced IFN-γ *in vitro* and not IL-4, a protective acquired immunity was developed. This evidence was also clear when the animals infected with *A. fumigatus* were treated with IFN-γ and a lower mortality rate was observed when compared to the nontreated group. The importance of monocyte-derived dendritic cells to drive CD4 T cell differentiation during *A. fumigatus* infection was also observed. In the absence of dendritic cells there is a prevalence of Th17 over Th1 phenotype, together with the lost of T-bet expression in CD4 specific T cells, suggesting that monocyte-derived dendritic cells are important for the maintenance and development of protective Th1 cell response against the fungus. In addition, the predominance of Th1 response against *A. fumigatus* was also demonstrated in healthy individuals [159].

For many years, until the discovery of Th17 cell subtype [160], the occurrence and immunopathogenesis of fungal infections was attributed only for the traditional balance/imbalance between Th1 and Th2 responses [161–163]. However, in the last years different studies have shown a controversy role for Th17 response (Figure 1) during *A. fumigatus* infection. In mice experimentally infected with *A. fumigatus* or *Candida albicans*, the production of Th17 signatures cytokines, such as IL-23 and IL-17, may play a nonprotective role during fungal infection due to its ability to negatively regulate the development of Th1 response and to impair the *in vitro* neutrophil-mediated killing or the *in vivo* fungal clearance [164]. To reinforce the negative influence of Th17 cells on fungal control, these authors blocked *in vivo* the IL-23 and IL-17 cytokines and observed an increased resistance to both infections, as evaluated by the observation of a decreased fungal growth in the lungs. Despite the permissive role of Th2 response in the progression of *A. fumigatus* infection, the deleterious participation of Th17 cells was also observed in an experimental model of chronic infection in which the inflammatory reaction was characterized by eosinophilia and Th2 cell-associated cytokine profile coupled to IL-17 production, which persisted even after Th2 response, had begun to resolve [165]. These data indicated that Th17 could be as detrimental as Th2-biased response for host during *A. fumigatus* infection. In this study, after repeated challenges with fungus conidia, when an IL-17 knockout mice was used, the inflammation was attenuated and fungus clearance was enhanced. On the other hand, the protective role of IL-17 in the host defense against this fungus was also observed in another experimental model of *A. fumigatus* infection. In this case, the *in vivo* neutralization of this Th17-related cytokine early during infection resulted in an impaired pathogen clearance that led to an increased fungal pulmonary burden [112]. Therefore, the exact role of IL-17 in the *A. fumigatus* infection is still not totally clear yet and may depend on the host, time of infection, or even contact with fungal specific wall components [151].

The expansion of regulatory T cells (Tregs) (Figure 1) during fungal infections may constitute an important tool to avoid deleterious or exacerbated inflammation due to Th1 response or hypersensitivity reactions associated with Th2 responses [154]. Tregs may play a relevant role early in the *A. fumigatus* infection by modulating proinflammatory activities of polymorphonuclear leukocytes through contact-dependent or independent mechanisms such as IL-10 production [166]. Otherwise, later during *A. fumigatus* infection

Tregs may work especially by inhibiting Th2 cell response and then preventing allergy to the fungus.

Besides CD4 T helper responses, it is know that $CD8^+$ T cells may also mediate protective immunity against *A. fumigatus* infection, especially through recognition of HLA-A^*0201 restricted fungal peptides [167]. Furthermore, dendritic cells may recognize fungal RNA by TLR3 and induce class I MHC restricted CD8 T-cell protective responses to *A. fumigatus* [122]. In addition, Templeton et al. observed increased IFN-γ producing $CD8^+$ T cells in bronchoalveolar lavage fluid of mice repeatedly challenged with *A. fumigatus* conidia coupled to the maintenance of airway memory phenotype $CD8^+$ T cells [168]. The maintenance of this $CD8^+$ T cells in airways could be attributed to the specific characteristic of *A. fumigatus* conidia to germinate and persist in lungs, thus pointing to cytotoxic cells as another important mechanism for fungal control.

4. Concluding Remarks

In summary, this review showed that the control of *A. fumigatus* infection is associated with different and interconnected mechanisms dependent on the interplay between the pathogen virulence factors and the host immune competence. Recently it was showed that the immune response induced by *Aspergillus* spp. may be dependent on variations of the fungus strain that could present diverse virulence factors and therefore increased or reduced infectivity [169]. Even though, in general, the infection control may begin early during pathogen invasion when the fungus crosses physical or chemical natural barriers which, if disrupted, may lead to the pathogen recognition and activation of innate immunity receptors and cells like macrophages and neutrophils that, in turn, may initiate the acquired immunity events represented by CD4 or CD8 T-cell activations. In this context, a consensus among the authors that Th1-biased response is protective seems to exist, due to its ability to recruit other cells and restrain fungal growth, while the Th2 cell response is harmful especially to be associated with higher fungal burden at lungs or the occurrence of allergy. In addition, a controversy remains regarding the role of Th17 responses during *A. fumigatus* infection, while the cytotoxic activity of CD8 lymphocytes may be essential in some circumstances of intracellular pathogen. Overall, the regulation of leukocyte excess inflammation and avoidance of tissue damage directed to the elimination of *A. fumigatus* is related to Tregs activity. However, further studies must be conducted in order to better clarify how certain fungal compounds may activate or escape from the immune system as well as to provide tools for the development of novel therapeutic approaches to control this fungal lung infection.

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Review Article

Aspergillus-Associated Airway Disease, Inflammation, and the Innate Immune Response

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Aspergillus moulds exist ubiquitously as spores that are inhaled in large numbers daily. Whilst most are removed by anatomical barriers, disease may occur in certain circumstances. Depending on the underlying state of the human immune system, clinical consequences can ensue ranging from an excessive immune response during allergic bronchopulmonary aspergillosis to the formation of an aspergilloma in the immunocompetent state. The severest infections occur in those who are immunocompromised where invasive pulmonary aspergillosis results in high mortality rates. The diagnosis of *Aspergillus*-associated pulmonary disease is based on clinical, radiological, and immunological testing. An understanding of the innate and inflammatory consequences of exposure to *Aspergillus* species is critical in accounting for disease manifestations and preventing sequelae. The major components of the innate immune system involved in recognition and removal of the fungus include phagocytosis, antimicrobial peptide production, and recognition by pattern recognition receptors. The cytokine response is also critical facilitating cell-to-cell communication and promoting the initiation, maintenance, and resolution of the host response. In the following review, we discuss the above areas with a focus on the innate and inflammatory response to airway *Aspergillus* exposure and how these responses may be modulated for therapeutic benefit.

1. Introduction

Aspergillus molds represent a significant proportion of total airway spores [1]. This ubiquitous species therefore may result in invasive disease in those hosts with predisposing risks such as structural lung disease or defects in immune host responses. The pulmonary manifestations of disease extend from hypersensitivity responses to invasive cavitation secondary to spore germination and hyphal infiltration [2]. Interestingly, while over two-hundred species of *Aspergillus* are described, approximately ten percent are pathogenic to humans, and interspecies variability in the antigenic response explains the varied disease spectrum encountered in clinical practice [3–5].

The most commonly isolated species is *A. fumigatus*. Accounting for ninety percent of systemic infection, it has been described that the human milieu in certain circumstances permits invasion by *A. fumigatus* but restricts it in cases of *A. flavus* and *niger* [6, 7]. *A. flavus* possesses a survival ability in higher temperatures hence its predominance in the Middle East, Africa, and parts of Southeast Asia [8]. Whilst this organism usually presents as invasive pulmonary aspergillosis (IPA) or an aspergilloma, minimal published evidence reports an implicating association with allergic bronchopulmonary aspergillosis (ABPA) [7]. The spectrum of pulmonary disease associated to *Aspergillus spp.* involves a complex interplay between the respiratory epithelium and the host response in the presence of inhaled spores (Table 1). Despite extensive research fungal conidial host interaction within the airway remains poorly understood [9]. Inhalation of *Aspergillus* spores triggers a cascade of consequences determined by the immunological state of those affected

Table 1: Clinical spectrum of disease associated with *Aspergillus* species.

Disease	Aspergillus species
(1) Atopic asthma	*A. fumigatus*
(2) Hypersensitivity pneumonitis	*A. clavatus*
(3) ABPA	*A. fumigatus*
(4) Aspergilloma (mycetoma)	*A. fumigatus, A. flavus*
(5) Invasive aspergillosis	*A. fumigatus, A. flavus*

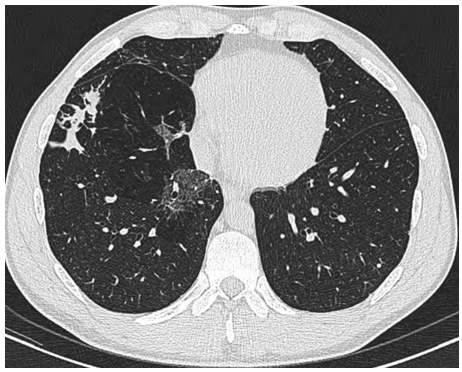

Figure 1: Focal area of ground glass change in the medial aspect of the right lower lobe in a patient with ABPA.

[10, 11]. For instance, if an individual is immunocompetent, an allergic ABPA or hypersensitization response can ensue. However during immunocompromised states IPA can occur resulting in invasive life-threatening septicaemia [12–14].

Reaching a diagnosis of *Aspergillus*-associated lung disease is based on a constellation of clinical observation, radiological findings, and immunological testing. *Aspergillus* species in sputum, bronchoalveolar lavage, or biopsy may be visualized under direct microscopy as septated hyaline hyphae and subsequently stained with Gomori methenamine-silver or periodic acid-Schiff (PAS) stains [15]. It is, however, crucial to note that other filamentous fungi including *Scedosporium* and *Fusarium* species possess similar appearances under direct visualisation. Adjunctive fungal cultures are occasionally helpful; however, the high prevalence of negative cultures diminishes their value; for instance, several multicenter surveillance studies in haemopoietic transplant recipients have shown that up to half of those with suspected invasive aspergillosis had documented negative fungal cultures [16, 17]. Furthermore, histopathologic-based diagnoses are limited due to an inability to biopsy at certain sites and dearth of adequate visualization of the fungi or its fragments; for instance, hyphael fragments distort the diagnosis and may result in absence of the classic 45° branching.

Chest radiology in *Aspergillus*-associated airway pathology varies widely depending on host immunocompetence and, consequently, the clinical manifestation of disease. However each manifestation has a well-described yet different range of radiological findings. Pathognomonic high-resolution computed tomography (HRCT) findings may be an early sign such as a central ground-glass opacification surrounded by a ring of consolidation termed the "reverse halo sign." In the setting of ABPA, HRCT may demonstrate proximal cylindrical bronchiectasis with upper lobe predominance combined with bronchial wall thickening. Other important suggestions of ABPA include mucus plugging, atelectasis, consolidation, ground glass attenuation, mosaic pattern perfusion, or air trapping (Figure 1) [18, 19]. Most critical, however, is the importance correlating radiology with clinical evaluation for a definitive diagnosis [18].

A recent development aiding a diagnosis of IPA is an assay for the detection of serum *Aspergillus* galactomannan (GM), a polysaccharide produced by the fungal cell wall during growth that illustrates moderate sensitivity and high specificity in pooled analyses [20]. BAL galactomannan assessment however has been shown to facilitate an even more rapid diagnosis of IPA in comparison to either serum GM testing or fungal BAL cytology and culture [21]. A major disadvantage is, however, the high rate of false positives, causing an inability to distinguish between invasive disease and airway colonization alone [22]. Furthermore, recent comparative analyses assessing the diagnostic accuracy of *Aspergillus* PCR versus GM assays in the diagnosis of IPA concluded that the diagnostic performance remains comparable between both tests [23]. Although the sensitivity of PCR increased when BAL was tested, the findings again may simply reflect colonization rather than invasive disease.

As the clinical manifestations of *Aspergillus*-associated pulmonary disease depends largely on the interaction between the inhaled conidia and immune effector cells, the role of inflammatory and immune responses is both critical in accounting for disease and eliminating sequelae. Alveolar macrophages play a key role in phagocytosis. The intra-cellular degradation of inhaled conidia occur following proinflammatory mediator secretion that, in turn, aids neutrophil degranulation further enhancing conidial clearance. *A. fumigatus* interacts with the innate immune system through pattern recognition receptors (PRRs) such as dectin-1 and toll-like receptors (TLRs) 2 and 4 [24–27]. Germinating conidia activates phosphatidylinositol3-kinase (PI3K), p38 mitogen-activated protein kinase (MAPK), and ERK1/2 resulting in interleukin-8 (IL-8) release [28]. Conidial swelling during germination exposes cell surface β-1,3-glucan that triggers dectin-1 recognition by receptors present on circulating macrophages eliciting protective responses [29]. For instance, dectin-1 (−/−) mice demonstrate impaired IL-1α, IL-1β, TNF-α, CCL3, CCL4, and CXCL1 responses that result in insufficient neutrophil recruitment and subsequent uncontrolled *A. fumigatus* growth [30]. Phagocytosed conidia fuse with lysosomes and via an endocytic pathway generates an acidic milieu to degrade conidia in an effort to eliminate the fungus [31]. In addition to the innate immune response, T cells initiate an adaptive immune cascade to *Aspergillus*. Interestingly, conidial phagocytosis results in a protective Th1 response by dendritic cells whilst hyphael phagocytosis generates an unfavorable Th2 response and subsequent CD4-driven IL-10 release [32, 33]. This review will highlight the main pulmonary manifestations of *Aspergillus*-associated disease with a focus on the innate immune and inflammatory responses encountered.

Figure 2: A pulmonary aspergilloma in a 24-year-old patient with cystic fibrosis. CT images show a fungus ball within the preexisting left upper lobe cavity, and the air-crescent sign is demonstrated in the nondependent part of the cavity on both CT imaging performed in the supine and prone position.

2. Aspergilloma

Aspergilloma remains an important pulmonary manifestation of the fungus. Described as a conglomeration of condensed hyphae, it usually presents as a ball projecting in a polypoid manner into a preexisting thoracic cavity. Most patients are asymptomatic; however, life-threatening episodes of haemoptysis can occur and are explained by hyphal invasion of the bronchial arteries. Typically identified as a spherical mass on chest radiography, aspergillomas can vary in both size and less commonly number and are seen to move into dependent portions of the cavity during supine and prone CT imaging aiding a diagnosis (Figure 2). The rim of air between the ball and the periphery of the cavity noted in these cases is termed the "air-crescent sign" [34].

The association between aspergillomas and ABPA remains poorly understood. Whilst most believe there is no association, a single study has demonstrated high concomitance between the two describing fungal ball formation several years preceding a subsequent diagnosis of ABPA [35]. Evidence-based data related to the treatment of aspergillomas is limited to case series. Whilst surgical removal remains the mainstay of treatment to prevent life-threatening haemoptysis, such resection is technically challenging with high intra- and postoperative complication rates [36]. It is important to note however that early intervention did minimize operative risk in asymptomatic patients [37]. Bronchial artery embolisation is the treatment of choice in patients with haemoptysis or those unfit for surgical resection; however, while initial outcomes are very good, relapse rates may be as high as fifty percent [38]. A newer approach showing promise is CT-guided intracavitatory instillation of antifungals such as amphotericin B. This may in some cases resolve the aspergilloma completely [39].

3. Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is a complex allergic pulmonary hypersensitivity to *A. fumigatus* [3]. Correlations exist between its development and airborne spore concentrations [40–43]. Historically described as presenting with sputum plugs, radiographic evidence of lung collapse or consolidation, and an elevated serum eosinophil count, ABPA typically demonstrates a peribronchial eosinophilic inflammatory pattern comparable to bronchiolitis obliterans with organizing pneumonia [44, 45].

The Rosenberg-Patterson criteria can be used to make a diagnosis of ABPA and include the following: the presence of bronchial asthma, positive skin response to an injected *A. fumigatus* antigen, elevated total and *A. fumigatus*-specific serum immunoglobulin-E (IgE), serum eosinophilia, pulmonary opacities and/or central bronchiectasis, and positive serum IgG precipitins against *Aspergillus* antigens [46–48]. Whilst the latter aids the diagnosis of ABPA, a doubling of the IgG/IgE ratio may differentiate ABPA from other disorders [49–52].

ABPA complicates some chronic pulmonary conditions with a varying prevalence including asthma, bronchiectasis [53], COPD [54], cystic fibrosis (CF) [4], and immunodeficiencies including chronic granulomatous disease and hyper-IgE syndromes [55]. While the association of ABPA with CF has received attention, its recognition remains challenging due to an overlap between symptoms and its similarity of clinical presentation to that of an infective bacterial exacerbation [56]. To overcome such difficulties, a consensus report with diagnostic criteria was developed to help distinguish ABPA from an infective exacerbation in CF; however, clinical difficulties continue to persist [57]. Our group has shown that frequency of isolation of *Aspergillus* species from sputum in CF does not correlate with occurrence of ABPA and furthermore that *Aspergillus* colonization in itself is associated with more severe radiological abnormalities undetectable by pulmonary function alone [58, 59]. The association of ABPA with asthma also remains poorly understood with a significant mean lag in diagnosis of up to a decade [60]. ABPA is characterized by an exaggerated Th-2-mediated response triggering release of inflammatory cytokines and growth factors leading to airway hyperresponsiveness, goblet cell hyperplasia, and subepithelial fibrosis [13, 14]. IgE sensitization to *A. fumigatus* is associated with reduced lung function in asthmatics, and the firm link between fungi and severe asthma is best summarized by the described condition severe asthma associated with fungal sensitivity (SAFS) [61, 62].

The foundation of effective ABPA treatment involves use of systemic glucocorticoids and azole antifungal therapies over a number of weeks. Whilst institutional protocols vary, corticosteroid response is monitored by serial serum IgE measurements: declines suggest remission while increases indicate relapses [63–65]. Higher doses of systemic glucocorticoids with slow titrations and longer durations are associated with better remission rates and a reduced prevalence of glucocorticoid-dependent ABPA [66].

Itraconazole therapy has been validated in two randomized controlled trials with reductions of serum IgE levels exceeding 25% when compared with placebo [67, 68]. Targeted anti-IgE therapy with omalizumab provides an alternate approach for patients with a concomitant diagnosis of ABPA and asthma [69]. In the context of CF, itraconazole is utilized in the setting of recurrent ABPA; however, its role in those colonized with *Aspergillus* species in the absence of ABPA remains unclear. We have shown that the Vitamin D receptor (VDR) is downregulated in the presence of *Aspergillus* colonization driven by the small molecule gliotoxin. Treatment with itraconazole decreases BAL gliotoxin concentrations, restoring VDR expression. Concurrently, a diminished systemic level of the Th2 cytokines IL-5 and IL-13 is detectable with concomitant improvement in clinical and radiological parameters [70]. Despite effective treatment options, an early diagnosis of ABPA with rapid initiation of therapy is key to prevent irreversible pulmonary damage irrespective of the underlying pulmonary condition [71]. Once therapy has been initiated, there is limited data available to outline the expected clinical course, and hence a personalized individual approach must be adopted for optimal patient management [72–75].

4. Invasive Pulmonary Aspergillosis (IPA)

IPA is a consequence of *A. fumigatus* invasion of the bronchial epithelium resulting in pneumonia, tracheobronchitis, and pleural effusions [12]. Inhaled conidia germinate within alveoli and migrate into the bloodstream, and this expanding fungal inoculum secretes chemokines attracting neutrophils for phagocytosis but concurrently inducing proinflammatory responses [9]. Such proinflammatory responses target conidia that manage to evade phagocytosis. In the immunocompetent, alveolar macrophages phagocytose inhaled conidia whilst, in those immunosuppressed, dysfunction of such host defenses increases the risk of developing IPA [76]. Once the fungus invades beyond the pulmonary epithelium and into the systemic circulation, it is termed invasive aspergillosis (IA) which carries with it, even higher rates of mortality.

The major risk factor associated with the development of IPA is immunosuppression; therefore, patients with prolonged neutropenia, undergoing haematopoietic stem cell transplantation, and/or those who use corticosteroids chronically are at greatest risk. IPA has particularly high mortality rates (~70%) in transplant recipients and occurs in up to fifteen percent of patients undergoing allogenic stem cell or solid-organ transplantation [77, 78].

Whilst IA most commonly invades the pulmonary system presenting with fever, chest pain, dyspnoea, cough, and haemoptysis, the clinical diagnosis remains challenging owing to nonspecific serology and radiologic testing. The culture of *Aspergillus* from a site normally sterile in tandem with histological evidence of tissue invasion makes the diagnosis of IPA [79]. The best noninvasive serologic marker remains galactomannan concentrations; however, false positive results are complicated by its presence in non-*Aspergillus* cell walls; for example, cross-reactive antigens are reported in disseminated *Fusarium* and histoplasmosis sepsis [80, 81].

Imaging modalities used in the context of IPA lack sensitivity due to the wide possible range of radiographic abnormalities encountered. Computed tomography (CT) findings in those with neutropenia compared to those without neutropenia revealed similar patterns; however, individuals with neutropenic IPA tended to present with segmental areas of consolidation [82]. More recently, CT pulmonary angiography (CTPA) has been touted a promising tool to highlight arterial vessel interruption secondary to angioinvasion from the fungus that may aid diagnosis [83]. Clear guidelines for the treatment of IPA have been published and recommend voriconazole as the first line agent where a diagnosis has been established or liposomal amphotericin B in suspected cases. Therapy is generally continued until all signs and symptoms of active disease have resolved and may be prolonged [84–86].

5. Innate and Inflammatory Defences against *Aspergillus* Species

The human airways are under constant exposure to *A. fumigatus* with daily inhalation of several hundred spores [87]. These asexual spinous conidia are usually harmless and readily cleared by the immunocompetent host, through a myriad of defence mechanisms and pattern recognition systems involving alveolar macrophages, neutrophils, and antimicrobial peptides [88, 89].

Components of the innate immune system that are involved in recognition and removal of *Aspergillus* from the airways therefore include (i) the physical and mechanical barriers of the respiratory tract, (ii) phagocytic cells (iii) antimicrobial peptides, and (iv) soluble and cell surface expressed pattern recognition receptors (PRRs).

5.1. Anatomical Barriers. The turbulent airflow that is generated via the nasal turbinates and branching of the lower airways facilitates the deposition of inhaled conidial spores onto the airway wall where they become trapped in mucus and can be removed from the respiratory tract by the mucociliary escalator, coughing or sneezing. Some *Aspergillus* conidia can bypass this system and due to their small size (2–5 microns) may be inhaled into the alveoli. When this occurs additional protective mechanisms are called into play such as phagocytosis.

5.2. Phagocytosis. Neutrophils and macrophages are phagocytic cells that can ingest and kill invading spores. In response to the presence of *Aspergillus* in the lower airways,

neutrophils are recruited to the alveolar spaces where they can either phagocytose the spores or release the contents of their granules leading to direct fungal killing. Alveolar macrophages in particular play a key role in anti-*Aspergillus* defences in the lung.

The alveolar macrophage (AM) remains the principle phagocytic cell within the lung and represents the first line of defence against conidia within the alveoli [87]. The recognition, phagocytosis, and killing of fungal pathogens are crucial in controlling microbial proliferation. AMs recognise conidia through pattern recognition receptors (PRRs) on their cell surface. Spore engulfment subsequently occurs via pseudopodial extensions that involve actin polymerisation. Following internalisation, the phagolysosome induces fungal killing by both oxygen-dependant and -independent methods [90, 91]. Nonoxidative killing involves acidification of the phagolysosome and conidial degradation by hydrolytic enzymes such as cathepsin D. Furthermore, chitinase a macrophage-based mammalian enzyme capable of degrading chitin, a component of the fungal cell wall additionally aids the killing ability of the AM [91–93]. Once conidia are taken up by AMs, swelling ensues activating the NADPH oxidase system. Reactive oxygen species (ROS) are therefore produced to kill microbes; for example, superoxide anions (O_2^-) are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [94]. Compelling evidence exists showing that NADPH oxidase inhibition allowed almost complete conidial germination illustrating the importance of ROS production in conidial killing [95]. Interestingly, in this context *A. fumigatus* has the propensity to cause infection in individuals with chronic granulomatous disease, a hereditary condition of impaired reactive oxygen species generation.

Neutrophils recruited to the site of fungal infection also possess the capacity to engulf and kill conidia. Unlike macrophages however, neutrophils produce mesh-like extracellular traps (NETs) to further control *A. fumigatus* proliferation. NET formation occurs (NETosis) where membranes of a dying neutrophil are disrupted and the granular contents couples with nuclear DNA to complex together. The result is released into the surrounding milieu in the form of a matrix ensnaring both conidia and hyphae of the fungal pathogen [96]. Trapped fungi are subsequently killed by cationic antimicrobial peptides embedded within the NET matrix [97].

5.3. Antimicrobial Peptides. Antimicrobial peptides (AMPs) are endogenous molecules that play a critical role in the innate immune response to fungal infection. They have particular importance in the early control of *A. fumigatus* proliferation.

Defensins are a group of small cationic AMPs found in humans involved in the nonoxidative killing of *A. fumigatus*. Their mechanism of action is the disruption of the fungal membrane resulting in cell lysis. There are four α-defensins exclusively synthesised in the primary granules of neutrophils whilst the larger β-defensins are associated with epithelial cells. Human β-defensin 2 (hBD2) is the defensin most commonly expressed in the lung [98]. Exposure of the airway to *A. fumigatus* induces expression of hBD2 [99].

Cathelicidins are another class of AMPs with potent killing activity against fungi. Highly expressed at sites and times of inflammation, the only human cathelicidin expressed by neutrophils and airway epithelial cells is LL-37 [100, 101]. Like defensins, cathelicidins permeabilise the fungal membrane inducing killing [102]. In addition to a direct target against the microbe, they also operate indirectly as "alarmins" to modulate other immune responses [102, 103].

Human secretory leukoprotease inhibitor (SLPI) is an 11.7 kDa protein critical to maintaining the protease: antiprotease balance within the lung [104]. SLPI, produced by neutrophils and macrophages displays potent antifungal properties against *A. fumigatus* [94]. Its cationic nature permits membrane perturbation and subsequent loss of fungal cell viability [105]. Lactoferrin, another AMP produced by neutrophils, inhibits fungal proliferation. By the sequestration of circulating bioavailable iron, a starvation of conidia occurs inducing death [88].

5.4. Pattern Recognition Receptors (PRRs). *Aspergillus* conidia can be recognised by a number of receptors of the innate immune system (Table 2). This occurs largely through recognition of components of the conidial cell wall such as β-glucan, chitin, mannan, or galactomannan. The mammalian cell receptors that are involved in these processes include secreted factors such as pentraxin-3, C-type lectins and complement, and cell-surface-expressed toll-like receptors (TLRs), lectins, and dectin-1.

5.4.1. Soluble PRRs. Pentraxin-3 is a soluble receptor that acts as an opsonin by recognising galactomannan on *Aspergillus* conidia. Its expression is upregulated in macrophages and dendritic cells in response to conidia leading to enhanced phagocytic function in these cells [106]. Pentraxin-3-deficient mice are highly susceptible to invasive aspergillosis [107]. Other soluble receptors that act as opsonins for *Aspergillus* are the surfactant proteins (SP) A and D. These collectins, secreted by type II pneumocytes and Clara cells, are members of the C-type lectin family and can bind *Aspergillus* carbohydrate structures causing conidial agglutination and enhanced neutrophil phagocytosis and killing [108, 109]. Although it is not known what component of the conidial cell wall is recognised by SP-A, β-1,6-glucan has been shown to be a ligand for SP-D [110]. Another collectin, mannose-binding lectin (MBL) as the name suggests, also acts as an *Aspergillus* opsonin and can activate the lectin complement pathway via C4bC2a or C2 [111, 112]. A selection of other complement proteins have been reported to participate in anti-*Aspergillus* defences including C3 [113, 114] and C5 [115]. Interestingly *Aspergillus* itself can inhibit complement activation by binding factor H and plasminogen [116, 117].

5.4.2. Cell-Associated PRRs. TLRs are a family of at least 12 mammalian germ-line encoded PRRs that recognise and discriminate various pathogen-associated molecular patterns (PAMPs). The activation of TLRs induces change in proinflammatory gene expression; for example, cytokines that lead to activation of the adaptive immune response. Of the TLR

Table 2: Receptors that recognise *Aspergillus* species.

Receptor type	Receptor family	Receptor	Ligand
Soluble	Long pentraxin	Pentraxin-3	Galactomannan
	C-type lectin/collectin	Surfactant protein A	Unknown
	C-type lectin/collectin	Surfactant protein D	β-1,6-glucan
	C-type lectin/serum collectin	Mannose-binding lectin	Mannose
Cell surface	Toll-like receptor	TLR2	Chitin
	Toll-like receptor	TLR4	Unknown
	Toll-like receptor	TLR9	Unmethylated
	C-type lectin	DC-SIGN	Unknown
	Lectin	Mannose receptor	Mannose
	Phagocytic receptor	Dectin-1	β-1,3-glucan

family, currently the best evidence points to roles for TLRs 2, 4, and 9 in *Aspergillus* recognition [24, 118, 119]. Whilst these receptors are commonly associated with the recognition of lipopeptides, lipopolysaccharide, and unmethylated CpG DNA, respectively; in the context of *Aspergillus* recognition TLR9 appears to be activated as normal by hypomethylated fungal DNA [120] whereas TLR2 participates in the recognition of chitin [121]. The *Aspergillus* PAMPs that activate TLR4 are not yet known.

The lectins DC-SIGN and the mannose receptor (MR), together with the transmembrane phagocytic receptor dectin-1, facilitate the binding and ingestion of *Aspergillus* by phagocytes [122–124]. Dectin-1 primarily recognises β-1,3-glucans on *Aspergillus* spores [26]; however, it can also interact with TLRs, specifically TLR2, to modulate the immune response to *Aspergillus* infection [125].

6. Cytokines in the Innate Pulmonary Defense against *Aspergillus* Species

Complex networks of cytokines play important roles in the innate pulmonary response against *A. fumigatus*. These soluble mediators assume responsibility for cell to cell communication within the innate arm of the immune system and promote initiation, maintenance, and resolution of the host response. Dependent on their predominant functional capabilities against *A. fumigatus*, they are best described in three distinct groups: recognition, recruitment, and activation cytokines [126].

6.1. Recognition Cytokines. This group of cytokine represents the initial response to pathogen recognition. Mediating the recruitment of additional immune cells to the site of infection remains their most important task and members include ligands of the interleukin-1 (IL-1) family such as IL-1β and tumor necrosis factor-α (TNF-α).

TNF-α is a proinflammatory cytokine that in the earliest innate response to *A. fumigatus* is released from pulmonary AMs and later on by recruited immune effector cells including neutrophils and monocytes [127, 128]. Its levels have been shown to significantly increase following intrapulmonary challenge with *A. fumigatus* in both the setting of normal and immunocompromised states. Conversely, suboptimal pulmonary concentrations and TNF-α neutralization are associated with increased fungal loads, decreased neutrophil recruitment, and increased mortality in animal models challenged with the fungus [128–131]. Consequently, TNF-α-deficient mice remain more susceptible to infection with *A. fumigatus* [129]. This critical and protective role that TNF-α orchestrates within innate defense systems against fungal infection is further corroborated by the observations that the use of TNF-α antagonists in clinical practice is associated with an increased incidence of aspergillosis [132–134]. The underlying mechanisms by which this occurs include an increase in ROS production, phagocytosis by pulmonary AMs, and the augmentation of hyphal damage by neutrophil activation [135]. TNF-α whilst not directly chemotactic in itself induces expression of cell adhesion molecules and promotes expression of CXC and CC chemokines including MIP-1α, JE, and MIP-2 which in turn recruit further immune effector cells to the infected site [128, 136].

6.2. Recruitment Cytokines. The efficient recruitment of leukocytes to the site of any pulmonary infection involves the process of rolling and adhesion of circulating cells to the vascular endothelium followed by extravasation and directional migration to the gradient of chemotactic molecules such as chemokines. Described as a superfamily of small secreted proteins, chemokines can be classified into four separate families; CXC, CC, C, and CX_3C based on the position of their cysteine residues relative to the N-terminus. They are secreted from a variety of cell types, including the leukocyte, airway epithelium and endothelial cells upon exposure to *A. fumigatus* [126, 137–143].

The CXC family is further divided into two subgroups, based on the presence or absence of an ELR (Glu-Leu-Arg) motif preceding the first cysteine. The ELR^+ CXC chemokines include neutrophilic chemoattractants such as IL-8 (CXCL8), growth-related oncogene (GRO) chemokines (CXCL1-3), macrophage inflammatory protein-2 (MIP-2), and KC. While IL-8 and GRO chemokines elicit their effects through binding human CXCR1 and CXCR2 receptors, MIP-2 and KC bind the sole murine CXCR2 receptor [137]. Antibody-mediated neutralization of CXCR2 interestingly leads to the development of invasive aspergillosis in neutropenic mice challenged with *A. fumigatus* and is associated with reduced pulmonary recruitment of neutrophils and increased mortality [128]. Furthermore, pulmonary overexpression of KC diminishes

fungal burden and increases resistance to IPA in mice challenged with *A. fumigatus* [144]. Unlike ELR^+ chemokines, the ELR^- group represents the major chemoattractants for mononuclear cells including CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC, all upregulated during early IPA [126, 145].

The CC chemokines such as CCL2/MCP-1 and CCL3/MIP-1α and their respective receptors are involved in the mononuclear recruitment during responses to *A. fumigatus* infection [146–150]. Levels of these chemokines increase in the lungs of both normal and neutropenic mice with *A. fumigatus* infection and following antibody mediated neutralization cause an increased mortality owing to an increased fungal load and lack of appropriate monocytic recruitment [146, 147]. Additionally, a lack of CCR6 (receptor for CCL20/MIP-3α) showed similar results following an *A. fumigatus* challenge [149]. Interestingly however, data has emerged illustrating that certain CC ligands and their respective receptors may impair the antifungal response to *A. fumigatus*. Antibody-mediated depletion of CCL17/TARC for instance increased CCL2/MCP-1, CCL3/MIP-1α, and TNF-α pulmonary concentrations reducing fungal burden and improving survival in the setting of *A. fumigatus* infection whilst mice lacking CCR4, the receptor for CCL17/TARC, exhibited enhanced resistance to *A. fumigatus* [151].

6.3. Activation Cytokines. Activation cytokines can be classified into Th1 and Th2 subgroups according to the T helper cell subtype to which they are associated during adaptive immunity [152]. Whilst these cytokines may also be produced by other T-cells, leukocytes, and other unrelated cells following *A. fumigatus* infection, they play critical roles in the innate immune armoury against the fungus [126].

Resistance to *A. fumigatus* infection is associated with high levels of Th1 cytokines including IFN-γ, IL-2, IL-12, and TNF-α whilst disease progression has been associated with Th2 cytokines IL-4 and IL-10 [153, 154]. Protective Th1 mechanisms include the induction of fungal killing abilities of particular immune effector cells; for example, IFN-γ and GM-CSF enhance ROS production in monocytes, bronchoalveolar macrophages, and neutrophils. IFN-γ alone has direct anti-*A. fumigatus* effects and increases the expression of chemokines such as CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC. This in turn further mediates recruitment to the infected site [145, 155–161].

Th2-associated cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 all inhibit a variety of innate host defense strategies and therefore contribute to poorer outcomes during IPA. The protective Th1-associated response is suppressed by IL-4 and IL-10 in IPA by the downregulation of IL-12 and IFN-γ. Simultaneous production of Th2 cytokines is concurrently promoted including IL-4, IL-5, and IL-10 in this setting [154, 162]. Our group and others have shown that elevated Th2 cytokine levels correlate with *A. fumigatus* positivity in people with CF (IL-5 and IL-13) and the development of IPA with particularly unfavorable outcomes in immunocompromised patients (IL-10) [70, 163]. In addition, Th2-associated cytokines suppress ROS production and hyphal damage of the fungi [164]. By corollary, IL-4- and IL-10-deficient mice show lower fungal burdens and increased survival rates compared to wild-type counterparts in the murine model of IPA [154, 165].

7. Modulating the Immune and Inflammatory Response for Therapeutic Purpose

While the treatment of *Aspergillus*-associated pulmonary disease has been established and is in the main effective, instances do arise where the side effects of such treatments become too great or they lack efficacy posing an enhanced clinical challenge. Chronic steroid use in cases of resistant ABPA is undesirable and the increasing challenge of fungal resistance to antimycotic therapies is becoming globally recognized. This results in the need to consider alternative approaches to management and promote their development in the forthcoming decades. One viable option may be to use our understanding of the innate and inflammatory responses *in vivo* to *A. fumigatus* to develop therapies that augment its killing and elimination and consequently prevent negative sequelae.

Improving the efficiency of mucociliary clearance, the major anatomical barrier to our fungal defence while general in approach may in fact be very effective in preventing the onset of *A. fumigatus*-associated disease by the removal of the inhaled fungi before it can cause disease. It may only be appropriate to use mucolytics such as DNAase and/or hypertonic saline in particular settings of chronic disease such as CF or advanced idiopathic bronchiectasis, and hence such an approach has to be individually tailored rather than routinely prescribed.

The augmentation of fungal killing through the immunological approach is attractive and circumvents the problems created by resistance and the overuse of antifungal therapies such as azoles. Immune cells such as neutrophils, monocytes, and macrophages all play critical roles in the killing of *A. fumigatus,* and this is in part executed by recognition via PRRs and release of AMPs. The development of synthetic antibodies against surface components of the fungi may in fact promote phagocytosis through their action as "synthetic opsonins." Potential approaches include pentraxin 3 analogues that recognize galactomannan or the enhancement of SP-D that recognizes β1,6 glucan on *A. fumigatus*. The latter can be achieved by the administration of cAMP analogs or more recently phosphodiesterase inhibitors such as Roflumilast although formal clinical work needs to evaluate its effects in the fungal setting [166]. The administration of recombinant human MBL (rhMBL) also remains a possibility owing to its role in activation of the lectin complement pathway; however, no work to date has been performed in the context of *A. fumigatus*-associated pulmonary disease. Although cell surface PRRs such as the TLRs 2, 4, and 9 have been implicated in the response to *A. fumigatus* in the airway, their roles and PAMPs to which they respond have not been fully established limiting the attractiveness of developing therapy on their basis.

A major potential route of treatment against *A. fumigatus* in the development pipeline is use of synthetic compounds

developed from naturally occurring AMPs [167]. Such AMP-like molecules would enhance the disruption of the fungal membrane similar to that caused by the defensins. Additionally, previous work from our group has shown that LL-37 undergoes complexation with glycosaminoglycans in cystic fibrosis lungs subsequently limiting its killing activity. This however importantly can be restored by the use of hypertonic saline [168]. This concept is a crucial one when considering fungi particularly in the context of a heavy protease burden such as that encountered in the CF lung. The use of recombinant SLPI (rSLPI) can also be considered in the *A. fumigatus* context; however, several pharmacological challenges do exist. One advance has been its theoretical delivery in a liposomal carrier for effective inhalation during *in vitro* experimentation [169].

Probably the most promising avenue for the development of therapeutics against *A. fumigatus* may be in augmenting and manipulating the cytokine response that ensues following exposure. It is important for clinicians to be aware that patients on TNF-α inhibitor therapy are at increased risk of aspergillosis due to the critical role that TNF-α plays in innate protection against *A. fumigatus*. Consequently, low thresholds should be maintained for the use of anti-fungal agents in the treatment of sepsis in these settings. While augmenting the cytokine response in the setting of *A. fumigatus* exposure may be beneficial, this must be performed in a controlled fashion as an exuberant cytokine storm will do more harm than good. Use of protease inhibitors such as $\alpha_1$antitrypsin can be considered. They are however mainly restricted to pulmonary conditions with a heavy protease burden such as CF or advanced idiopathic bronchiectasis. Dampening the Th2 response is possibly a more global approach and may be considered directly through the development of agents that inhibit the major cytokines IL-5, IL-10 and IL-13; however, an alternative indirect approach may be the use of anti-fungal agents such as itraconazole which decreases IL-5 and IL-13 in the *A. fumigatus* colonized setting in CF [70]. Furthermore, adjuvant therapy using IFN- γ and GM CSF in addition to antifungals has shown a marked clinical improvement of pulmonary aspergillosis in immunocompetent patients with a concomitant increase in the Th1 cytokine response and decrease in IL-4 production [170].

While our understanding of the consequences and responses of *A. fumigatus*-associated lung disease has exponentially developed over the last two decades, our difficulties in making a diagnosis and the limited available treatment options continue to present major clinical challenges. With the global increase in anti-fungal resistance, there exists a need for further work and particularly well designed clinical trials in this area. Perhaps the next generation of therapy will be focused on the manipulation of the immune and inflammatory response that has innately existed for decades but whose potential is yet uncovered within this complex field.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

CTLA-4 Expression and Polymorphisms in Lung Tissue of Patients with Diagnosed Non-Small-Cell Lung Cancer

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Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a potent immunoregulatory molecule that downregulates T-cell activation and thus influences the antitumor immune response. CTLA-4 polymorphisms are associated with various cancers, and CTLA-4 mRNA/protein increased expression is found in several tumor types. However, most of the studies are based on peripheral blood mononuclear cells, and much less is known about the relationship between CTLA-4 expression, especially gene expression, and its polymorphic variants in cancer tissue. In our study we assessed the distribution of CTLA-4 two polymorphisms (+49A/G and −318C/T), using TaqMan probes (rs231775 and rs5742909, resp.), and *CTLA-4* gene expression in real-time PCR assay in non-small-cell lung cancer (NSCLC) tissue samples. The increased *CTLA-4* expression was observed in the majority of NSCLC patients, and it was significantly correlated with TT genotype (−318C/T) and with tumor size (T2 versus T3 + T4). The presence of G allele and GG genotype in cancer tissue (+49A/G) was significantly associated with the increased NSCLC risk. Additionally, we compared genotype distributions in the corresponding tumor and blood samples and found statistically significant differences. The shift from one genotype in the blood to another in the tumor may confirm the complexity of gene functionality in cancer tissue.

1. Introduction

The incidence and development of cancer are closely related to dysfunction of immune function. Clinical data indicate an increased risk for tumor development in individuals who are immunesuppressed [1], and, on the other hand, the improved overall and progression-free survival associated with dense intratumoral lymphocyte infiltration of lesions [2]. Human can initiate immune response towards tumors by means of several different mechanisms. Among them the most well documented is cytotoxicity of T cells. As cancer cells have developed multiple mechanisms to evade the immune response [3] proper function of cytotoxic T lymphocytes (CTLs) is critical for immunosurveillance. CTLs after activation are able to recognize and kill autologous cancer cells. The first activating signal is delivered by the tumor-associated antigens (TAAs) displayed by major histocompatibility complex (MHC) class I molecules on antigen-presenting cells (APCs) [4, 5], and it provides specificity to the response. The second, so-called the "costimulatory signal", as it stimulates T cells in conjunction with antigen, is provided by molecules on APCs that engage particular costimulatory receptors on T cells. The best known is the CD28 receptor, which binds to two costimulatory molecules, B7-1 (CD80)

and B7-2 (CD86) [6]. The B7-CD28 interactions stimulate T-cell proliferation, differentiation, and survival.

However, in patients with cancer, the weaken immune response is observed. In this regard cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is of significant importance. This molecule is a homodimeric glycoprotein receptor on CTLs and CD28 homologue that also binds to B7-1 and B7-2, expressed after T-cell activation [6]. By inhibiting interleukin-2 production CTLA-4 blocks cell-cycle progression, leading to induction and maintenance of T-cell tolerance. Under physiological conditions, it reduces T-cell response to foreign antigens as well as to autoantigens. In the presence of the tumor's microenvironment, the CTLA-4 molecule is upregulated on the T cells with the help of TGF-beta, a suppressive cytokine secreted by the tumor cells. It has been hypothesized that during the early stage of tumorigenesis, CTLA-4 may elevate the T-cell activation threshold, thereby attenuating the antitumor response and increasing cancer susceptibility [7]. Additionally, the results of several studies suggest that CTLA-4 molecule might be involved in the control of other functions, not only in the inactivation of T-cell response. CTLA-4 mRNA or/and protein expression has been observed on different types of non-T cells, for example, placental fibroblasts, cultured muscle cells, monocytes, and, moreover, neoplastic cells, including leukemic and solid tumor-derived cells [8].

The *CTLA-4* gene is located on chromosome 2q33. The function of gene can be influenced by number of genetic variations, including single nucleotide polymorphisms (SNPs), and resulting in disease phenotypes. Indeed, as evidenced in our previous study and in numerous others, several *CTLA-4* polymorphisms that may influence gene expression, lead to amino acid substitution, and alter mRNA splicing have been linked with susceptibility to autoimmune diseases, including autoimmune thyroid diseases, systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes [9–15]. Thus, it is hypothesized that in cancer disease antitumor responses such as proliferation and activation of tumor-specific CTLs may also be altered by genetic polymorphisms in *CTLA-4* gene. However, despite the large number of studies of *CTLA-4* SNPs in autoimmune diseases, so far there is still little published data regarding human cancers [16–18]. Although the most recently published meta-analysis proved the association between *CTLA-4* SNPs and the risk of multiple cancer it included only two reports on lung cancer [19].

Lung cancer is a leading cause of cancer-related death worldwide, and the morbidity and the mortality rates are similar, which is a huge clinical problem [20]. The prognosis in lung cancer is poor and limited by the difficulties of diagnosis at early stage of the disease. Therefore, the advance in identification of genetic factors that influence the susceptibility to lung cancer, especially to non-small-cell lung cancer (NSCLC), which is the most frequent lung cancer type, is of great value.

The aim of our study was to evaluate the level of *CTLA-4* mRNA expression in lung cancer tissue and to assess its relationship with two chosen *CTLA-4* polymorphisms, affecting the leader sequence of CTLA-4 protein (+49A/G) and the promoter (−318C/T) of the gene. Additionally, we were interested in the question of whether the distribution of *CTLA-4* genotypes was the same in the blood and the corresponding cancer tissue. We also focused on the associations between *CTLA-4* expression and gene polymorphic variants and NSCLC patients characteristics, as well as tumor clinical staging.

Table 1: Tumor characteristics.

Cancer staging system	n (%)	NSCC		
		SCC $n = 41$ (58%)	AC $n = 23$ (32%)	LCC $n = 7$ (10%)
TNM				
T1	19 (26.76%)	8	11	0
T2	33 (46.48%)	18	11	4
T3-T4	19 (26.76%)	15	1	3
AJCC				
IA	13 (18.31%)	7	6	0
IB	14 (19.72%)	4	9	1
IIA	16 (22.53%)	12	3	1
IIB	8 (11.27%)	6	0	2
IIIA/IIIB	20 (28.17%)	12	5	3

AC: adenocarcinoma; LCC: large cell carcinoma; NSCC: nonsquamous cell carcinoma; SCC: squamous cell carcinoma.

2. Material and Methods

The study has been approved by the Ethical Committee of the Medical University of Lodz, Poland.

2.1. Patients Characteristics. Seventy-one patients with diagnosed NSCLC (25 women, mean age 63 ± 8.717, range 47–79 and 46 men, mean age 65 ± 8.234, range 47–87) have been enrolled into the study. They underwent lobectomy at the Department of Chest Surgery, General and Oncological Surgery University Hospital No. 2, Medical University of Lodz. During the surgery, tumor tissue samples (100–150 mg) were obtained, collected in lysis buffer (Buffer RNAlater, Qiagen Sciences, USA), and frozen at −70°C until use.

According to the pathomorphological reports, the study material included adenocarcinoma (AC, $n = 23$), squamous cell carcinoma (SCC, $n = 41$), and large cell carcinoma (LCC, $n = 7$). Table 1 shows the studied tumor samples characteristics according to TNM (tumor, node, and metastasis) and AJCC (American Joint Committee on Cancer) staging systems.

Regarding the smoking history, 90% of the NSCLC patients ($n = 64$) were smokers. They were divided into two groups: one with smoking level ≤30 pack-years ($n = 26$) and the other with smoking level >30 pack-years ($n = 38$).

Control group consisted of 104 healthy persons without lung cancer in the history. Peripheral blood samples (5 mL) were collected on EDTA and frozen at −70°C until use.

2.2. Relative Expression. Total RNA was isolated from biological material (cancer tissue obtained from the center of lung lesion and macroscopically unchanged lung tissue obtained from the most distant site from the resected lesion), using Universal RNA Purification Kit (Eurix, Poland), according to the manufacturer's procedure. The quality and quantity assessments of total RNA samples were determined by minielectrophoreses in polyacrylamide gel (Agilent 2100 Bioanalyzer, Agilent, USA), using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies, USA). Reverse transcription reaction was performed using 100 ng of total RNA, in a total volume of 20 μL, using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), in the following conditions: 10 minutes at 25°C, followed by 120 minutes at 37°C; then the samples were heated to 85°C for 5 seconds and hold at 4°C. Gene relative expression was assessed using TaqMan probes for the studied *CTLA-4* gene and the reference gene β-actin (Hs00175480_m1 and Hs99999903_m1, resp.; TaqMan Gene Expression Assays, Applied Biosystems) in 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). PCR reactions were carried out for 40 cycles with annealing temperature of 60°C. The reaction was repeated 3 times. The expression level (RQ value) was calculated based on the delta-delta C_T method adjusted to β-actin expression, for each sample. Macroscopically unchanged lung tissue served as calibrator sample.

2.3. SNP Genotyping. Total genomic DNA was isolated from lung tissue and peripheral blood samples of NSCLC patients as well as peripheral blood samples of healthy controls using Qiagen QIAamp DNA Mini Kit (Qiagen Sciences, USA), according to the manufacturer's protocol. *CTLA-4* gene polymorphisms were examined using TaqMan Universal PCR Master Mix, DNA (20 ng/μL) and TaqMan probes (rs231775 and rs5742909) in 7900HT Fast Real-Time PCR System using TaqMan 5′ allelic discrimination assay (Applied Biosystems, USA).

2.4. Statistical Analysis. For statistical analysis, STATA version 10 (State College, TX, USA) was used. Compliance with Hardy-Weinberg equilibrium (HWE) was assessed using the chi-square test (χ^2) and Fisher's test. Odd ratios (OR) and its 95% confidence intervals (95% CI) were calculated as an assessment of the strength and direction of association between the studied individual genotypes and susceptibility to NSCLC. Kruskal-Wallis or Mann-Whitney U tests were used to assess the associations between gene polymorphic variants as well as expression levels and NSCLC histological subtypes, tumor staging (TNM and AJCC systems), patient age, gender, and smoking history. To compare allele/genotype distributions between the tumor tissue and blood samples χ^2 test was used. Linkage disequilibrium (LD) in the two studied polymorphic sites was evaluated using Fisher's exact test. In all tests P value below 0.05 ($P < 0.05$) was considered as statistically significant.

3. Results

3.1. CTLA-4 Expression. The obtained RQ values, calculated based on the delta-delta C_T method, adjusted to the β-actin expression, and relative to the expression level of calibrator (for which RQ = 1), were compared among NSCLC patients in regard to histopathological NSCLC subtypes, tumor staging (TNM, AJCC), patients' age, gender, and smoking history, as well as to *CTLA-4* polymorphic variants. Generally, *CTLA-4* expression was increased in the majority of NSCLC patients (74.65%), with the mean RQ value of 6.91. Table 2 specifies the results of relative expression analysis, indicating mean RQ values, as well as number of samples with the increased (RQ value >1) and decreased (RQ value <1) *CTLA-4* expression in comparison with calibrator.

Statistical analysis revealed significant association between the increased gene expression level and tumor staging according to TNM staging: in T2 tumors *CTLA-4* expression was significantly higher than in T3 + T4 group ($P = 0.007$, Newman-Keuls test). There were no statistically significant differences in gene expression in T1 tumors versus T2 tumors ($P = 0.12$) and versus T3 + T4 group ($P = 0.50$). Regarding the studied polymorphisms, the significantly increased expression of *CTLA-4* was found for *CTLA-4* −318C/T SNP, namely, for TT genotype as compared with CC genotype ($P = 0.008$, Newman-Keuls test). There were no statistically significant associations between other −318C/T genotypes (CT versus TT, $P = 0.84$; CT versus CC, $P = 0.80$) and between +49A/G genotypes and *CTLA-4* expression levels ($P = 0.53$).

There was no statistically significant differences ($P > 0.05$) for the other variables studied, that is, histopathological subtypes (SCC versus AC versus LCC; SCC versus NSCC), AJCC staging, patients' age, gender, and the history of smoking.

3.2. CTLA-4 +49A/G and −318C/T Polymorphisms

3.2.1. NSCLC Tissue. The studied polymorphisms were in Hardy-Weinberg equilibrium both in the NSCLC patients and in the controls.

Statistical analysis confirmed the presence of significant differences between the studied groups (NSCLC versus control) concerning the distribution of *CTLA-4* +49A/G genotypes (AA, AG, and GG) and the individual alleles. The obtained results summarized in Table 3 indicate that the presence of G allele and GG genotype may lead to the increased NSCLC risk, while the presence of A allele and AA genotype may reduce the risk of lung cancer development.

Regarding −318C/T polymorphism, there were no statistically significant differences in genotypic or allelic distributions between NSCLC patients and the control group (see Table 3).

We did not reveal any statistically significant associations ($P > 0.05$) between the studied *CTLA-4* polymorphisms and patients' gender, age, smoking history, individual NSCLC subtypes, TNM, and AJCC classification.

3.2.2. Peripheral Blood Mononuclear Cells. The studied polymorphisms were in Hardy-Weinberg equilibrium both in the NSCLC patients and in the controls. The results of statistical analysis concerning the distribution of the studied genotypes in PBMCs are summarized in Table 4.

Table 2: RQ values, reflecting the relative expression levels of *CTLA-4*, in NSCLC subtypes and according to the tumor size.

Histopathological NSCLC subtype	Mean RQ value ± SEM	RQ value >1 *n* (%)	RQ value <1 *n* (%)
SCC ($n = 41$)	8.34 ± 16.86	32 (78%)	9 (22%)
AC ($n = 23$)	5.47 ± 9.92	15 (65.22%)	8 (34.78%)
LCC ($n = 7$)	6.93 ± 1.23	6 (85.71%)	1 (14.29%)
Tumor size (TNM system)			
T1 ($n = 19$)	8.29 ± 7.56	13 (68%)	6 (32%)
T2 ($n = 33$)	14.16 ± 18.24	24 (73%)	9 (27%)
T3 + T4 ($n = 19$)	2.13 ± 14.37	11 (58%)	8 (42%)
Total ($n = 71$)	6.91 ± 17.79	53 (74.65%)	18 (25.35%)

Table 3: *CTLA-4* +49 A/G and −318 C/T genotype and allele distribution in tumor tissue of NSCLC patients in comparison with the control group.

CTLA-4 genotypes and alleles	NSCLC patients $n = 71$		Healthy controls $n = 104$		OR (CI 95%)	*P* value
	Number	Frequency	Number	Frequency		
+49 AA	19	0.27	49	0.47	0.41 (0.21–0.78)	0.02
+49 AG	25	0.35	33	0.32	1.16 (0.62–2.21)	0.74
+49 GG	27	0.38	22	0.21	2.23 (1.17–4.48)	0.015
	$\chi^2 = 5.83$ $P = 0.63$		$\chi^2 = 2.41$ $P = 0.83$			
+49 A allele	63	0.44	131	0.63	0.47 (0.30–0.72)	0.028
+49 G allele	79	0.56	77	0.37	2.13 (1.38–3.29)	0.028
−318 CC	24	0.33	36	0.34	0.96 (0.91–1.82)	0.15
−318 CT	19	0.27	37	0.36	0.66 (0.34–1.28)	0.97
−318 TT	28	0.40	31	0.30	1.53 (0.81–2.89)	0.41
	$\chi^2 = 3.42$ $P = 0.37$		$\chi^2 = 8.55$ $P = 0.42$			
−318 C allele	67	0.47	109	0.60	0.81 (0.53–1.24)	0.32
−318 T allele	75	0.53	99	0.40	1.23 (1.80–1.89)	0.32

Table 4: *CTLA-4* +49 A/G and −318 C/T genotype and allele distribution in blood samples of NSCLC patients in comparison with the control group.

CTLA-4 genotypes and alleles	NSCLC patients $n = 71$		Healthy controls $n = 104$		OR (CI 95%)	*P* value
	Number	Frequency	Number	Frequency		
+49 AA	56	0.80	49	0.47	4.19 (2.11–8.33)	0.58
+49 AG	13	0.18	33	0.32	0.48 (0.23–1.02)	0.23
+49 GG	2	0.02	22	0.21	0.11 (0.02–0.48)	0.03
	$\chi^2 = 1.23$ $P = 0.73$		$\chi^2 = 3.26$ $P = 0.37$			
+49 A allele	63	0.88	131	0.63	4.32 (2.42–7.72)	0.47
+49 G allele	79	0.12	77	0.37	0.23 (0.13–0.41)	0.47
−318 CC	59	0.83	36	0.34	9.29 (0.42–9.48)	0.76
−318 CT	10	0.14	37	0.36	0.30 (0.14–2.65)	0.57
−318 TT	2	0.03	31	0.30	0.10 (0.02–0.30)	0.02
	$\chi^2 = 2.67$ $P = 0.25$		$\chi^2 = 4.65$ $P = 0.35$			
−318 C allele	128	0.90	109	0.60	8.30 (0.5–15.36)	0.23
−318 T allele	14	0.10	99	0.40	0.12 (0.65–2.22)	0.23

Table 5: *CTLA-4* SNP shift between blood and tumor tissue.

+49 *CTLA-4* SNP genotype shift (n = 47; 72.31%)			
Genotype shift	AA → AG	AG → GG	GG → AA
No. of patients (%)	24 (51.06)	13 (27.66)	10 (21.28)
−318 *CTLA-4* SNP genotype shift (n = 41, 63.10%)			
Genotype shift	CC → CT	CT → TT	CC → TT
No. of patients (%)	17 (41.46)	10 (24.39)	14 (34.15)

No statistical significances were found ($P > 0.05$) regarding the association between the studied *CTLA-4* polymorphisms in PBMCs and patients' characteristics (age, gender, smoking history), NSCLC subtypes (SCC, AC, and LCC) and tumor staging (TNM and AJCC systems).

3.3. CTLA-4 Genotype Distributions in NSCLC Tissue versus Blood Samples. Comparing the results of *CTLA-4* SNP genotyping in the tumor tissue and the corresponding blood sample, a shift from one genotype to another in more than 60% of patients was observed, for both studied *CTLA-4* polymorphisms. Statistically significant difference was found in case of +49 *CTLA-4* SNP ($P = 0.01$, χ^2 test) when comparing the samples with and without genotype shift, while in case of −318 *CTLA-4* SNP it did not reach the statistical significance ($P = 0.08$, χ^2 test). Regarding the individual genotypes, statistically significant differences were found for +49 AA → AG shift ($P = 0.011$, χ^2 test) and −318 CC → CT shift ($P = 0.017$, χ^2 test). Table 5 summarizes the differences between the distribution of the studied genotypes in blood and paired tissues in NSCLC patients.

3.4. Linkage Disequilibrium Analysis. No linkage disequilibrium (LD) was found between the two studied polymorphic sites ($P > 0.05$, Fisher's exact test).

4. Discussion

It is widely accepted that *CTLA-4* plays a crucial role in the maintenance of the immune response by its expression on activated and regulatory T cells. Accordingly, it is speculated that extraordinary *CTLA-4* expression could be associated with enhanced susceptibility to tumor growth and/or progression, attenuating the antitumor immune response. And although the significance of CTLA-4 molecule in antitumor immunity has been well recognized, proving its role in T-cell down regulation [21, 22], its expression in a non-immunogenic context has not been well studied.

4.1. CTLA-4 Expression in Cancer Tissue. There are only a few publications that focused on this subject, and they confirm the expression of *CTLA-4* mRNA and/or protein on tumor cells [23–25] and tumor-derived cell lines, although Contardi et al. [8] found no correlation with tissue origin. Recently, CTLA-4 protein expression has been reported to be significantly higher in primary lung cancer samples and precancerous lesions than in normal lung tissue [24].

The aim of our work was to assess the level of *CTLA-4* mRNA expression in lung cancer tissue, and we confirmed the increased gene expression in nearly 75% of NSCLC samples. To our knowledge, it is the first such study performed on mRNA level using real-time PCR method. The other available studies performed in lung cancer cells focused on CTLA-4 protein expression [24, 26, 27]. Among them, the report of Salvi et al. [26] was the first one suggesting a prognostic role of CTLA-4 expression in NSCLC.

In our analysis we observed statistically significant association among the increased *CTLA-4* mRNA expression and tumor size. It was significantly higher in T2 group as compared to the group combining T3 and T4 samples. Also, the findings of others [26] indicate a tendency of favourable independent influence of CTLA-4 protein overexpression on patients' overall survival with better prognosis in those with higher CTLA-4 expression. The study of Contardi et al. [8] undoubtedly indicated that CTLA-4 is able to specifically transduce an apoptotic signals into tumor cells, comparable with the ones observed in T cells. In this way, CTLA-4 molecule as a negative regulator of tumor cell proliferation might influence the clinical outcome of patients with cancer. Our results indicating *CTLA-4* overexpression in T2 versus T3 and T4 tumors could support in a sense this hypothesis.

The levels of *CTLA-4* expression in our study did not differ significantly between the individual NSCLC histopathological subtypes. Similar results, although on protein level, were obtained by Zheng et al. [24], but other study revealed higher CTLA-4 immunoexpression level in nonsquamous as compared to squamous histological type of NSCLC [26]. In our study, we did not observe any differences in *CTLA-4* mRNA expression in NSCC versus SCC.

We also did not find any associations between *CTLA-4* mRNA expression and patients' age and gender. Generally, it is in accordance with the results of others [24, 26] with the exception for patients' age that was closely related with the positive CTLA-4 protein expression in the study of Zheng et al. [24].

Summarizing the previous considerations, it should be emphasized that it is difficult to compare the results of CTLA-4 expression obtained on mRNA and protein level, having regard to the posttranscriptional or posttranslational modifications that could take place.

4.2. Functional Significance of CTLA-4 Polymorphisms. The altered expression of *CTLA-4* may be influenced by genetic variations in the gene, including single nucleotide polymorphism. There are numerous reports assessing the association between *CTLA-4* genotypes and cancer risk: +49A/G SNP

has been found in breast, esophageal, gastric and colorectal cancers, oral squamous cell carcinoma, and osteosarcoma [17, 27–31], while −318C/T SNP, although less frequently analyzed, has also revealed associations with breast, cervical, gastric and colorectal cancers, and B-cell chronic lymphocytic leukemia [29, 32–35]. Polymorphisms in *CTLA-4* are regarded as genetic susceptibility factors; however, there are reports that could not establish any associations between different *CTLA-4* SNPs and several types of cancers [29, 36].

4.2.1. CTLA-4 +49A/G Polymorphism. The available data analyzing the functionality of *CTLA-4* SNPs are limited. Polymorphism at position +49A/G is associated with adenine (A) to guanine (G) transition within exon 1 of *CTLA-4* gene coding the leader sequence of the CTLA-4 protein, and resulting in (17)Thr to (17)Ala amino acid substitution. This functionally affects T-cell activation: CTLA-4-(17)Ala homozygotes (+49GG genotype) express one-third less CTLA-4 protein on the surface of their T cells than CTLA-4-(17)Thr homozygotes (+49AA genotype) [37], thus exerting less profound inhibitory effect on T-cell proliferation [38]. This could be associated with better antitumor immune response and decreased cancer risk. In our study group only two patients had +49GG genotype in PBMCs, and it is too small to regard the results of OR analysis as reliable. On the other hand, we did not observe any significant association between +49AA genotypes—constituting the majority in the studied group—and increased NSCLC risk. Another study conducted in Polish population revealed a trend toward overrepresentation of A allele carriers (AA, AG) among patients as compared with controls, however, the statistical significance was reached only in women [16]. The studies performed in Asian population established +49AA genotype as an independent adverse prognostic factor in NSCLC patients [27, 39]. However, population-based differences cannot be excluded.

In our study, lack of association between +49 *CTLA-4* SNP and gene expression may indicate the significance of posttranscriptional or posttranslational modifications, with similar amounts of starting mRNA irrespective of the genotype. The results of several studies indicate that the polymorphism in *CTLA-4* exon 1 may influence the rates of endocytosis or surface trafficking, glycosylation of CTLA-4 or lower capability to bind B7.1 [27, 37, 38], resulting in lower CTLA-4 expression on the surface of T cells and thereby forming the mechanism responsible for weaker inhibitory effect of GG genotype on T-cell activation.

Interestingly, a genotype shift observed in our study between the blood and the corresponding tumor samples revealed the presence of G allele in the majority of tissue samples (73.24%). The differences regarding genotype distribution in PBMCs and tumor specimens were statistically significant. Indeed, there are reports describing a shift from one genotype in the blood to another genotype in the tissue and proving its role as a prognostic factor, as in ovarian cancer patients [40]. To our knowledge, the result of our study revealing the genotype shift in blood versus NSCLC tissue is the first one. Moreover, the genotypes found in our tumor samples were significantly associated with NSCLC risk.

4.2.2. CTLA-4 −318C/T Polymorphism. The other studied polymorphism, −318C/T, is associated with cytosine- (C-) thymine (T) single-base substitution within the promoter sequence and is likely to affect the gene expression. Indeed, it has been reported that the presence of −318T allele is associated with higher promoter activity [41–43] and significantly increased expression of mRNA and surface CTLA-4 [44], enabling more potent inhibition of T-cells activation. This may eventually lead to the decreased antitumor immunity and to cancer susceptibility.

In fact, our analysis of *CTLA-4* expression revealed the significantly higher mRNA expression level in the lung cancer tissue samples carrying −318TT genotype. This confirms the functionality of the T allele substitution in the place of the C allele in the promoter of *CTLA-4*.

In our study we could not establish any significant association between −318C/T SNP and NSCLC risk, neither in tumor tissue nor in blood. However, in another study involving Polish patients, statistical significance for T allele and T-allele carrying genotypes (TT and CT) was obtained [16], although observed only for NSCLC female patients. The results of meta-analysis involving several types of cancer and including lung cancer [19] revealed a significantly increased risk of cancer in Europeans carrying T allele (TT and TC genotypes) but not in Asians or Americans. Indeed, the studies performed in Asian populations found no association between polymorphic variants in the promoter of *CTLA-4* gene and NSCLC [27, 45].

Considering the association between the studied *CTLA-4* polymorphisms and clinicopathological features of NSCLC, including histopathological type of cancer, tumor stage, and patients' gender, we did not observe any significant differences. Similar findings were obtained by others [39, 45] with the exception of one study [16] regarding patients' gender (women) and +49 *CTLA-4* SNP.

5. Conclusions

The presented study carried out in NSCLC tissue and blood samples, combining the CTLA-4 expression analysis on mRNA level with gene polymorphic variants, seems valuable as it expands a small amount of data available in lung cancer patients. It should be noted that till now only four studies investigating *CTLA-4* polymorphisms in NSCLC have been published [16, 27, 38, 39]. In our study we showed allelic and genotypic distribution of +49 and −318 *CTLA-4* SNPs in a group of NSCLC patients of Caucasian origin and revealed *CTLA-4* genotype distribution shift from one genotype in the blood to another genotype in the tumor. This phenomenon may reflect the complexity of gene functionality in cancer tissue. Our results, indicating the correlation between the C/T substitution in the promoter of the gene and its increased expression, and the observed significant association between the gene expression and the tumor size might provide additional evidence confirming the proapoptotic effect of CTLA-4 on tumor cells. Additionally, the described association between GG genotype in tumor tissue and the increased risk of NSCLC development might confirm the weaker inhibitory effect of this genotype on cell proliferation.

Although the obtained results are promising, more investigations in the future in a larger group of NSCLC patients are needed. Particularly, if further research—focusing on the association between CTLA-4 SNPs and altered gene expression—could definitively confirm its role as a negative regulator of tumor cell proliferation.

Authors' Contribution

Adam Antczak and Dorota Pastuszak-Lewandoska have equally contributed to this work.

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Research Article

Interleukin-33 Drives Activation of Alveolar Macrophages and Airway Inflammation in a Mouse Model of Acute Exacerbation of Chronic Asthma

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We investigated the role of interleukin-33 (IL-33) in airway inflammation in an experimental model of an acute exacerbation of chronic asthma, which reproduces many of the features of the human disease. Systemically sensitized female BALB/c mice were challenged with a low mass concentration of aerosolized ovalbumin for 4 weeks to induce chronic asthmatic inflammation and then received a single moderate-level challenge to trigger acute airway inflammation simulating an asthmatic exacerbation. The inflammatory response and expression of cytokines and activation markers by alveolar macrophages (AM) were assessed, as was the effect of pretreatment with a neutralizing antibody to IL-33. Compared to chronically challenged mice, AM from an acute exacerbation exhibited significantly enhanced expression of markers of alternative activation, together with enhanced expression of proinflammatory cytokines and of cell surface proteins associated with antigen presentation. In parallel, there was markedly increased expression of both mRNA and immunoreactivity for IL-33 in the airways. Neutralization of IL-33 significantly decreased both airway inflammation and the expression of proinflammatory cytokines by AM. Collectively, these data indicate that in this model of an acute exacerbation of chronic asthma, IL-33 drives activation of AM and has an important role in the pathogenesis of airway inflammation.

1. Introduction

Asthma is one of the most common chronic diseases affecting children and young adults, especially in economically developed nations. Acute exacerbations of asthma account for a large fraction of the health care costs and morbidity of this illness [1]. Most childhood asthma is allergic, and children hospitalized for severe asthma exacerbations are typically markedly atopic [2]. Exacerbations are characterized by increased airway inflammation, which extends further distally [3] and is associated with recruitment of both eosinophils and significant numbers of neutrophils [4, 5]. In parallel, patients develop worsening airflow obstruction and its consequences, which may be difficult to manage and can be life threatening [6, 7]. Although usually triggered by viruses, exposure to high levels of allergens is synergistic in the induction of acute exacerbations [8, 9]. Furthermore, these factors appear to converge on a "final common pathway" in which the allergic inflammatory response, including bystander aeroallergens, may be enhanced as a consequence of the infection [10–12]. However, the cellular and molecular events underlying these changes remain incompletely defined.

To investigate pathogenetic mechanisms of asthmatic exacerbations, we have developed a model of acute-on-chronic asthmatic inflammation of the airways [13]. This is based on our well-characterized model of chronic asthma in BALB/c mice, which are systemically sensitized to ovalbumin (OVA) and repeatedly challenged with a low mass concentration ($\approx 3\,mg/m^3$) of aerosolized OVA for 4 weeks. These

challenges induce chronic inflammation of the airways, as well as changes of remodeling such as subepithelial fibrosis and goblet cell metaplasia [14]. Importantly, low-level challenge induces minimal parenchymal inflammation, so that lesions are confined to the conducting airways, and the model thus closely resembles mild chronic human asthma [15]. We then use a subsequent single moderate-level challenge ($\approx$30 mg/m^3) to trigger acute inflammatory changes simulating an allergen-induced acute exacerbation. The moderate-level challenge, which still employs a much lower mass concentration than in conventional animal models of allergic airway inflammation, triggers rapid and enhanced accumulation of eosinophils and neutrophils around intrapulmonary airways, similar to that seen in clinical acute exacerbations [13]. However, the overall severity of inflammation remains much less marked than in conventional short-term models. This has been recognized as a valid murine model that resembles acute asthma in patients more closely than conventional short-term experimental models [16].

Using this model, we have recently shown that the following the final moderate-level challenge, alveolar macrophages (AM) are activated to express enhanced levels of proinflammatory cytokines, including tumour necrosis factor- (TNF-) α, interleukin- (IL-) 1β, IL-6, and CXCL-1. Significantly, unlike naïve AM or AM from mice that received chronic challenge alone, activated AM from an acute exacerbation can stimulate Th2 cytokine secretion by primed CD4+ T cells, via a mechanism involving expression of CD80/86 costimulatory molecules by AM [17]. Thus, activated AM could contribute to the pathogenesis of an acute exacerbation of asthma.

In allergy, Th2 cytokines such as IL-4 and IL-13 drive the differentiation of macrophages towards a phenotype referred to as M2 (alternative) activation, which is associated with enhanced expression of characteristic markers, notably including arginase-1, the inflammation-associated protein FIZZ1 (also known as resistin-like α), the chemokine eotaxin-2 (CCL24), and the chitinase-like protein Ym1 [18–20]. In the present study, we have defined the phenotypic characteristics of activated AM in an experimental acute exacerbation. We have also investigated the role of interleukin-33 in the activation of macrophages in vivo, and its contribution to the development of airway inflammation. IL-33 is a novel member of the IL-1 family which is expressed by a variety of cell types, notably epithelial cells, and signals via the ST2 receptor, also known as IL-1 R4 [21, 22]. In vitro, polarization of macrophages towards an alternatively activated phenotype by treatment with IL-4 or IL-13 is amplified by IL-33, leading to enhanced expression of markers of alternative activation [23]. Whether similar activation occurs in vivo is unknown. Studies in a short-term challenge model in mice suggest that IL-33 may also be important in promoting asthmatic inflammation, because pretreatment with a polyclonal antibody to IL-33 suppressed the Th2-biased inflammatory response [24], and similar results have recently been reported in a multiple-challenge chronic model [25]. However, the role of IL-33 in airway inflammation in a model that simulates a clinical acute exacerbation of chronic asthma has not previously been studied.

Here, we show that induction of an acute exacerbation is associated with marked upregulation of the expression by AM of markers of alternative activation, as well as of proinflammatory cytokines and of cell surface proteins associated with antigen presentation to T cells; that expression of IL-33 in the airway wall is enhanced; and that IL-33 plays a key role both in the activation of AM and the development of airway inflammation.

2. Materials and Methods

2.1. Mice, Sensitization, and Challenge. The protocols we employed for sensitization and inhalational challenge have previously been described [13]. Briefly, specific pathogen free female BALB/c mice aged 7-8 weeks (Monash Animal Research Platform, Melbourne, Australia) were systemically sensitized by intraperitoneal injection of 50 μg of alum-precipitated chicken egg ovalbumin (OVA) (Grade V, $\geq$ 98% pure, Sigma, Australia) 21 and 7 days before inhalational challenge then exposed to aerosolized OVA in a whole body inhalation exposure chamber (Unifab Corporation, Kalamazoo, MI, USA) [14]. Chronic low-level challenge involved exposure to $\approx$3 mg/m^3 aerosolized OVA for 30 min/day on 3 days/week for 4 weeks. At the end of this period, a single moderate-level challenge ($\approx$30 mg/m^3) was used to induce an acute exacerbation. Particle concentration within the chamber was continuously monitored using a DustTrak 8520 instrument (TSI, St Paul, MN, USA). Experimental groups each comprised 6–8 animals. Control groups included sensitized mice that received chronic challenge with aerosolized OVA for 4 weeks but no additional single moderate-level challenge; sensitized mice that received a single moderate-level exposure to aerosolized OVA without prior chronic challenge; and naïve animals. All experimental procedures complied with all relevant legislation and codes of practice, and with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (Reference number 08/09B).

To assess the role of IL-33 in the development of an experimental acute exacerbation, mice received an intraperitoneal injection of 100 μg of either a blocking monoclonal antibody to IL-33 (clone 1F11, MBL, Nagano, Japan) [26] or a control monoclonal antibody to β-galactosidase (βGL-113) [27], at 30 minutes prior to the final moderate-level challenge (Figure 1).

2.2. Assessment of Inflammatory Response. At 4 hours after the final airway challenge, mice were euthanized by exsanguination following an overdose of sodium pentobarbital. This time point was selected on the basis of our earlier studies using this model [13, 17]. Bronchoalveolar lavage (BAL) fluid was collected for assessment of proinflammatory cytokines. Tissue accumulation of eosinophils was quantified using a colorimetric assay for eosinophil peroxidase, adapted from previously described methods [28]. Tissue accumulation of neutrophils was quantified by immunostaining in frozen sections, using rat anti-Gr-1 (RB6-8C5, BD Bioscience, Sydney, Australia) as previously described [29].

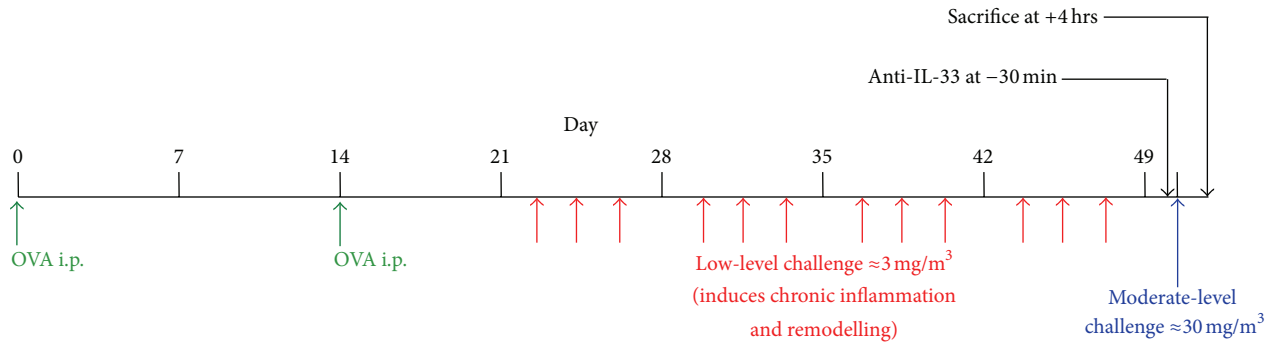

Figure 1: Model of an acute exacerbation of chronic asthma: timeline for sensitization, inhalational challenges, and antibody treatment.

2.3. RNA Isolation and PCR Analysis. To purify AM, BAL cells from individual animals were resuspended in RPMI-1640 and incubated at 37°C in 12- or 24-well plates for 30 min. Plates were washed at least 4 times to remove nonadherent cells, after which AM were lysed using TriReagent (Sigma) for extraction of RNA. Adherent cells were >90% AM by morphological criteria and immunostaining for F4/80, as previously demonstrated [17].

For assessment of IL-33 mRNA expression in the airways, proximal airway tissue was isolated by blunt dissection, using two pairs of forceps to separate lung parenchyma from the larger airways and leaving several generations of airway attached to the trachea [30]. Tissue was frozen in liquid nitrogen until RNA extraction was performed using TriReagent.

Extracted RNA samples were treated with DNase (Turbo DNase, Ambion, Scoresby, Australia) and reverse transcribed into cDNA using Superscript III (Invitrogen). Quantitative real-time PCR was used to assess expression of cytokines, with detection of amplified products using SYBR Green (BioLine, Tauton, MA, USA). Primers were custom-designed in house to allow the use of identical thermocycler conditions, thus permitting simultaneous assessment of multiple cytokines and activation markers. Reactions were performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Melbourne, Australia), and expression was normalized to HPRT.

2.4. Flow Cytometry. Expression of surface markers on AM was assessed by staining with saturating amounts of fluorochrome-conjugated antibody to the macrophage marker F4/80, in combination with fluorochrome-conjugated antibodies to the surface receptors/activation markers CD11b, CD11c, CD14, CD16, CD23, CD64, CD80, CD86, and MHCII (eBioscience, San Diego, CA, USA, and BD Bioscience). Nonspecific binding was blocked by incubation for 10 min in PBS containing 5% normal rat serum (Sigma) and 2 μg/mL unconjugated rat IgG2a and IgG2b (eBioscience). Staining was for 30 min at 4°C in the dark, after which cells were washed twice with PBS containing 1% BSA and fixed with 1% paraformaldehyde. Negative controls were cells incubated with the corresponding labeled and isotype-matched immunoglobulins.

A 4-channel FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to acquire fluorescence data. Compensation for each channel was determined using cells stained with single fluorochromes. FlowJo version 8.8.6 (Treestar Inc, Ashland, OR, USA) was used to analyze the percentage of positively stained cells and the mean fluorescence intensity (MFI).

Intracellular cytokine staining was assessed using a fluorochrome-conjugated antibody to TNF-α in combination with the surface markers F4/80 and MHCII. This was performed using the BD Cytofix/Cytoperm fixation/permeabilization kit according to the manufacturer's instructions, with GolgiStop (BD Bioscience) plus 5 μg/mL Brefeldin A (Sigma) to inhibit secretion.

2.5. Immunostaining for IL-33 Expression. Immunoperoxidase staining of formalin-fixed, paraffin-embedded sections of tracheas was performed as previously described [31] using an affinity-purified goat antibody to mouse IL-33 (R&D Systems, Minneapolis, MN, USA). Intensity of immunoreactivity was semiquantitatively scored as grade 0: no staining, grade 1: weak staining, grade 2: moderate staining, and grade 3: strong staining.

2.6. In Vitro Stimulation of Macrophages with IL-33. BAL cells were collected from naïve mice or from animals that had received repeated low-level challenge. Cells from pairs of mice were pooled, and 3.5×10^5 cells were dispensed into polyethylene tubes (Minisorp, Nunc, ThermoFisher Scientific, Australia) in 1 mL of RPMI-1640 containing 10% FCS. Cells were incubated in nonadherent culture for 4 hours either with or without 20 ng/mL of recombinant mouse IL-33 (eBiosciences, San Diego, CA, USA). AM were then purified by adherence as mentioned earlier and mRNA was extracted and reverse transcribed.

Confirmatory experiments were performed using MH-S cells, which are derived from BALB/c alveolar macrophages and retain morphological and functional characteristics of highly differentiated macrophages [32]. MH-S cells were similarly incubated in adherent culture overnight either with or without 20 ng/mL of IL-33.

2.7. Cytokine Assays. The concentration of proinflammatory cytokines in BAL fluid and in culture supernatants was assessed either using enzyme immunoassay kits (R&D Systems, Sydney, Australia) or using a multiplex immunoassay (Mouse 23-Plex panel, Biorad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

2.8. Statistical Analysis. Data are presented as arithmetic means ± SEM. In general, results were analyzed by a one-way ANOVA followed by a Newman-Keuls multiple comparison test. Where only two experimental groups were involved, an unpaired *t*-test was used. The software package GraphPad Prism 5.04 (GraphPad Software, San Diego, CA, USA) was used for all data analyses and preparation of graphs.

3. Results

3.1. Alternative Activation of AM in an Acute Exacerbation. Following induction of an experimental acute exacerbation of chronic asthma, expression by AM of mRNA for the alternative activation markers arginase-1, FIZZ1, eotaxin-2/CCL24, and Ym1 was markedly and significantly elevated (Figures 2(a)–2(d)). In contrast, AM from mice that received chronic low-level challenge alone, or a single moderate-level challenge alone, did not demonstrate significantly increased levels of expression of mRNA for alternative activation markers. There was no change in levels of expression of mRNA for mannose receptor, another marker of alternative activation, and expression of mRNA for other markers of macrophage activation (iNOS, IL-10, and IL-12) was virtually undetectable in all of the samples (data not shown).

3.2. Enhanced Expression of Cytokines by AM from an Acute Exacerbation. We have previously shown that AM from an acute exacerbation have significantly increased expression of mRNA for proinflammatory cytokines including IL-1β, TNF-α, and CXCL-1 [17]. We confirmed that AM with upregulated expression of markers of alternative activation also had enhanced expression of TNF-α by intracellular staining in F4/80+ macrophages. Compared to naïve animals, a significantly increased percentage of AM from an acute exacerbation exhibited positive staining for TNF-α (naïve = 6.18 ± 0.52 versus acute exacerbation = 11.45 ± 1.79, $P < 0.05$), and the relative MFI of staining was also increased (naïve = 9.04 ± 0.11 versus acute exacerbation = 10.41 ± 0.34, $P < 0.05$).

3.3. Enhanced Expression of Other Activation Markers by AM from an Acute Exacerbation. Flow cytometric analysis revealed that the relative to naïve animals, F4/80+ AM from an acute exacerbation exhibited significant increases in the proportions of cells that expressed molecules associated with antigen presentation, including MHCII, CD11b, CD11c, and CD86. In parallel, the relative MFI of staining for these markers was significantly increased (Table 1). There was also a significant increase in the proportion of F4/80+ AM simultaneously expressing CD11b, CD11c, and MHCII (naïve = 0.62 ± 0.03 versus acute exacerbation = 2.71 ± 0.34, $P < 0.001$) as well as the relative MFI for cells positive for all three markers (naïve = 633 ± 83.7 versus acute exacerbation = 1882 ± 152, $P < 0.001$).

Other noteworthy changes exhibited by F4/80+ AM included a decrease in both the percentage and relative MFI of cells expressing CD16, a decrease in the percentage of cells expressing CD64, an increase in the percentage of cells expressing the Fc receptor CD23, and an increase in the relative MFI for the LPS coreceptor CD14 (Table 1).

3.4. Upregulated Expression of IL-33 in an Acute Exacerbation. Blunt-dissected proximal airway tissue from mice that received chronic low-level challenge alone did not demonstrate significantly increased levels of expression of mRNA for IL-33. In contrast, levels of mRNA were elevated 7-8-fold in mice in which an experimental acute exacerbation had been induced, as well as in sensitized mice that received a single moderate-level challenge alone (Figure 3(a)). Immunostaining of sections of trachea demonstrated that tracheal epithelial cells in the acute exacerbation group exhibited significantly greater immunoreactivity than in either naïve animals or animals that received a single moderate-level challenge (Figure 3(b)). However, levels of IL-33 in BAL fluid were below the limits of detection in all experimental groups.

3.5. IL-33 Activation of Primed AM. AM from animals previously subjected to repeated inhalational challenge exhibited increased levels of expression of mRNA for Ym1, a marker of alternative activation, and this was significantly upregulated following incubation in vitro with IL-33 (naïve = 1.4 ± 0.5 versus naïve + IL-33 = 2.0 ± 0.7 versus chronic = 20.2 ± 6.5 versus chronic + IL-33 = 44.1 ± 13.5, $P < 0.05$ for chronic AM compared to chronic AM + IL-33). Arginase-1 and FIZZ1 were also increased in AM from challenged animals, but treatment with IL-33 in vitro did not further increase the expression of these markers (not shown).

3.6. IL-33 Activation of MH-S Cells. Because of limited availability of AM, we assessed the ability of IL-33 to directly stimulate cytokine production using alveolar macrophage-like MH-S cells. Following treatment with IL-33, these cells exhibited markedly upregulated levels of expression of mRNA for TNF-α, IL-1β, IL-6, CXCL-1, IL-12p40, and G-CSF (Table 2). Increased expression of cytokine mRNA was paralleled by increased protein concentrations in culture supernatants, as assessed by multiplex immunoassay (Table 2). In contrast, in cells which had not been pretreated with IL-4 and/or IL-13, there was no change in the levels of expression of the alternative activation markers arginase-1, FIZZ1, and Ym1 (not shown).

3.7. Effects of Inhibiting IL-33 on Airway Inflammation. Compared to treatment with the control antibody, treatment of animals with anti-IL-33 prior to induction of an acute exacerbation inhibited inflammation in the lung tissue associated with an acute exacerbation. There was significant inhibition of the recruitment of eosinophils into the lung tissue associated with an exacerbation, assessed using the assay for

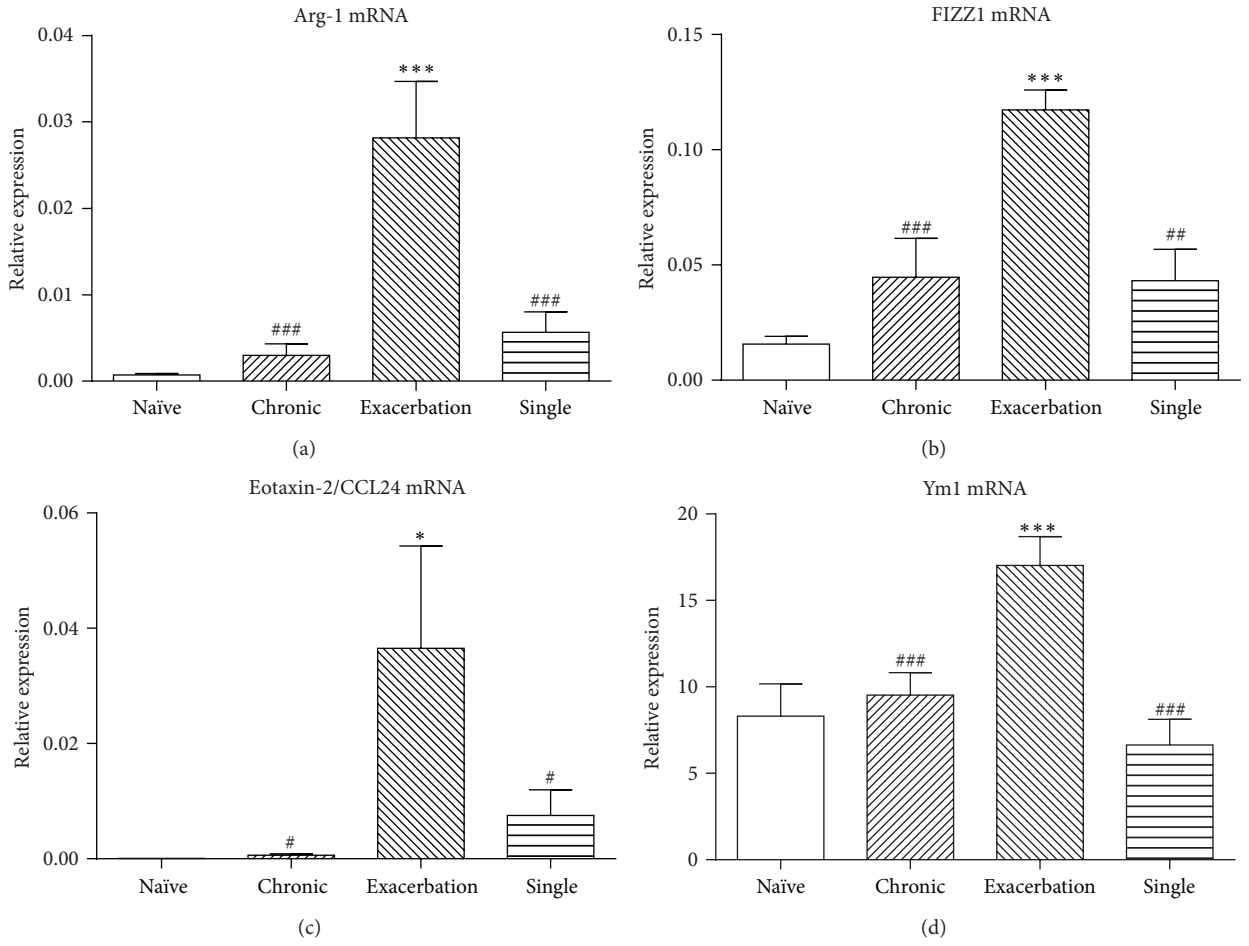

Figure 2: Expression of mRNA for markers of alternative activation, relative to HPRT, by AM from the acute exacerbation and control groups. (a) Arginase-1. (b) FIZZ1. (c) Eotaxin-2/CCL24. (d) Ym1. Data are mean ± SEM ($n = 6$ samples per group). Significant differences relative to the naïve group are as shown as $^{*}(P < 0.05)$ and $^{***}(P < 0.001)$ and those relative to the acute exacerbation group are shown as $^{\#}(P < 0.05)$, $^{\#\#}(P < 0.01)$, and $^{\#\#\#}(P < 0.001)$.

Table 1: Flow cytometric assessment of activation markers by AM.

Marker	Percentage of F4/80^{+} AM staining positive		Mean fluorescence intensity (relative units)	
	Naïve	Acute exacerbation	Naïve	Acute exacerbation
CD11b	3.43 ± 0.16	9.72 ± 1.62**	48.73 ± 5.27	95.53 ± 13.49**
CD11c	88.37 ± 1.06	92.05 ± 0.61*	81.97 ± 1.62	140.0 ± 4.93***
CD14	98.90 ± 0.12	98.17 ± 0.15	45.67 ± 0.68	190.8 ± 2.68***
CD16	98.18 ± 0.15	54.48 ± 1.70***	32.37 ± 0.82	19.97 ± 0.58***
CD23	4.31 ± 0.33	5.85 ± 0.14**	28.94 ± 0.31	28.38 ± 0.28
CD64	74.08 ± 1.74	62.77 ± 1.52**	15.93 ± 0.28	16.37 ± 0.37
CD80	81.83 ± 1.06	76.42 ± 0.58	17.12 ± 0.24	17.75 ± 0.17
CD86	61.35 ± 1.40	70.60 ± 1.92**	19.58 ± 0.21	29.83 ± 0.47***
MHCII	31.33 ± 1.62	55.93 ± 1.88***	171.8 ± 2.12	460.8 ± 22.34***

Data are mean ± SEM ($n = 6$ animals per group). Significant differences relative to naïve animals are shown as $^{*}(P < 0.05)$, $^{**}(P < 0.01)$, and $^{***}(P < 0.001)$.

eosinophil peroxidase activity ($P < 0.001$) (Figure 4(a)). There was also a significant decrease in the numbers of neutrophils in lung tissue following treatment with anti-IL-33 ($P < 0.05$) (Figure 4(b)). However, the blocking antibody had no effect on the increase in number of cells in BAL fluid, which was primarily the result of recruitment of neutrophils at this early time point.

Expression of mRNA for proinflammatory cytokines by AM was decreased by treatment with anti-IL-33 prior to induction of an acute exacerbation (Table 3). However, there

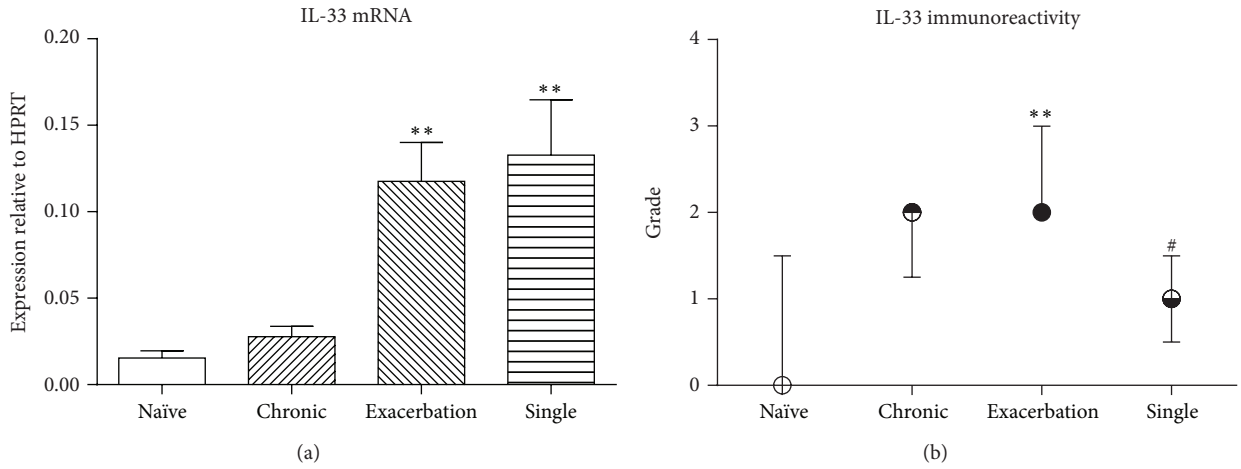

Figure 3: (a) Expression of mRNA for IL-33, relative to HPRT, in airway wall tissue from the acute exacerbation and control groups (mean ± SEM). (b) Intensity of immunoreactivity for IL-33 in the airway epithelium (median ± interquartile range). Significant differences relative to the naïve group are shown as $^{**}(P < 0.01)$ and those relative to the acute exacerbation group are shown as $^{\#}(P < 0.05)$ ($n = 8$ samples per group).

Table 2: Expression of proinflammatory cytokine mRNA and protein by IL-33-stimulated MH-S cells.

Cytokine	Expression of mRNA (relative to HPRT $\times 10^3$)		Concentration of protein in culture supernatant	
	Unstimulated	IL-33 stimulated	Unstimulated	IL-33 stimulated
TNF-α	3.43 ± 0.33	6.72 ± 0.86**	197.3 ± 38.2	398.3 ± 23.8**
IL-1β	0.66 ± 0.04	3.88 ± 0.48***	51.6 ± 28.4	93.1 ± 9.7
IL-6	9.9 ± 6.2	85.5 ± 9.5***	124.0 ± 8.5	709.7 ± 38.8***
CXCL-1	0.001 ± 0.000	0.002 ± 0.000**	10.6 ± 0.4	19.2 ± 0.6***
IL-12p40	0.003 ± 0.000	0.228 ± 0.004***	11.2 ± 1.4	49.2 ± 0.6***
G-CSF	0.001 ± 0.000	0.004 ± 0.001	87.9 ± 8.5	538.5 ± 33.2***

Data are mean ± SEM ($n = 4$–6 replicates per group). Significant differences relative to unstimulated control are shown as $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$.

was no effect on the upregulation of expression of mRNA for markers of alternative activation (not shown).

Treatment with the neutralizing antibody also diminished levels of IL-6 and CXCL-1 proteins in BAL fluid (Figures 4(c) and 4(d)), but did not decrease the concentrations of TNF-α (not shown).

4. Discussion

In this study, we have characterized the activated macrophages in an experimental acute exacerbation of chronic asthma and shown that they have a phenotype that includes features of alternative activation; provided evidence that expression of IL-33 in the airways is enhanced in an acute exacerbation and that IL-33 can promote expression of proinflammatory cytokines by macrophages; and demonstrated that inhibition of IL-33 reduces the distal airway inflammation which characterizes an acute exacerbation of asthma.

In the first part of this study, we demonstrated that induction of an experimental acute exacerbation of chronic asthma is associated with striking upregulation of the expression by AM of arginase-1, FIZZ1, eotaxin-2/CCL24, and Ym1, all of which are markers characteristic of alternative activation. However, alternatively activated macrophages are often described as being anti-inflammatory, expressing low levels of costimulatory molecules related to antigen presentation and thus being more likely to have roles in wound healing and/or immunoregulation [20, 33]. In contrast, we confirmed our previous finding [17] that these cells also exhibit upregulated expression of proinflammatory cytokines. Furthermore, we demonstrated enhanced expression by these AM of cell surface molecules associated with antigen presentation.

The division of macrophages into M1 and M2 (and M2a/b/c subtypes) is based on their profile of chemokine and cytokine expression [18, 19]. However, the boundaries between these phenotypes are poorly defined [20]. Moreover, they have largely been described on the basis of in vitro studies, most of which have used bone-marrow- or monocyte-derived macrophages, and little work has been done to examine gene expression profiles in macrophages freshly isolated from tissues in disease [34]. It is becoming increasingly clear that M2 or alternatively activated macrophages may be important effector cells in inflammatory states. For example, they appear to play significant roles in antifungal defense [35, 36] and exhibit enhanced production of proinflammatory chemokines and cytokines when exposed to various

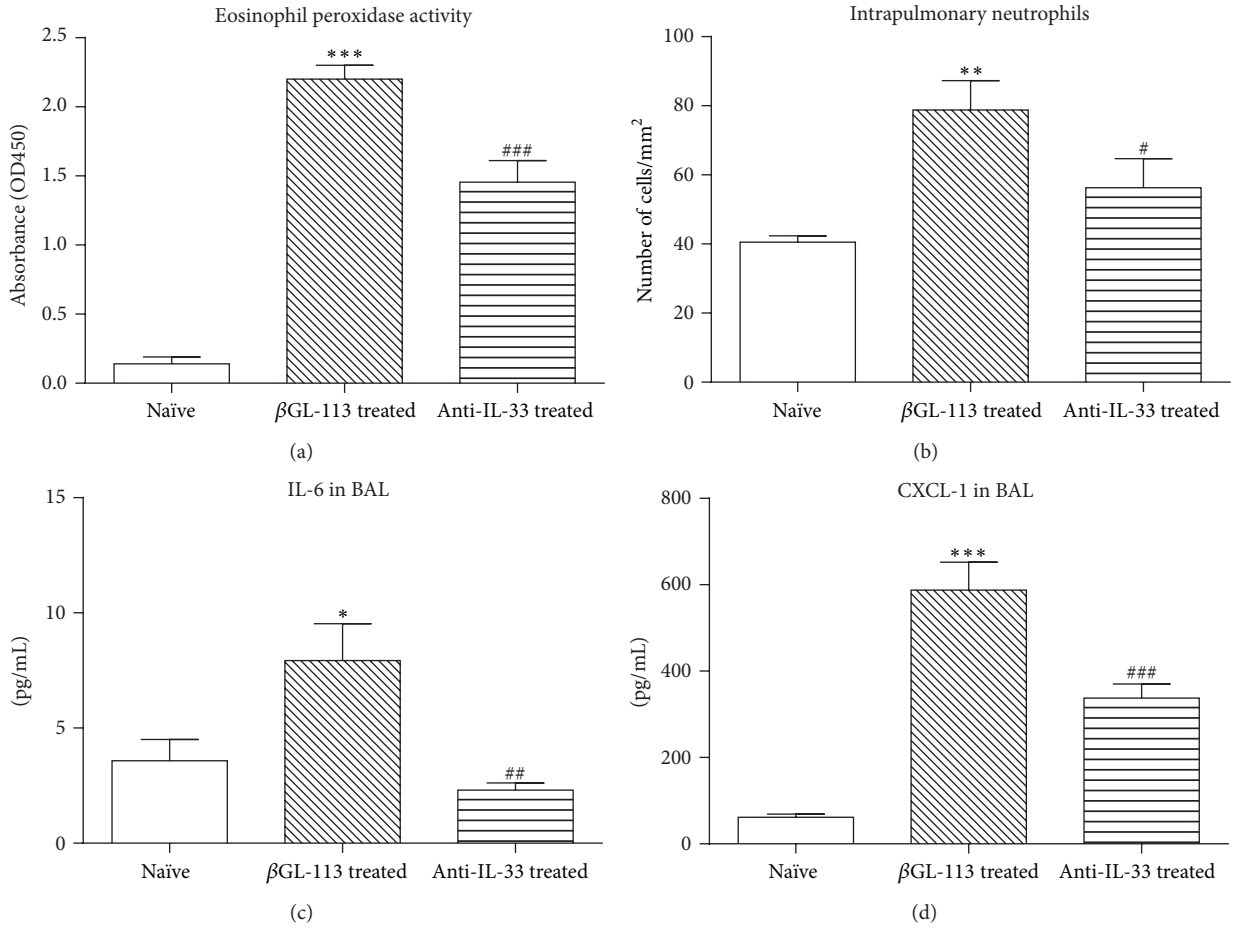

Figure 4: Effect of pretreatment with anti-IL-33 or a control antibody to β-galactosidase on the airway inflammatory response. (a) Relative accumulation of eosinophils, assessed by eosinophil peroxidase activity. (b) Number of neutrophils in lung tissue, assessed by immunostaining. (c) Concentration of IL-6 in BAL fluid. (d) Concentration of CXCL-1 in BAL fluid. Data are mean ± SEM ($n = 6$ samples per group). Significant differences relative to the naïve group are shown as $^{*}(P < 0.05)$, $^{**}(P < 0.01)$, and $^{***}(P < 0.001)$ and those relative to the control antibody-treated group are shown as $^{\#}(P < 0.05)$, $^{\#\#}(P < 0.01)$, and $^{\#\#\#}(P < 0.001)$.

Table 3: Expression of proinflammatory cytokine mRNA by AM.

Cytokine	Naïve	βGL-113	Anti-IL-33
TNF-α	0.01 ± 0.01	$1.20 \pm 0.35^{**}$	$0.29 \pm 0.14^{\#}$
IL-1β	0.03 ± 0.00	1.95 ± 0.74	0.76 ± 0.36
IL-6	0.00 ± 0.00	$0.37 \pm 0.11^{**}$	$0.02 \pm 0.01^{\#}$
CXCL-1	0.05 ± 0.02	$0.35 \pm 0.05^{**}$	$0.09 \pm 0.05^{\#\#}$

Data are mean ± SEM (n = 4–6 animals per group) relative to HPRT. Significant differences relative to the naïve group are shown as $^{**}(P < 0.01)$ and those relative to the acute exacerbation group are shown as $^{\#}(P < 0.05)$ and $^{\#\#}(P < 0.01)$.

additional stimuli [37–39]. Animal experimental studies have also demonstrated a role for alternatively activated AM in driving allergic airway inflammation [40, 41]. Furthermore, investigations in children have revealed that, during acute exacerbations of asthma, circulating monocytes and dendritic cell populations display upregulation of genes indicative of an alternatively activated phenotype [42].

Our findings are concordant with these latter observations and indicate that, in the setting of an experimental acute exacerbation, AM exhibit a phenotype including not only expression of characteristic markers of alternative activation, but also enhanced production of proinflammatory cytokines and an enhanced capacity for antigen presentation. This phenotype does not exactly align with any of the described subsets of macrophages, reinforcing the concept of the phenotypic plasticity of macrophages [34, 43]. We note that the upregulated markers of alternative activation are likely to be relevant to the inflammatory response, because eotaxin-2/CCL24 cooperates with IL-13 in the recruitment of eosinophils [44], Ym1 may also have a role in eosinophil recruitment [45], while FIZZ1 may contribute to the development of airway hyperresponsiveness [46]. We also note that markers of classical activation were not upregulated in our model and that not all markers associated with alternative activation were upregulated to the same extent. In particular, expression of mRNA for the macrophage mannose receptor was not significantly increased, while the magnitude

of increase for Ym1 was lower than for the other markers assessed. These findings are consistent with published data on AM, which constitutively express much higher basal levels of these mRNAs than do macrophages from other sources [47].

In the second part of this work, we investigated whether activation of AM in an experimental acute exacerbation of murine chronic asthma might be related to increased expression of IL-33. This cytokine is a relatively recently identified member of the IL-1 family [22] that appears to have a role in airway inflammation [24, 48]. IL-33 has been demonstrated to drive upregulated expression of markers of alternative activation in IL-4/13-primed bone-marrow-derived macrophages in vitro, as well as to promote AM-dependent airway inflammation in vivo [23].

We found that levels of mRNA for IL-33 in the airways were markedly increased both in the acute exacerbation and single moderate-level challenge groups of animals, suggesting that moderate-level inhalational challenge with OVA in sensitized mice was responsible for this upregulation. Immunohistochemistry revealed that expression of IL-33 protein by epithelial cells lining the airway lumen was also significantly increased. However, we were unable to demonstrate enhanced release of IL-33 protein because levels in BAL fluid were below the limits of detection of our assay.

To establish that enhanced expression of IL-33 could drive the activation of AM, we collected AM from mice that had received repeated low-level inhalational challenge and stimulated them with IL-33 in short-term culture. We found evidence of significantly upregulated expression of mRNA for Ym1, although in this in vitro setting other markers of alternative activation were not increased in parallel. We extended these studies using the MH-S alveolar macrophage-like cell line and showed that IL-33 could directly stimulate these cells to express a variety of proinflammatory cytokines, independent of any effect on expression of markers of alternative activation. Collectively, our data reinforce the notion that IL-33 can activate macrophages of different phenotypes and are consistent with an earlier report suggesting that IL-33 can drive both M1 and M2 patterns of chemokine expression by macrophages, depending on the cytokine environment [49].

In the third part of this study, we evaluated the role of IL-33 in the inflammation associated with an acute exacerbation of asthma. Prior to the final allergen challenge used to trigger an exacerbation, we treated mice with a neutralizing antibody to IL-33, which significantly diminished the recruitment of pulmonary eosinophils and neutrophils. These cells are key players in the inflammatory response in lung tissue, and our data provide novel evidence that IL-33 drives the distal airway inflammation associated with an acute exacerbation of chronic asthma. Our results are consistent with a role for alternatively activated macrophages in the recruitment of eosinophils via production of eotaxin-2/CCL24, as noted previously, as well as with evidence that IL-33 may be chemoattractant for neutrophils [50].

We then investigated whether the anti-inflammatory effect of anti-IL-33 was related to inhibition of the production of proinflammatory cytokines by AM. We demonstrated significantly reduced expression of cytokine mRNA by these cells, which was paralleled by decreased levels of at least some proinflammatory cytokines in BAL fluid. In the context of our previous report that activated AM from an acute exacerbation are able to stimulate Th2 cytokine secretion by primed CD4+ T cells [17], these results strongly suggest a role for these AM in driving IL-33-dependent inflammation in an acute exacerbation of asthma. This notion is also supported by a recent study demonstrating that depletion of AM decreases pulmonary inflammation induced by IL-33 [25]. Unexpectedly, we did not observe any reduction in the upregulation of expression of markers of alternative activation by AM from animals treated with anti-IL-33.

Overall, our findings are consistent with accumulating evidence that IL-33 has an important role in driving allergic inflammatory responses. We suggest that in the setting of acute asthma, IL-33 is likely to play an important role in the distal airway inflammation which is responsible for functional impairment.

Conflict of Interests

The authors confirm that they have no conflict of interest that would influence the validity of their research.

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Review Article

Update on Anticytokine Treatment for Asthma

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Current advances in the knowledge of asthma pathobiology suggest that anticytokine therapies can be potentially useful for the treatment of this complex and heterogeneous airway disease. Recent evidence is accumulating in support of the efficacy of anti-IL-4, anti-IL-5, and anti-IL-13 drugs. Therefore, these new developments are now changing the global scenario of antiasthma therapies, especially with regard to more severe disease. Current findings referring to variability of individual therapeutic responses highlight that the different asthma subtypes need to be well characterized, in order to implement phenotype-targeted treatments which in the near future will hopefully be mainly based on cytokine-directed biologics.

1. Introduction

Asthma is a chronic disease of the airways, characterized by inflammatory, structural, and functional changes responsible for bronchial hyperresponsiveness and usually reversible airflow limitation [1, 2]. It constitutes a heavy medical, social, and economic burden because its prevalence is continuously increasing worldwide [3]. Indeed, asthma affects over 300 million people around the world, and some epidemiologic projections estimate that such a number will further increase during the next decades [4]. Although a good control of asthma symptoms can be achieved in a vast majority of patients by current standard therapies mainly based on combinations of inhaled corticosteroids and β_2-adrenoceptor agonists, eventually associated with oral leukotriene inhibitors [5, 6], a small percentage (about 5–10%) of asthmatic subjects who are affected by the most severe subtypes of the disease, though receiving the best available conventional treatments remain symptomatic and inadequately controlled, thus having a poor quality of life. In these patients, asthma symptoms can be further worsened by concomitant comorbidities including rhinitis, sinusitis, gastrooesophageal reflux, obesity and obstructive sleep apnoea [7–10]. Patients with uncontrolled asthma exhibit a high risk of serious morbidity and mortality, thereby representing the most severe sector of the overall phenotypic asthma spectrum, and thus being characterized by the greatest unmet medical needs [11, 12]. Therefore, though being a minority of the global asthmatic population, patients with severe asthma are those who use the largest share of economic resources and health care services, including emergency visits, hospitalizations, and additional consumption of drugs utilized for recurrent exacerbations. A further social and economic impact of difficult-to-treat asthma arises from the frequent loss of school and work days, due to such a disabling condition. Moreover, patients with severe asthma often show

a tendency to anxiety and depression, which can further impair disease control by reducing their compliance to prescribed medications. Therefore, for the poorly controlled asthma phenotypes, additional therapeutic approaches are absolutely required.

In this regard, a large body of evidence also suggests that many cytokines released by both immune-inflammatory and airway structural cells significantly contribute to shape the several different disease phenotypes [13–15]. Indeed, basic and clinical research has identified several potentially suitable cytokine targets for antiasthma therapies [16, 17]. Such considerations highlight the potential importance of biologic treatments directed against proinflammatory cytokines, including monoclonal antibodies and small-molecule inhibitors. In particular, biologics may represent useful adjunctive therapies, especially for patients with more severe asthma which is not fully responsive to conventional treatments alone [18–20]. Variable responses have been observed using experimental cytokine-directed therapies, probably because of the significant differences occurring among distinct asthma phenotypes. This implies that biologic drugs need to be addressed against the molecular targets which are relevant to each phenotypic subgroup of asthma. In this regard, the aim of the present review is to outline, after recalling the most recent advances in asthma pathobiology, the newly developing anticytokine therapies for asthma.

2. Pathobiology of Asthma

Asthma is a complex and heterogeneous disease characterized by various pathologic and clinical phenotypes, based on different patterns of airway inflammation involving immune-inflammatory cell types such as T and B lymphocytes, mast cells, eosinophils, basophils, neutrophils, and dendritic cells, as well as structural cellular elements including both epithelial and mesenchymal cells. This widespread respiratory disease, which originates from multiple interactions between genetic factors and environmental agents such as allergens, respiratory viruses, and airborne pollutants, is characterized by recurrent episodes of dyspnoea, wheezing, chest tightness, and cough, usually associated with a reversible airflow limitation and an exaggerated bronchoconstrictive response to several different stimuli (airway hyperresponsiveness). Since many decades ago, asthma has been classified into two major phenotypes known as allergic (atopic) or "extrinsic" asthma and "intrinsic" (nonatopic) asthma [21]. Atopic asthma is the dominant disease manifestation during early life and young adulthood, whereas the nonatopic form is more frequent in older patients, thus often characterizing the so-called late-onset subtype of asthma.

The main pathologic feature of asthma is chronic inflammation, frequently associated with structural changes of airway wall, collectively defined as tissue remodelling [22]. Atopic asthma is widely believed to be triggered by an immune-inflammatory response driven by T-helper type 2 (Th2) lymphocytes. This so-called "Th2-high" subphenotype of asthma arises from a complex interplay between the innate, and adaptive branches of immune system [23, 24]. In particular, aeroallergens that cause atopic asthma including pollens, house dust mite and animal dander, often have proteolytic properties and also contain trace amounts of bacterial constituents such as lipopolysaccharide (LPS) [25]. By virtue of these features, once penetrated into airway epithelium inhaled allergens can activate the Toll-like receptor (TLR) class of pattern recognition receptors involved in innate immunity. TLR activation induces the synthesis of innate cytokines such as thymic stromal lymphopoietin (TSLP) and interleukins-25 (IL-25) and -33 (IL-33), able to elicit the development of Th2 adaptive responses. Furthermore, TLR stimulation also promotes the epithelial release of C-C chemokine ligands 2 (CCL2) and 20 (CCL20), which favour the recruitment and maturation of dendritic cells [15]. The latter extend their intraepithelial processes into the airway lumen and capture aeroallergens. This uptake of inhaled antigens is stimulated by IgE bound to high affinity receptors (FcεRI) located on the surface of dendritic cells. Interaction of IgE with FcεRI receptors expressed by dendritic cells facilitates allergen internalization inside their cytoplasm [26], where antigens are processed by cathepsin S whose action, thus, results in the generation of allergenic peptide fragments. The latter are then loaded within the context of HLA molecules belonging to the class II of the major histocompatibility complex (MHC class II) expressed by dendritic cells, that migrate to T-cell areas of regional thoracic lymph nodes where antigen presentation to T lymphocytes takes place. Recognition of specific antigenic peptides by T-cell receptors triggers sensitization and the following adaptive immune response. Allergen-dependent activation of naïve T lymphocytes requires the interaction of their costimulatory molecules (CD28, ICOS, and OX40) with the respective counter-ligands expressed by dendritic cells (CD80/B7.1, CD86/B7.2, ICOS ligand, and OX40 ligand) [27]. The type of antigen presentation-dependent differentiation of T lymphocytes is critically determined by the cytokine milieu. In particular, IL-12 produced by dendritic cells promotes Th1 polarization whereas commitment towards the Th2 lineage is driven by IL-4, probably released from mast cells, basophils, eosinophils, and T cells [28]. Moreover, TSLP is secreted in large amounts by bronchial epithelial cells and mast cells of asthmatic patients, thus inducing dendritic cells to release CCL17 and CCL22 chemokines, that recruit Th2 cells upon binding to their CCR4 receptor [29]. Activated Th2 lymphocytes then leave lymph nodes and enter the airways, where further allergen deposition and antigen presentation by local dendritic cells occur. As a consequence, Th2 cells expressing the CCR4 chemokine receptor synthesize large amounts of cytokines encoded by the gene cluster located on the long arm of chromosome 5, including IL-3, IL-4, IL-5, IL-6, IL-9, IL-13, and granulocyte macrophage colony stimulating factor (GM-CSF). These cytokines and growth factors stimulate maturation and recruitment of other immune cells involved in the allergic cascade, such as eosinophils and mast cells [30]. In particular, eosinophil differentiation in the bone marrow is promoted by IL-5, whose action is synergized by eosinophil-recruiting chemokines like eotaxin, released by both inflammatory and airway resident cells [31]. IL-4 and IL-13 act on B lymphocytes by driving immunoglobulin class

switching towards the production of IgE [12]. IL-9, secreted by a further subset of T lymphocytes (Th9) derived from Th2 cells, attracts mast cells and triggers their differentiation [32].

Whereas Th2 lymphocytes are mainly involved in the development of an inflammatory phenotype referred to as eosinophilic asthma, other Th cell subsets induce airway neutrophilic inflammation, often associated with the most severe clinical phenotypes. In particular, a specific lineage of $CD4^+$ effector T lymphocytes, expressing IL-17 and thus named Th17, appears to play a pivotal role in airway neutrophilia [33, 34]. Indeed, in lung tissue sections from asthmatic patients there is an overexpression of IL-17A and IL-17F, whose levels correlate with asthma severity, especially in subjects with neutrophilic, steroid-resistant disease [35]. In mice, Th17 lymphocyte differentiation from uncommitted cell precursors requires IL-6 and transforming growth factor-β (TGF-β), and IL-17 expression is further enhanced by IL-23 [36]. IL-17A and/or IL-17F stimulate airway structural cellular elements, like bronchial epithelial cells and subepithelial fibroblasts, to secrete powerful neutrophil chemoattractants such as IL-8 and CXCL1/GRO-α [36]. Th17 cells may contribute to the pathogenesis of allergic asthma, thus worsening its severity [37]. Therefore, it is reasonable to speculate that a predominantly Th2-mediated airway eosinophilia is likely responsible for mild and moderate atopic asthma, whereas concomitant activation of both Th2 and Th17 cells can be frequently associated with a mixed eosinophilic/neutrophilic inflammatory phenotype underlying more severe disease.

Another cytokine that is implicated in the pathogenesis of severe neutrophilic asthma is tumour necrosis factor-α (TNF-α), produced by $CD4^+$ T lymphocytes, monocytes and/or macrophages as well as several other cell types, which exerts pleiotropic effects on inflammatory and structural airway cells [38, 39]. Combined patterns of both neutrophilic and eosinophilic airway infiltrates may occur in recurrent acute relapses of asthma, that characterize the so-called exacerbation-prone asthmatic phenotype [40]. These exacerbations can be caused by allergens and especially by respiratory viruses, whose pathogenic effects within the airways of asthmatic patients are favoured by a deficient epithelial synthesis of antiviral cytokines such as interferons β (IFN-β) and λ (IFN-λ) [41].

Across the wide severity spectrum of allergic asthma phenotypes, defective numbers and/or functions of specific regulatory T lymphocytes (T_{reg} cells) have been detected [42, 43]. Several different T_{reg} lymphocyte subsets have been identified, including naturally occurring $CD4^+CD25^+$ cells expressing the transcription factor FOXP3 (forkhead box P3). T_{reg} cells exert their immune-modulatory functions through direct and indirect mechanisms. In particular, T_{reg} cells produce anti-inflammatory cytokines like IL-10 and TGF-β, express inhibitory factors such as CTLA4 (cytotoxic T lymphocyte antigen 4), and also downregulate MHC class II proteins and CD80/CD86 costimulatory molecules expressed by antigen presenting cells [44]. A defective function of T_{reg} cells is probably also a feature of intrinsic, nonatopic asthma triggered by microbial superantigens. The latter can indeed suppress the immune-modulatory role of T_{reg} cells, thus enhancing the activity of both $CD4^+$ and $CD8^+$ T lymphocytes [45].

In asthma, chronic inflammation is frequently associated with dynamic structural changes that involve all airway wall layers and extend from proximal, large-to-distal, small airways. Such a tissue remodeling occurs in both atopic and nonatopic asthma [46], and includes epithelial shedding, goblet cell, and mucous gland hyperplasia, enhanced deposition of extracellular matrix proteins leading to subepithelial fibrosis, and increased angiogenesis and hypertrophy/hyperplasia of smooth muscle cells, which acquire a highly proliferative, secretory, and contractile phenotype [47]. It is currently believed that airway remodeling in asthma is largely due to complex interactions between bronchial epithelium and the underlying mesenchyme, resulting from a reactivation of the developmental epithelial-mesenchymal trophic unit (EMTU) responsible for lung morphogenesis during fetal life [48]. Within the context of EMTU reactivation, a crucial role is played by TGF-β [49], a fibrogenic growth factor whose levels are upregulated in asthmatic airways because of an increased release from immune-inflammatory cells, as well as from damaged epithelial cells and activated mesenchymal cells. Other growth factors contributing to airway remodeling in asthma include endothelin-1 (ET-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [50–57]. Overall, airway remodeling results in thickening of bronchial and bronchiolar walls, leading to reduction of airway calibre and fixed airflow limitation that is correlated with a progressive decline of respiratory function.

These recent advances in the understanding of asthma pathobiology, and especially a better knowledge of the cellular and molecular mechanisms that underlie uncontrolled asthma, may have important prospective therapeutic implications. In particular, the improved awareness of the inflammatory and immune events involved in cytokine cascades is unravelling potential targets for the development and implementation of new biological therapies. Several different antiasthma biologics are currently under different stages of investigation, including molecules directed against IL-5, IL-4, IL-13, IL-9, GM-CSF, and TNF-α. Moreover, IL-17 and IL-23, as well as the innate cytokines TSLP, IL-25, IL-33, and IL-27, are additional interesting targets for future asthma therapies. The main anticytokine strategies under current development for asthma treatment are schematically illustrated in Figure 1.

3. Anti-IL-5

IL-5 plays a crucial role in the growth, maturation, and activation of eosinophils [31]. Therefore, anti-IL5 therapeutic strategies may potentially be effective in the treatment of eosinophilic asthma phenotypes [58]. In this regard, several preclinical studies have been carried out in experimental animal models of asthma. In particular, pretreatment with the anti-IL-5 blocking antibody TRFK-5 was able to inhibit eosinophil influx into the airways of allergen-sensitized mice [59]. Furthermore, TRFK-5 also suppressed airway eosinophilic infiltration and bronchial hyperresponsiveness

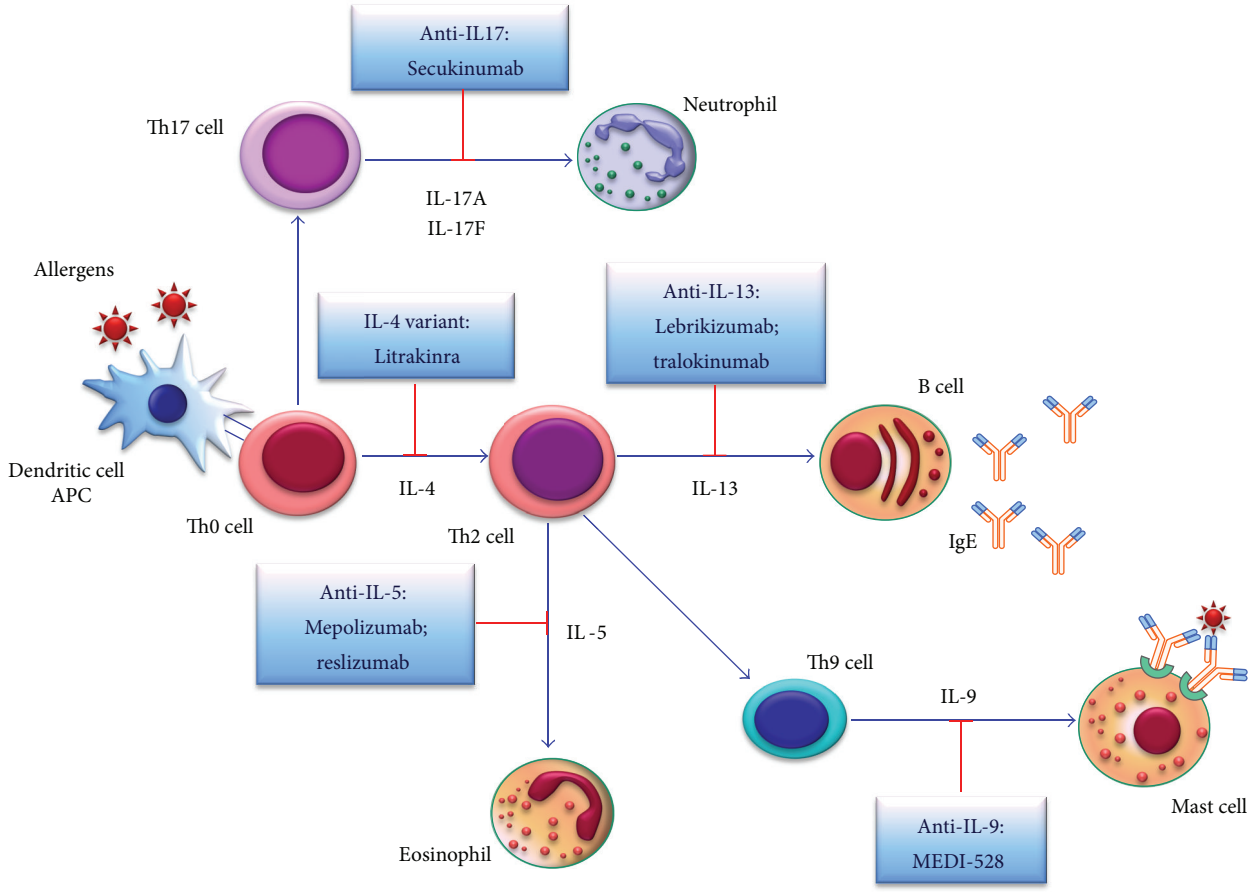

Figure 1: Anticytokine therapies for asthma. The main suitable cytokine targets for developing antiasthma biologics are depicted. See text for further details.

in a nonhuman primate model of asthma [60]. More recently, other antibodies such as mepolizumab, reslizumab, and benralizumab have been developed [61].

Some clinical trials performed in heterogeneous populations of patients with mild or moderate chronic persistent asthma have shown that mepolizumab, a humanized monoclonal antibody against IL-5, is safe and can effectively reduce eosinophil numbers in airways and blood [62, 63]. However, these effects were not paralleled by significant improvements in asthma symptoms, lung function, and bronchial hyperresponsiveness. More recently, mepolizumab has been tested in selected subtypes of chronic severe asthma, characterized by frequent exacerbations and airway eosinophilia refractory to inhaled and systemic corticosteroid therapies [64, 65]. Taken together, the results of these two small trials demonstrate that mepolizumab was well tolerated during a 12-month treatment period and dramatically decreased asthma exacerbations and eosinophil levels in both blood and induced sputum. Such findings have been further corroborated by the large, multicentre DREAM study, carried out in 621 patients with severe, exacerbation-prone, eosinophilic asthma who were randomly assigned to four groups receiving at 4-week intervals 13 intravenous infusions of placebo or one of three doses of mepolizumab (75 mg, 250 mg, or 750 mg) [66]. At all dosages used, mepolizumab was well tolerated and effectively decreased the frequency of asthma exacerbations, as well as blood and sputum eosinophil counts [66].

In addition to mepolizumab, another interesting anti-IL-5 biologic drug is reslizumab, an IgG4/κ humanized monoclonal antibody. When compared to placebo in patients with poorly controlled eosinophilic asthma, reslizumab has been recently shown to significantly decrease sputum eosinophils and improve lung function, as well as inducing a positive trend toward better asthma control [67]. The antiasthma effects of reslizumab were most pronounced in a subgroup of patients characterized by the highest levels of blood and sputum eosinophils, which were associated with the presence of nasal polyposis [67].

Therefore, all such findings further emphasize the importance of accurate phenotype selection, in order to tailor antiasthma treatments targeted to the peculiar biologic and clinical features of the individual disease expressions. These concepts will eventually also apply to the use of benralizumab, an IgG1 monoclonal antibody directed to IL-5 receptor, that

in preliminary investigations has been reported to be quite safe and to effectively reduce peripheral blood eosinophils [68].

4. Anti-IL-4

IL-4 contributes to asthma pathophysiology by inducing Th2 cell differentiation and expansion, isotype switching of B cells to IgE synthesis, as well as eosinophil recruitment, development of mast cells and mucous metaplasia [50]. Moreover, IL-4 is also involved in airway remodeling by upregulating collagen and fibronectin production. Several studies aimed to evaluate the effects of anti-IL-4 therapies in asthma treatment have yielded conflicting results [69]. In murine models of allergen-induced asthma, blockade of either IL-4 or its receptor has been shown to inhibit eosinophil influx into the airways and IL-5 release from T cells, as well as decreasing lung inflammation, serum IgE levels, and airway hyperresponsiveness to methacholine [70, 71]. However, although the humanized anti-IL-4 monoclonal antibody pascolizumab is well tolerated, it lacks clinical efficacy in asthmatic patients [16]. Similarly, despite some promising preliminary findings regarding the soluble recombinant human IL-4 receptor altrakincept, no significant clinical efficacy has been later confirmed [72]. More effective appears to be pitrakinra, a bioengineered variant of IL-4 that acts as an antagonist at the heterodimeric receptor complex (IL-4Rα/IL-13Rα1) shared by both IL-4 and IL-13 [73]. In particular, when administered by either subcutaneous or inhaled route, pitrakinra is safe and inhibits allergen-induced early and late asthmatic responses, as well as disease exacerbations in selected phenotypes of eosinophilic asthma [50, 74]. Moreover, in the first large pharmacogenetic, placebo-controlled investigation of the IL-4/IL-13 pathway, three doses (1 mg, 3 mg, or 10 mg twice daily for 12 weeks) of inhaled pitrakinra were tested in patients with moderate-to-severe asthma [75]. Although this trial failed to demonstrate clinical efficacy in the whole study population, at the 10 mg dosage pitrakinra significantly lowered the frequency of asthma exacerbations in individuals carrying specific single nucleotide polymorphisms in the gene encoding IL-4Rα, located within the 3′ untranslated region (rs8832GG and rs1029489GG genotypes) [75].

More recently, a fully human monoclonal antibody directed against the α subunit of the IL-4 receptor (dupilumab) has been tested in patients with persistent, moderate-to-severe asthma and blood or sputum eosinophilia. When compared with placebo, dupilumab induced a significant decrease in asthma exacerbation rate during withdrawal of inhaled therapy with corticosteroids and long-acting β_2-adrenergic agonists, paralleled by a marked improvement of respiratory function and by reduced levels of Th2-associated biomarkers such as eosinophils, exhaled NO, and eotaxin-3 [76].

5. Anti-IL-13

IL-13 is a key target for the development of new antiasthma therapeutic strategies because of its involvement, together with IL-4, in several different aspects of airway inflammation and remodeling, including mucus production, IgE synthesis, recruitment of eosinophils and basophils, and proliferation of bronchial fibroblasts and airway smooth muscle cells [50]. Anti-IL-13 treatments performed in experimental animal models of chronic allergic asthma can markedly attenuate IgE synthesis, airway eosinophilia, and bronchial structural changes.

In clinical trials, the anti-IL-13 monoclonal antibody anrukinzumab has been able to significantly inhibit allergen-induced late asthmatic responses within 14, but not 35 days after administration [77]. Furthermore, it has also been shown that the anti-IL-13 monoclonal antibody lebrikizumab exerts an effective antiasthma action in the so-called "Th2-high" asthmatic phenotype, characterized by an overexpression of IL-13-inducible genes such as periostin, an extracellular matrix protein [78]. In this study, the overall frequency of adverse events resulted to be similar in the two groups of asthmatic subjects undergoing treatment with lebrikizumab or placebo, respectively, in addition to standard inhaled therapy. With regard to efficacy, lebrikizumab induced a relevant improvement of lung function in patients with moderate-to-severe asthma displaying high serum levels of periostin. In particular, at week 12 the reported percentage increases in forced expiratory volume in one second (FEV_1), with respect to baseline values, being 5.5% in the whole lebrikizumab-treated group, 8.2% in the high-periostin subgroup, and 1.6% (not significant) in the low-periostin subgroup [78]. This implies that easily detectable biomarkers could be routinely used in clinical practice to identify specific asthmatic phenotypes, characterized by an important pathogenic role of IL-13, and thus being potentially responsive to therapeutic strategies targeted against such a pleiotropic cytokine.

Another humanized anti-IL-13 antibody currently in clinical development is tralokinumab, characterized by favourable pharmacokinetic properties and a good safety profile [79]. Tralokinumab has been recently evaluated in a trial involving 194 adult patients with moderate-to-severe uncontrolled asthma randomly assigned to receive, every 2 weeks through the subcutaneous route in addition to currently available controller therapy, either placebo or one of three different doses (150, 300, and 600 mg) of the IL-13 neutralizing antibody, respectively [80]. Although when compared to placebo tralokinumab did not affect symptom score assessed by the Asthma Control Questionnaire (ACQ), this anti-IL-13 drug reduced the need for rescue medication and significantly improved lung function by eliciting percentage increases in FEV_1 from baseline ranging from 8.1% (150 mg) to 16.1% (600 mg) [80]. With respect to subjects lacking IL-13 in induced sputum, FEV_1 changes were far greater in patients having detectable IL-13 sputum levels. Tralokinumab was well tolerated and did not induce any serious adverse event.

6. Anti-IL-9

IL-9 is overexpressed in asthmatic airways, where this cytokine stimulates mast cell proliferation and mucus hyperplasia [81]. In mice, IL-9 blockade reduced airway inflammation and hyperresponsiveness [82]. Moreover, in two

randomized phase 2a trials carried out in subjects with mild-to-moderate asthma, the humanized anti-IL-9 monoclonal antibody MEDI-528 exhibited an acceptable safety profile and also evoked a trend toward improvement in asthma symptom scores and disease exacerbation rates [83]. The second of these two clinical studies showed that 50 mg of MEDI-528, administered subcutaneously twice weekly can exert a protective effect against exercise-induced bronchoconstriction [83].

7. Anti-GM-CSF

GM-CSF is a growth factor overexpressed in asthmatic airways, which plays a key role in eosinophil differentiation and survival [12]. In a murine model of allergic asthma, intranasal administration of a goat anti-mouse GM-CSF polyclonal antibody exerted a significant inhibitory effect on airway inflammation, mucus production, and bronchial hyperresponsiveness [84]. Later, a human anti-GM-CSF monoclonal IgG1 antibody (MT203) has been developed, capable of significantly decreasing survival and activation of peripheral human eosinophils [85].

8. Anti-TNF-α

In murine models of allergen-dependent asthma, the proinflammatory cytokine TNF-α, produced by Th1 lymphocytes, macrophages and mast cells, induced airway recruitment of neutrophils and eosinophils via upregulation of epithelial and endothelial adhesion molecules [38]. TNF-α is overexpressed in the airways of patients with severe asthma and also directly stimulates airway smooth muscle contraction through changes in intracellular calcium fluxes [86]. Therefore, several drugs targeting TNF-α have been evaluated for asthma treatment, including anti-TNF-α blocking antibodies such as infliximab and golimumab, as well as the soluble TNF-α receptor fusion protein etanercept. Overall, conflicting results have been obtained and serious concerns have been raised with regard to the safety of TNF-α blockade, which may cause susceptibility to the development of respiratory infections and human cancers.

Etanercept was preliminarily shown to significantly improve lung function, airway hyperresponsiveness, and quality of life in asthmatic patients expressing high monocyte levels of both TNF-α and TNF-α receptor [87]. More recently, however, no significant differences between etanercept and placebo have been observed with regard to lung function, airway hyperresponsiveness, quality of life, and exacerbation rate, during a larger randomized trial performed in patients with moderate-to-severe persistent asthma, exhibiting a good drug tolerability [88]. In subjects with moderate asthma, the humanized anti-TNF-α monoclonal antibody infliximab was able to reduce the circadian oscillations in peak expiratory flow and the related disease exacerbations [89]. However, a larger study carried out in patients with persistent severe asthma receiving golimumab, another TNF-α blocking antibody, did not detect any significant improvement in lung function and disease exacerbations [90]. Moreover, serious adverse infectious and neoplastic events like active tuberculosis, pneumonia, sepsis, and several different malignancies (breast cancer, B-cell lymphoma, metastatic melanoma, cervical carcinoma, renal cell carcinoma, basal cell carcinoma, and colon cancer) were reported. Therefore, the trial was interrupted and it appears to be currently very unlikely that anti-TNF-α antibodies will be soon further evaluated the for treatment of severe asthma. However, a subgroup analysis of the patients enrolled in the golimumab trial demonstrated that the drug was beneficial in older patients with late-onset asthma and a history of hospitalizations or emergency hospital visits during the year before screening, who also had lower baseline FEV_1 levels and a postbronchodilator FEV_1 increase of >12%.

9. Anti-IL-17 and Anti-IL-23

IL-17A and IL-17F, which are proinflammatory cytokines released by Th17 cells and crucially involved in neutrophilic inflammation as well as in airway remodeling, are significantly upregulated in bronchial biopsies obtained from patients with severe asthma [35]. In this regard, it is noteworthy that in mouse models of allergic asthma an anti-IL-17 antibody lowered the numbers of neutrophils, eosinophils, and lymphocytes detected in bronchoalveolar lavage fluid [91]. Ongoing phase II clinical trials are currently evaluating the efficacy and safety of a fully human IL-17A-specific monoclonal antibody (secukinumab), as well as of a human IL-17-receptor-specific monoclonal antibody (brodalumab), in patients with severe asthma that is not adequately controlled by inhaled corticosteroids and long-acting β_2-adrenergic receptor agonists [16].

Another potential therapeutic approach can be provided by the use of antibodies directed against the IL-17 regulating cytokine IL-23, whose blockade resulted in a significant inhibition of antigen-dependent recruitment of neutrophils, eosinophils, and lymphocytes into the airways of sensitized mice [92]. However, these experimental strategies should be considered with extreme caution because IL-17 is also involved in immune protection against infectious and carcinogenic agents [93]; hence, inactivation of this cytokine could result in an increased risk of opportunistic infections and cancer development.

10. Anti-IL-25, Anti-IL-33, and Anti-TSLP

IL-25, IL-33, and TSLP, mainly released from airway epithelium, are overexpressed in asthmatic airways and play a crucial role in driving and stimulating Th2-mediated immune-inflammatory responses [22]. Therefore, these cytokines are currently believed to be suitable targets for novel antiasthma therapies. In mice, an anti-IL-25 monoclonal antibody has been reported to suppress Th2-dependent allergic airway inflammation [94]. Moreover, murine allergic inflammation and airway hyperresponsiveness can be inhibited by antibodies directed against the IL-33 receptor [95]. These antibodies can also markedly inhibit IL-17F expression in human bronchial epithelial cells [96]. A relevant attenuation of mouse allergic airway inflammation has also been observed as a consequence of antibody-induced neutralization of the TSLP receptor [97].

11. Anti-IL-27

IL-27 is a monocyte- and macrophage-derived innate cytokine that is probably involved in the pathogenesis of severe, corticosteroid-resistant asthma. Indeed, IL-27 levels are increased in the airways of patients with severe neutrophilic asthma [98]. Moreover, in mouse lung macrophages, IL-27 inhibited nuclear translocation of glucocorticoid receptors [98], which is an essential cellular event for the biological and pharmacological effects of corticosteroids. Therefore, IL-27 may represent a potential target for new therapeutic strategies aimed to provide a better control of severe, steroid-refractory asthma.

12. Potential Problems Related to Anticytokine Therapies

Most anticytokine strategies under current development for the treatment of asthma mainly target Th2-derived cytokines, including IL-5, IL-4, and IL-13, crucially involved in the pathobiology of allergic phenotypes. Therefore, these anticytokine approaches may be beneficial in the management of atopic asthma and other allergic diseases such as allergic rhinitis and atopic dermatitis. Indeed, by significantly reducing eosinophilic tissue infiltration, Th2 cytokine antagonists interfere with a key cellular component of allergic inflammation. However, both patients and physicians should be aware of the time required for anti-Th2 cytokine treatments to alleviate asthma and allergy symptoms, usually ranging from one to a few weeks; this time is necessary to achieve a significant inhibition of the cellular and molecular mechanisms implicated in allergy pathophysiology. On the contrary, some concerns can arise from the consideration that anti-Th2 cytokine therapies can be potentially detrimental in patients with autoimmune disorders. Indeed, it has been recently suggested that IL-4, IL-5, and eosinophils can play a suppressive role in autoimmune mouse models of multiple sclerosis [99]. Thus, the possibility of administering anti-Th2 cytokine therapies to subjects with autoimmune diseases should be considered with extreme caution. Because of the widespread convincement that eosinophils exert a protective action against parasitic diseases, traditional medical judgement could lead to exclude patients with these infections from receiving Th2 cytokine-targeted treatments. However, this mental approach should probably be reconsidered in view of recent studies suggesting that eosinophils can be involved in parasite survival, rather than in parasite killing [100, 101]. Overall, anti-Th2 cytokine therapies are safe and well tolerated, and their main side effects include minor events such as mild injection site reactions, nasopharyngitis, nausea, and headache. Hypersensitivity responses including oedema, papular rashes, and urticaria can rarely occur during treatment with biologic drugs targeting Th2 cytokines; however, these unwanted effects are usually not severe and often resolve after immediate discontinuation of the study drug and nonurgent symptomatic treatment with corticosteroids and antihistamines.

Different from Th2 cytokine-targeted treatments, much more important problems can arise in asthmatic patients from the use of drugs directed against Th1 cytokines such as TNF-α. In particular, the occurrence of dramatic adverse events including serious infections and neoplastic disorders, observed during treatment with golimumab [90], currently discourages the further development of anti-TNF-α strategies for asthma therapy. A very careful attention should also be paid to the recruitment of asthmatic patients for clinical studies evaluating the effects of antibodies targeting IL-17A and IL-17F, given the physiologic role of these cytokines in the immunological surveillance against infections and cancer; of course, patients with immunodeficiencies must be excluded from such trials.

13. Conclusions

During the last years, both basic and clinical research strategies have identified many attractive molecular targets for asthma treatment. In particular, anticytokine therapies, added to conventional treatments and eventually used in various combinations, according to patient's individual requirements, could lead to significant improvements in the control of severe asthma. The remarkable variability observed in the individual responses to these novel biologic therapies further emphasizes the necessity of accurate asthma phenotyping, in order to achieve the best possible patient-focused management. In consideration of the frequently reported, only partial efficacy of the blockade of a single cytokine, the next research challenge might be represented by the opportunity of exploring, in carefully selected asthmatic subjects, the effects of different cocktails of biologics targeting the key pathogenic pathways underlying the various phenotypic subgroups of asthma. Of course, ongoing advances in our understanding of asthma pathobiology could make it possible, in the near future, to further extend the already promising scenario of anticytokine therapies for this sometimes hard-to-manage disease.

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Research Article

Role of M2 Muscarinic Receptor in the Airway Response to Methacholine of Mice Selected for Minimal or Maximal Acute Inflammatory Response

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Airway smooth muscle constriction induced by cholinergic agonists such as methacholine (MCh), which is typically increased in asthmatic patients, is regulated mainly by muscle muscarinic M3 receptors and negatively by vagal muscarinic M2 receptors. Here we evaluated basal (intrinsic) and allergen-induced (extrinsic) airway responses to MCh. We used two mouse lines selected to respond maximally (AIRmax) or minimally (AIRmin) to innate inflammatory stimuli. We found that in basal condition AIRmin mice responded more vigorously to MCh than AIRmax. Treatment with a specific M2 antagonist increased airway response of AIRmax but not of AIRmin mice. The expression of M2 receptors in the lung was significantly lower in AIRmin compared to AIRmax animals. AIRmax mice developed a more intense allergic inflammation than AIRmin, and both allergic mouse lines increased airway responses to MCh. However, gallamine treatment of allergic groups did not affect the responses to MCh. Our results confirm that low or dysfunctional M2 receptor activity is associated with increased airway responsiveness to MCh and that this trait was inherited during the selective breeding of AIRmin mice and was acquired by AIRmax mice during allergic lung inflammation.

1. Introduction

The cholinergic system plays a role in the regulation of many pathophysiological mechanisms. In the lung, the vagal parasympathetic nervous system via muscarinic receptors represents the dominant autonomic control of airway smooth muscle tone. Acetylcholine released at neuromuscular junctions binds to M3 muscarinic receptors in the smooth muscle and promotes airway contraction through a number of well-defined intracellular signaling mechanisms [1, 2]. An important negative feedback mechanism is given by the neuronal M2 muscarinic receptor that inhibits acetylcholine release [3, 4]. Airway hyperresponsiveness (AHR) is an exaggerated smooth muscle constriction observed in certain individuals among a population, which can occur in response to a variety of stimuli such as histamine, exercise, cold air, and methacholine (MCh). AHR is a cardinal feature of asthma [5]. Although there are exceptions [6], studies in asthmatic patients indicate a positive correlation between AHR and allergic eosinophilic inflammation [7]. Accordingly, susceptibility to develop asthma is associated with IL-5, a key cytokine for eosinophil differentiation, activation, and survival [8, 9].

One of the proposed mechanisms for allergic AHR to MCh is loss of function of vagal M2 receptors caused by eosinophilic inflammation [10–14]. Indeed, blockage of M2 receptors results in an increased acetylcholine release from vagal nerve endings that ultimately enhances bronchoconstriction [4, 15]. In this sense, AHR can be viewed as an integrated immune-neural circuit. Another well-established immune-mediated neural circuit is the cholinergic anti-inflammatory pathway [16–18]. This efferent pathway is triggered by proinflammatory cytokines via afferent sensory neurons and culminates in the release of acetylcholine by the vagus nerve. In turn, acetylcholine signals thorough nicotine acetylcholine receptor subunit $\alpha 7$ ($\alpha 7$nAChR), expressed on immune cells, inhibiting proinflammatory cytokine production [19].

Because the cholinergic system is involved in bronchoconstriction as well as regulation of inflammation, the present study aimed to evaluate the airway responses to MCh and airway allergic responses in two mouse lines selected to respond maximally (AIRmax) or minimally (AIRmin) to an innate inflammatory stimuli [20], focusing mainly on the role of M2 receptors. These mouse lines were produced by bidirectional selective breeding from a genetically heterogeneous population and behave differently in several experimental models such as arthritis [21], lung cancer [22], and bacterial infection [23]. Here we evaluated whether an innate response that selected the gene combinations determining AIRmax and AIRmin phenotypes would affect basal (intrinsic) or allergic (extrinsic) airway responses to MCh as well as an immune-adaptive T-helper-type-2- (Th2-) mediated stimuli in an experimental model of lung eosinophilic inflammation.

2. Results

2.1. Respiratory Pattern and Expression of M2 and M3 Muscarinic Receptors. It was previously reported that the basal airway responsiveness varies markedly among inbred strains of mice [24, 25]. Indeed, under basal noninflammatory condition multiple quantitative trait loci linked to AHR were identified in inbred mice strains [26, 27]. Therefore, we first determined the basal respiratory pattern of AIRmin and AIRmax in response to increasing doses of MCh in conscious unrestrained mice using a noninvasive whole-body barometric plethysmography. AIRmin mice showed a marked increase in Penh values when compared to AIRmax mice (Figure 1(a)). The dose of MCh required to elicit a 200% increase over baseline values (PC200) was 2.5-fold higher in AIRmax than in AIR min (Figure 1(b)). Our results indicate that the genetic selective process markedly influenced airway response to MCh. In this context, AIRmin animals can be considered as hyperresponsive whereas AIRmax animals are hyporresponsive to MCh.

The contractile response of airway smooth muscles to muscarinic agonists is induced mainly by activation of muscle M3 receptor subtype and inhibited by activation of vagal M2 receptor subtype. Thus, we determined the expression of M3 and M2 muscarinic receptors in lung tissue. We found that M3 receptor expression was lower in AIRmin than in AIRmax animals, as revealed by real-time PCR and Western blot analysis (Figures 1(c)-1(d)), thus, it could not explain the hyperresponsiveness of AIRmin mice to MCh. Therefore, we evaluated M2 muscarinic receptor expression by PCR and Western blot analyses. M2 receptor expression was lower in AIRmin than in AIRmax mice (Figures 1(e)-1(f)). Altogether, these results indicate that low expression of muscarinic M2 receptor in AIRmin mice might be associated with the increased AHR to MCh observed in these animals.

2.2. In Vivo M2 Muscarinic Receptor Activity. To evaluate the role of M2 receptor subtype on the airway responses *in vivo,* we treated the animals with gallamine, a selective M2 receptor antagonist. Gallamine treatment increased significantly Penh values in AIRmax but not in AIRmin mice when compared with untreated mice (Figures 2(a)-2(b)). However, gallamine treatment increased the responses to MCh in AIRmin animals indicating some activity of M2 receptor, but this activity was not sufficient to generate statistically significant data when compared to untreated group. In our study, we only used one dose of gallamine based on a previous report [28]. Taken together, these results indicate that the negative modulatory effect exerted by vagal M2 receptors was present in AIRmax mice but minimally in AIRmin animals. Therefore, the robust response to MCh observed in AIRmin mice and the lack of significant effect of gallamine indicate dysfunctional vagal M2 activity in this mouse line.

2.3. Ex Vivo M2 Receptor Activity. The results obtained *in vivo* with the noninvasive method indicated a major role of vagal M2 receptors in inhibiting airway constriction. In order to determine the role of muscle M2 receptors, we evaluated *ex vivo* the effect of gallamineonsmooth muscle constriction to MCh in isolated trachea. In this scenario, vagal receptors are excluded, and only muscarinic receptors of airway smooth muscle responded to MCh. We found that tracheal smooth muscle constriction induced by MCh was more intense in AIRmax when compared with AIRmin animals (Figure 3(a)). Gallamine treatment did not affect tracheal smooth muscle constriction induced by MCh (Figures 3(b)-3(c)), indicating that muscle M2 receptors have no role in smooth muscle contraction induced by MCh.

2.4. Allergic Lung Inflammation and Expression of Muscarinic Receptors. Loss of M2 receptor function due to allergic inflammation has been well documented [29, 30]. It has been shown that eosinophilic major basic protein plays a key role in M2 receptor dysfunction (for review see [10]). We first determined the magnitude of allergic inflammation developed in AIRmin and AIRmax mice referred as OVA group. Figure 4 shows that allergic lung inflammation, as revealed by total number of inflammatory cells (Figure 4(a)) and eosinophils (Figure 4(b)) in BAL, was more intense in AIRmax than in AIRmin animals. Accordingly, the levels of Th2 cytokines (IL-4 and IL-13) and mucus production were higher in AIRmax than in AIRmin mice (Figures 4(c)–4(e)). Histological analysis confirmed the more intense allergic inflammation in AIRmax when compared with AIRmin

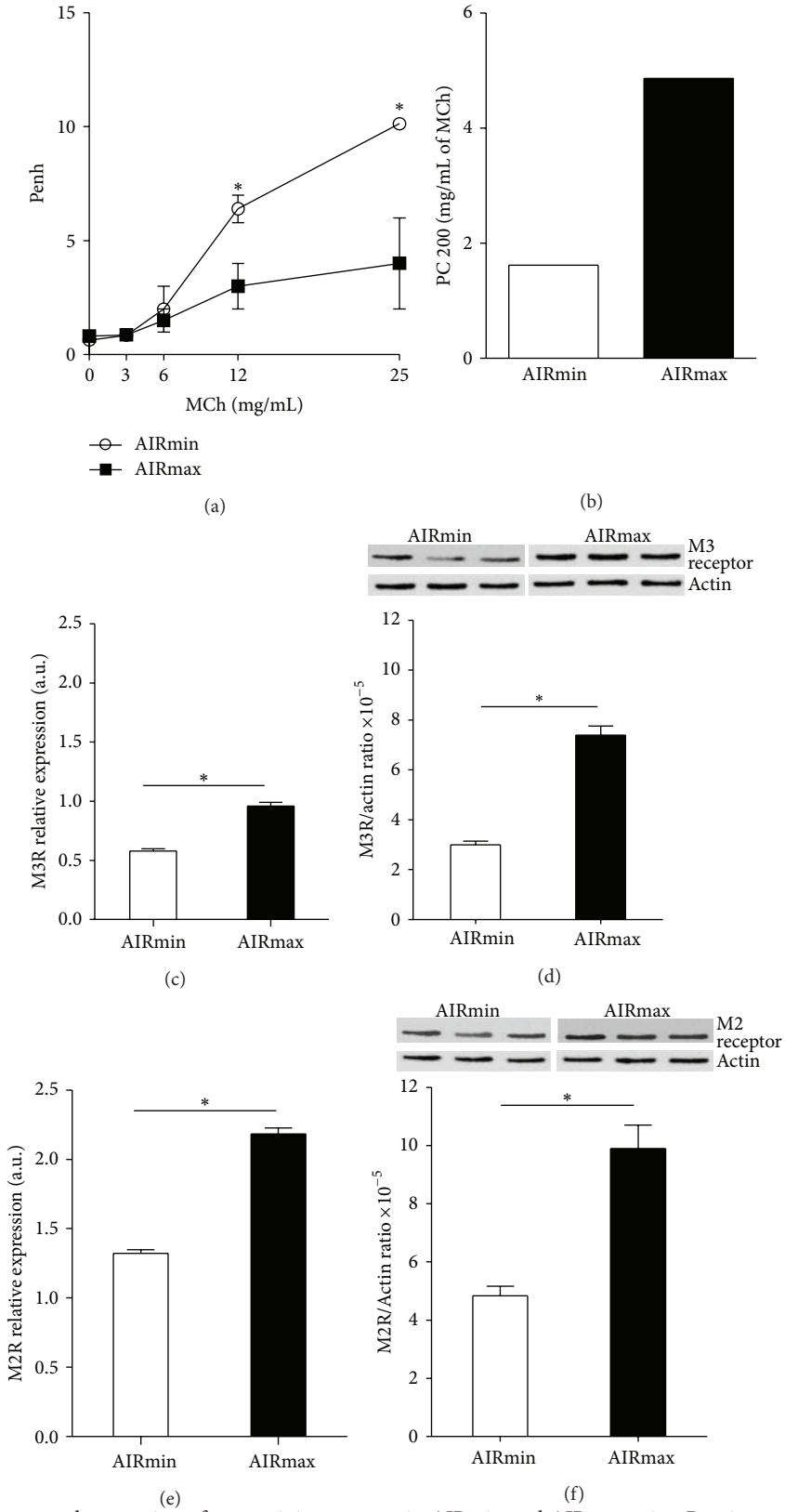

Figure 1: Respiratory pattern and expression of muscarinic receptors in AIRmin and AIRmax mice. Respiratory pattern was determined in awake, unrestrained mice by noninvasive whole-body barometric plethysmography. (a) Penh values were used as an index of bronchoconstriction induced after sequential delivery of increasing concentrations of MCh (3, 6, 12, and 25 mg/mL) and (b) provocative concentration of aerosol MCh at a 200% increase (PC200) over baseline values. Gene (c and e) or protein (d and f) expression of M2 and M3 muscarinic receptors was evaluated by real-time PCR or Western blot analysis in lungs from AIRmin and AIRmax mice. Real-time PCR was carried out using β-actin gene expression as internal control for normalization of M3R (c) and M2R (e) mRNA transcription levels. All PCR reactions were quantitative reactions made by real-time PCR. In Western blot analysis the density of M3R (d) and M2R (f) protein expression was nor aerosol at a malized to actin expression in each sample. Western blots were quantified by densitometry using the ImageJ software (NIH). Real-time PCR and Western-blot analyses were performed using pooled lungs from 5 mice. Data are expressed as mean ± SEM of five mice per group and are representative of three experiments; $^*P < 0.05$.

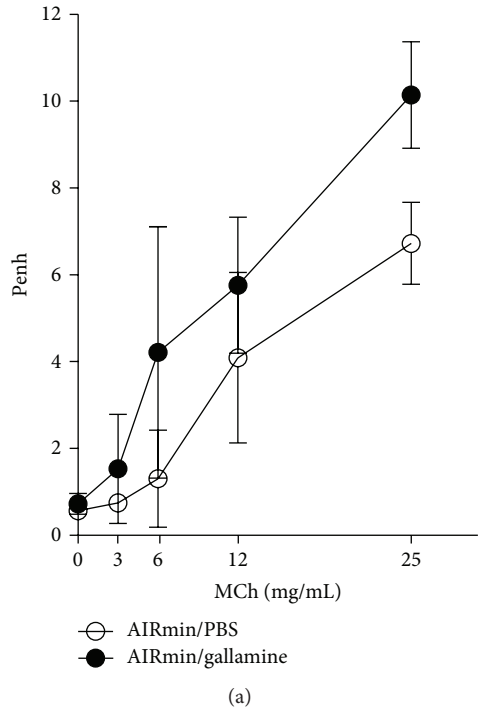

Figure 2: Respiratory pattern and airway resistance of AIRmin and AIRmax mice in the presence or absence of gallamine, a specific M2 receptor antagonist. Respiratory pattern to inhaled MCh was measured using noninvasive method in AIRmin (a) and AIRmax (b) mice. Airway resistance was measured by invasive method in AIRmin (c) and AIRmax (b) mice. Measurements were performed in the presence (black symbols) or absence (open symbols) of gallamine, in response to increasing concentrations of MCh. Results are reported as Penh values (a-b). Penh data represent the means ± SEM of five mice per group and are representative of three experiments (unpaired t-test, $^{*}P < 0,05$ as compared to PBS group).

mice as shown by peribronchovascular cellular infiltrate and increased mucus (Figure 4(f)).

We next determined the respiratory pattern of allergic AIRmin and AIRmax animals in the absence or presence of gallamine. Firstly, we found that allergic AIRmin and AIRmax mice showed higher Penh values when compared with control mice (Figures 5(a)-5(b)). Statistical analysis performed on data, represented by area under the curve, confirmed that the responses to MCh were significantly increased in allergic mice when compared with control non-allergic mice (Figures 5(c) and 5(d)). However, no significant differences were observed in Penh values of allergic mice treated or not with gallamine (Figures 5(a)–5(d)).

Because allergic mice showed an enhanced response to MCh when compared with control mice and gallamine treatment was ineffective in increasing Penh values, we asked whether the expression of muscarinic receptors has changed during allergic inflammation. We found that allergic inflammation did not change the expression of M2 or M3 receptors (Figures 5(e)–5(h)). Altogether, these data suggest that M2 muscarinic receptors dysfunction rather than altered muscarinic expression might be responsible for the increased response to MCh observed in allergic AIRmax or AIRmin mice.

3. Discussion

Our work shows that AIRmin and AIRmax mouse lines represent a model to study lung muscarinic receptor functions and its relation with the development of an allergic lung disease. The main cholinergic pathways are depicted in Figure 6. We found that AIRmin and AIRmax animals develop different patterns of airway reactivity under basal or inflammatory conditions. Notably, respiratory responses to increasing doses of MCh observed in AIRmin mice in basal condition were significantly higher than that obtained in AIRmax animals. Previous studies identified multiple quantitative trait loci (QTL) linked to basal, noninflammatory airway hyperreactivity in specific inbred mouse strains [26, 27, 31]. It was found in A/J mice that the enhanced baseline airway reactivity was independent of lymphocytes or bone marrow-derived cells [32]. Airway responses to MCh are regulated by muscarinic M3 receptor present on muscle cells, which induces muscle constriction, and negatively regulated by the activation of muscarinic M2 receptors on vagal nerve endings, which reduce acetylcholine release at the neuromuscular junction [2, 3, 33, 34]. Here we present evidence that enhanced basal (intrinsic) airway reactivity of AIRmin mice is associated with the low expression and activity of M2 receptors. This assumption is based on the following findings: firstly, mRNA and protein expression of M2 receptors were decreased in AIRmin mice compared to AIRmax animals; secondly, although mRNA and protein expression of M3 receptors, the major muscarinic pathway for airway smooth muscle constriction, were increased in AIRmax mice, these animals did not show enhanced basal airway reactivity; thirdly, the use of gallamine, a selective M2 receptor antagonist significantly increased the airway

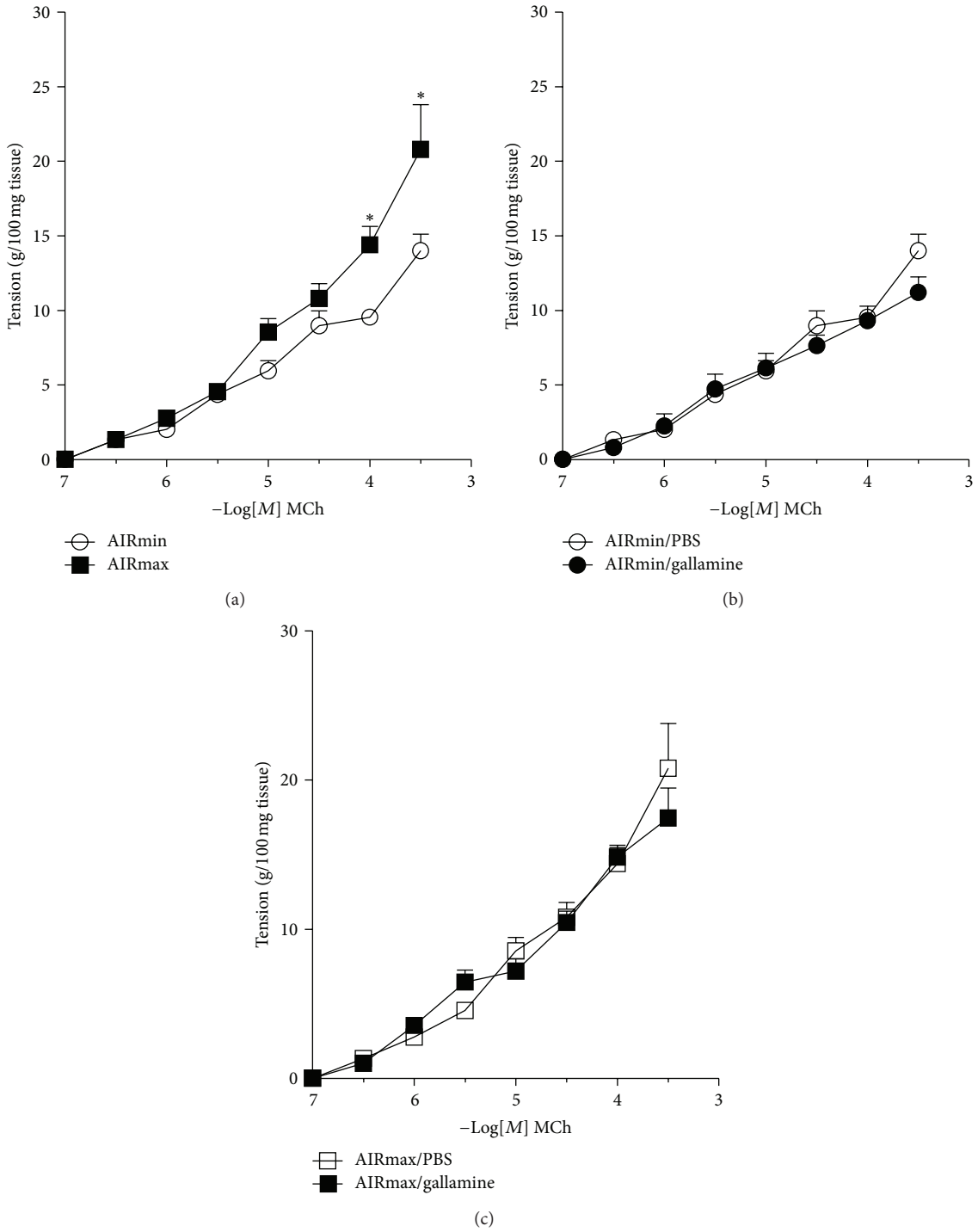

Figure 3: Tracheal responsiveness to MCh in AIRmin or AIRmax mice. Tracheal responsiveness of AIRmin or AIRmax to inhaled MCh was evaluated in the absence (a) or presence (b and c) of gallamine. Mouse tracheal segments were cultured in PBS or with gallamine for 20 min. Thereafter, the segments were maintained in the organ bath, and the contractile responses to MCh were recorded. Data are expressed as mean ± SEM of six mice per group $^{*}P < 0.05$.

responses of AIRmax mice, but affected minimally the responses of AIRmin mice. These results indicated that gallamine treatment impaired M2 function in AIRmax but not in AIRmin mice. Importantly, in conditions where the participation of vagal M2 receptors were precluded such as in *ex vivo* experiments with isolated tracheal rings, the tracheal responsiveness of AIRmin mice to MCh was not affected by gallamine treatment, reinforcing the predominant

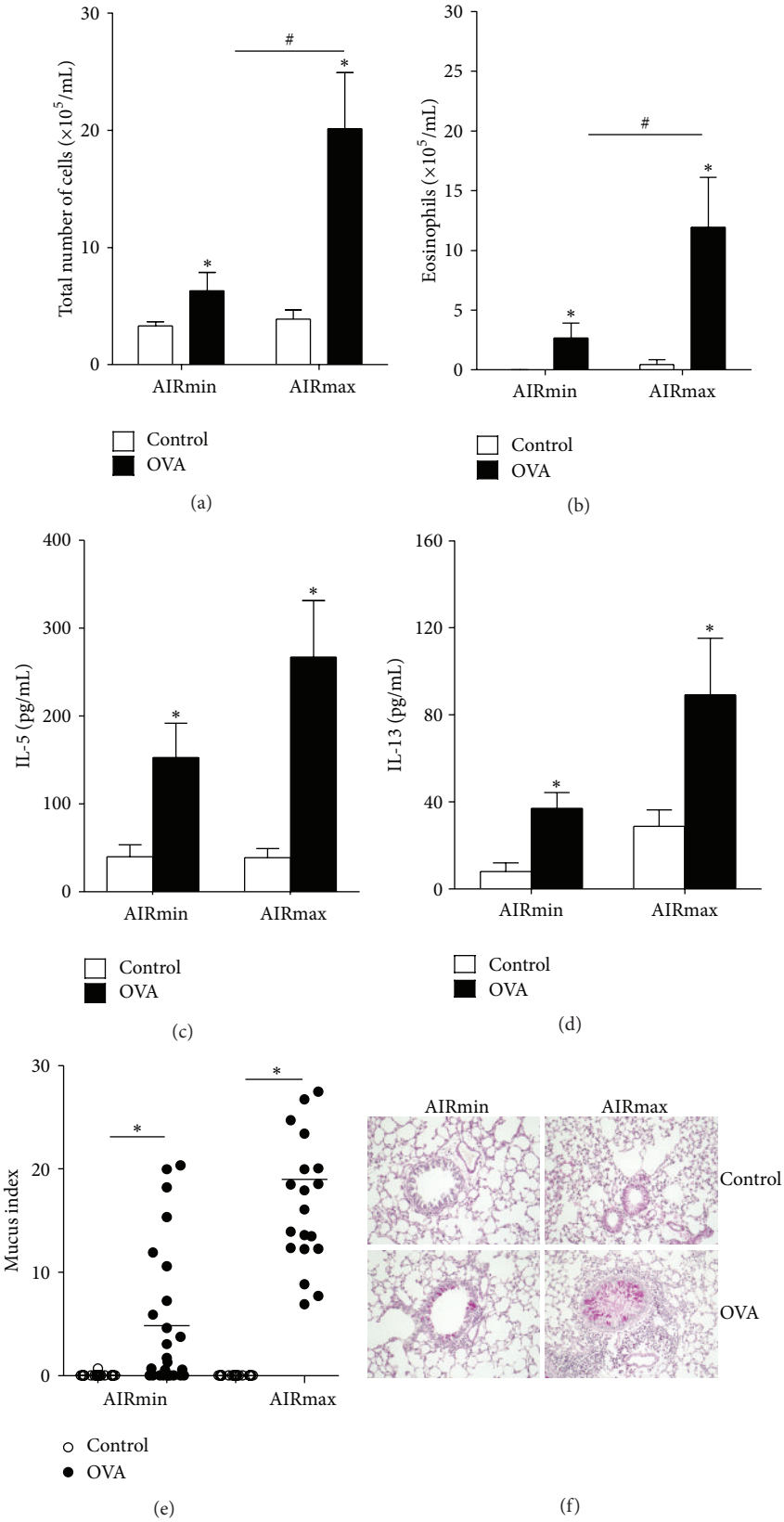

FIGURE 4: Allergic lung inflammation in AIRmin and AIRmax mice. AIRmin or AIRmax mice were sensitized twice (s.c.) with ovalbumin adsorbed on alum and challenged twice with ovalbumin (i.n.) (OVA group). Control group consisted of nonmanipulated animals. The experiments were performed 24 h after the last OVA challenge. The total inflammatory cells numbers (a), the number of eosinophils (b), and the levels of cytokines IL-5 (c) and IL-13 (d) were determined in bronchoalveolar lavage (BAL) fluid. The mucus index was determined by histocytometry of lung sections stained with periodic acid-Schiff (e) representative lung micrographs stained with HE of control and allergic AIRmin or AIRmax mice (original magnification ×200) (f). Data are expressed as mean ± SEM of four mice per group and are representative of two experiments; Statistical analyses of Student's t-test for (a), (b), (c), and (d). Statistical analyses of Mann Whitney-test for (e). $^{*}P < 0.05$ relative to control group. $^{\#}P < 0.05$ relative to AIRmin mice.

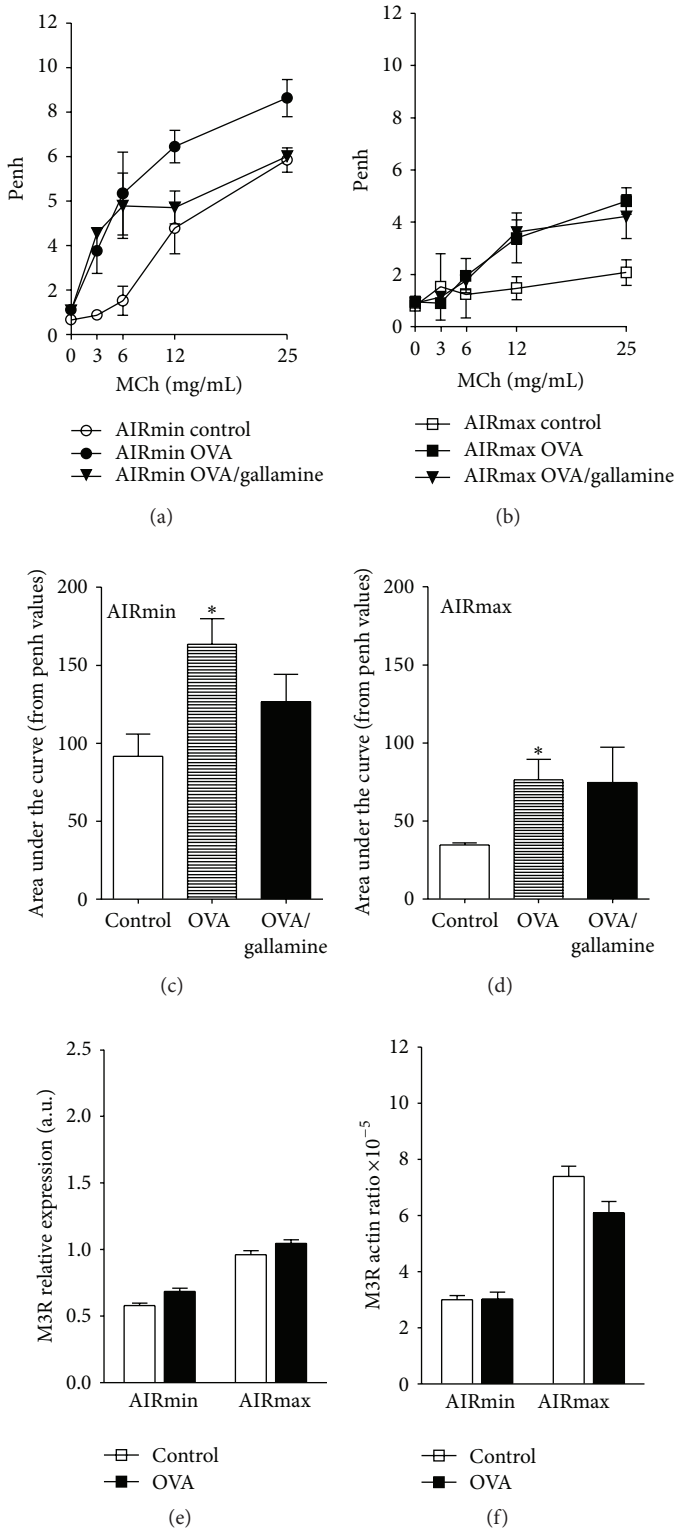

Figure 5: Continued.

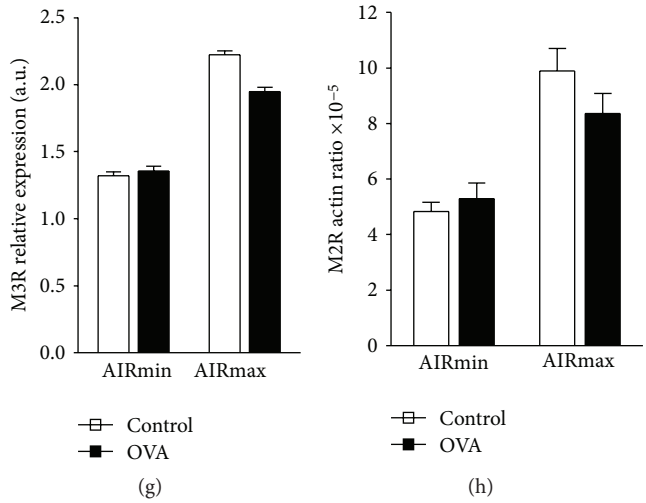

Figure 5: Pulmonary muscarinic receptors function and expression in allergic AIRmin and AIRmax mice. AIRmin or AIRmax mice were sensitized and challenged with OVA as in Figure 4. Control group consisted of nonmanipulated animals. The experiments were performed 24 h after the last OVA challenge. Respiratory pattern of allergic AIRmin (a and c) or AIRmax (b and d) to inhaled MCh was evaluated in the presence or absence of gallamine. Penh values were used as an index of bronchoconstriction induced after sequential delivery of increasing concentrations of MCh. Area under the curve was obtained from Penh values (c and d). Gene (e and g) or protein (f and h) expression of the M2 and M3 muscarinic receptors was evaluated by real-time PCR or Western blot analysis in lungs from OVA-sensitized AIRmin and AIRmax mice. The real-time PCR was carried out using β-actin gene expression as internal control for normalization of M3R (e) and M2R (g) mRNA transcription levels. In Western blot analysis the density of M3R (f) and M2R (h) protein expression was normalized to actin expression in each sample. Data are expressed as mean ± SEM of four mice per group and are representative of two experiments. Western blots data were quantified by densitometry using the ImageJ software (NIH). Statistical analyses of Student's t test for (a), (b), (e), (f), (g), and (h). Statistical analyses of ANOVA following Tukey HSD for (c) and (d). $^*P < 0.05$ relative to control group; NS, not significant.

role of M3 receptors in smooth muscle constriction. Our *ex vivo* experiments with tracheal rings are in line with previous studies in M2 receptor KO mice, which showed that M2 receptors do not exert an inhibitory effect on tracheal constriction induced by cholinergic stimuli [35, 36]. Several studies have shown that the loss of M2 receptor function increases acetylcholine release and potentates vagally mediated bronchoconstriction (reviewed in [15]). Similar to our findings, experiments performed with estrogen receptor-α KO mice showed enhanced airway responsiveness to inhaled MCh and serotonin under basal conditions and this was associated with reduced M2 receptor expression and function [28, 36]. In addition, our results are in line with the work of Fisher et al. showing that M2-deficient (KO) mice display a significantly enhanced *in vivo* bronchoconstrictor response to vagal stimulation [37].

In allergic conditions, AIRmin and AIRmax mice showed a significant increase over baseline response (control group) in Penh value. The change in respiratory pattern of AIRmax animals after allergic inflammation could not be attributed to increased expression of M3 receptors. Because gallamine treatment had no effect, we favor the notion that the major mechanism for the increased respiratory responses of allergic AIRmax mice was due to decreased expression and/or dysfunction M2 receptor. This finding is in accordance with previous studies in allergic mice or asthmatic patients indicating that M2 receptor dysfunction rather than M3 receptor overexpression is associated with airway hyperreactivity (reviewed in [3, 4, 33]). It was shown by Ricci et al. (2002, 2008) that patients with asthma or rhinitis present different expression M2 and M3 receptors on lymphocytes, but the role of these receptors in these cells is still elusive [38, 39].

A striking difference between AIRmin and AIRmax animals was related to the intensity of T-helper-type-2-cells- (Th2-) mediated allergic responses such as eosinophilic inflammation, type 2 cytokines production, and mucus production that were all significantly higher in AIRmax when compared with AIRmin animals. It is known that inflammatory mediators play a role in enhancing bronchoconstriction by several pathways. The major basic protein from eosinophils can bind directly to M2 receptors, blocking the interaction with acetylcholine that leads to a higher release of acetylcholine in airway smooth muscles [10]. In addition, type 2 cytokines play a critical role in the development of bronchial responsiveness. IL-13, for instance, may increase bronchoconstriction by directly modulating smooth muscle contractibility and promoting airway hyperreactivity through multiple molecular mechanisms [40, 41]. The participation of these signaling pathways in our asthma murine model in AIRmin and AIRmax animals remains to be determined.

Interestingly, AIRmin and AIRmax mice that were selected by an innate stimuli maintained the polarized inflammatory profile with a stimulus derived from T-cell-dependent adaptive inflammation. We speculate that the enhanced Th2 responses of AIRmax animals was due to an enhanced production of inflammatory cells in bone marrow, as shown previously for neutrophils [42].

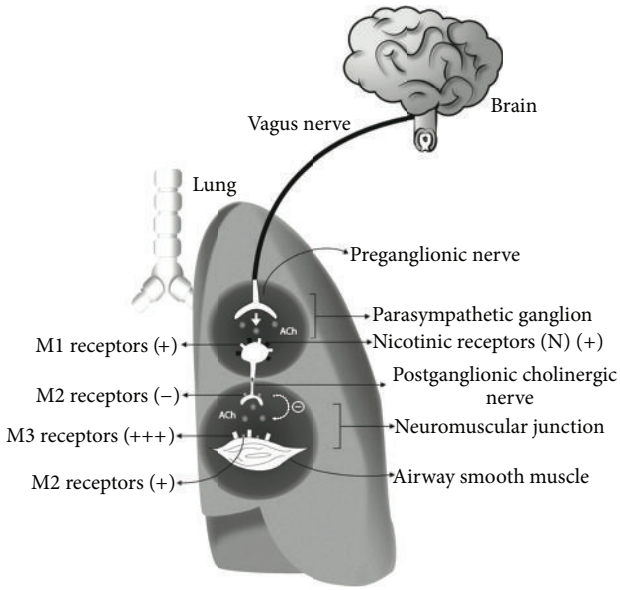

Figure 6: A model illustrating cholinergic receptors on pulmonary parasympathetic nerves and airway smooth muscle. Acetylcholine (ACh) released at parasympathetic ganglion binds to nicotinic (N1) and muscarinic (M1) receptors and activates postganglionic cholinergic nerve to release ACh at neuromuscular junction. ACh at neuromuscular junctions binds to M3 receptors in the airway smooth muscle, inducing muscle contraction, and to the autoinhibitory M2 receptors located in the vagus nerve, that limits ACh release at the neuromuscular junction and decreasing smooth muscle constriction.

How M2 receptors could be associated with two contrasting situations? On one side, M2 dysfunction was associated with AIRmin animals that have been selected for low inflammatory response and on the other side with AIRmax animals that develop an intense allergic inflammation. These apparently conflicting findings can be reconciled by considering that acetylcholine can also regulate inflammation via the nicotinic acetylcholine receptor subunit alpha7 (α7nAchR) known as the "cholinergic anti-inflammatory pathway" [16, 17, 43, 44]. It follows that cholinergic anti-inflammatory pathway would favor the selective process of AIRmin animals. The α7nAchR is coded by *Chrna7* gene, which is mapped closed to inflammatory response modulator 1 (*Irm1*) locus on chromosome 7 [45]. The *Irm1* locus region contains about 230 known genes that are involved in inflammatory response regulation [45]. Interestingly, AIRmin mice present the same *Imr1* locus haplotype as A/J MCh hyperresponsive mice. The genotype of AIRmin at the rs32017050 (SNP at the peak of the *Irm1* locus) is "TT" similar to A/J mice, which is one of the 8 parental strains used in F0 population of those selected mice. On the other hand, AIRmax mice are "CC", showing identical genotypes of C57BL/6J, CBA/J, DBA/2J, P/J, and SWR/J mice (mouse genomic informatics) that are hyporesponsive to MCh. Thus, M2 receptor dysfunction in AIRmin animals can be viewed as an additional mechanism inherited during the selective process that contributed for the low inflammatory phenotype. In the same vein, the loss of M2 receptor function observed in AIRmax animals during allergic inflammation could be interpreted as a neural homeostatic mechanism of the organism to control allergic inflammation [46]. Overall, the neuronal circuits that operate in inflammation or allergic asthma can be viewed as homeostatic mechanisms to counterbalance the inflammatory process.

Since asthma is a complex syndrome, the AIRmin/AIRmax model might mirror clinical cases where airway reactivity is more prominent than airway inflammation as described in fatal asthma and in clinical conditions where airway inflammation is more robust than airway reactivity [47].

4. Material and Methods

4.1. Mice. The AIRmax and AIRmin original mice were obtained by bidirectional selective breeding starting from a high polymorphic population produced by intercross between eight inbred mouse strains (A, DBA-2, P, SWR, CBA, SJL, BALB/c, and C57Bl/6) [20]. The selective breeding of these animals for maximal or minimal acute inflammatory response was based on both cellular infiltrate and protein contents in the inflammatory exudate 24 h after subcutaneous implant of polyacrylamide beads (Biogel). The main interline difference was characterized by the strong differential neutrophil PMN count in inflammatory exudate. Several quantitative trait loci (QTL) were recently detected in these mouse lines [46, 48]. Animals were obtained and maintained at the animal facilities of the Immunogenetics Laboratory at Butantan Institute on standard pellet food and water *ad libitum*. All experiments were performed following the guidelines for animal use approved by the Ethics Committee in Animal Experimentation of the University of São Paulo, Brazil, which is in accordance with the Ethical Principals of the Brazilian College of Animal Experimentation (COBEA), ethics protocol number 26, page 30, Book 2, ICB, USP.

4.2. Ovalbumin (OVA) Sensitization and Challenge. Mice were sensitized and boosted subcutaneously (s.c.) with 4 μg of chicken OVA (Sigma-Aldrich, St. Louis, MO, USA) and 1.6 mg of aluminum hydroxide gel in 0.2 mL of sterile PBS on days 0 and 7. Removal of lipopolysaccharides from OVA was performed as previously described [49]. After LPS removal, the endotoxin level of OVA was below the limit of detection in the LAL (Limulus amoebocyte lysate) assay (QCL-1000 kit, BioWhittaker, Walkersville, MD, USA). Airway inflammation was induced by two intranasal (i.n.) challenges with 10 μg OVA on days 14 and 21, animals were anaesthetized with ketamine (50 mg/kg body weight) and xilazine (20 mg/kg body weight) intraperitoneally in 200 μL of PBS for intranasal instilations. Experiments were performed 24 h after the last i.n. OVA challenge, on day 22.

4.3. Airway Responsiveness to Methacholine by Noninvasive Barometric Plethysmography. Respiratory parameters were determined before and after administration of increasing doses of inhaled MCh (3, 6, 12, and 25 mg/mL) by noninvasive method in conscious unrestrained mice using whole-body plethysmograph (Buxco Electronics Inc. Wilmington, North

Carolina, USA), as previously described [50, 51]. Signals were analyzed using BioSystem XA software to derive whole-body flow parameters including respiratory frequency, tidal volume, minute ventilation, peak inspiratory flow, peak expiratory flow, and enhanced pause (Penh). Penh is a dimensionless value that takes into account box pressure recorded during inspiration and expiration and the timing comparison of early and late expiration, which was used to define the breathing pattern. Linear interpolation was used to determine the provocative concentration of MCh aerosol at which a 200% increase (PC200) over baseline values was observed for Penh values. To test M2 muscarinic receptor function, mice were exposed or not to M2 muscarinic receptor antagonist gallamine (30 μM/animal) [28], nebulized 30 minutes before recording of respiratory parameters.

4.4. Ex Vivo Trachea Responsiveness to Methacholine. To test M2 muscarinic receptor function, rings of tracheal tissue were removed and mounted using two steel hooks in a 15 mL organ bath for the measurement of isometric force contraction [47] in presence or absence of the M2 muscarinic receptor antagonist gallamine (30 μM), added 20 minutes before administration of MCh. The force contraction was determined using a force displacement transducer and a chart recorder (Powerlab Labchart, AD Instruments, Colorado Springs, CO, USA). The tracheal tissue was maintained in an organ bath filled with Krebs-Hanseleit (KH) buffer at 37°C continuously aerated (95% O_2 and 5% CO_2) for 40 min (equilibrium period). After this period, the tracheal tension was adjusted to 0.5 g, and the tissue viability was assessed by replacing KH solution with KCl buffer (60 mM). The cumulative dose-response curve to MCh was constructed according to Van Rossum (1963) [52].

4.5. Bronchoalveolar Lavage Fluid. Mice were deeply anaesthetized by intraperitoneal injection of xilazine (50 mg/kg body weight) and ketamine (100 mg/kg body weight), folowed by cervical dislocation. Blood samples from the retro-orbital plexus were collected for serum antibody determinations. The trachea was cannulated, and lungs were washed twice with 0.5 and 1.0 mL PBS, respectively. Total and differential cell counts of bronchoalveolar lavage (BAL) fluid were determined by haemocytometer and cytospin preparation stained with Instant-Prov, hematoxylyn-eosin (Newprov, Pinhais, PR, Brazil).

4.6. Cytokines Measurements. The levels of cytokines (IL-5 and IL-13) in the BAL fluid were assayed by sandwich kit ELISA according to the manufacturer's instruction (PharMingen, San Diego, CA, USA), as previously described [53]. For IL-13 determinations, the pair used was 38 213.11 and biotinylated goat polyclonal anti-IL-13 from R&D Systems (Minneapolis, MN, USA). Values were expressed as pg/mL deduced from standards that run in parallel with recombinant cytokines. The limit of detection was 10 pg/mL for IL-5 and 31 pg/mL for IL-13.

4.7. Histological Analyses. After BAL collection, lungs were perfused via the right ventricle with 10 mL of PBS to remove residual blood, then immersed in 10% phosphate-buffered formalin for 24 h, and in 70% ethanol until embedded in paraffin. Lung sections with 5 μm were stained with hematoxylin/eosin for evaluation of peribronchial and perivascular lung inflammation or with periodic acid-Schiff (PAS)/haematoxylin for the evaluation of mucus production, as previously described [51]. Briefly, a quantitative digital morphometric analysis was performed using the application program Metamorph 6.0 (Universal Images Corporation, Downingtown, PA, USA). The circumference area of bronchi and the PAS-stained area were electronically measured, and the mucus index was determined by the following formula: (PAS-stained area/bronchial circumference area) × 100.

4.8. Western-Blot Assays. Lungs from mice were homogenized in ice-cold lysis buffer containing protease inhibitors, 10 mM TrisHCl (pH 7.5), 8 M urea, 20 mM EDTA, 150 mM NaCl, and 1% Triton X-100. Membrane proteins (40 μg) were fractionated by differential centrifugation, separated by SDS-PAGE and transferred to nitrocellulose membranes. The incubation procedure was performed using primary and secondary antibodies, as previously described [54–57]. The following polyclonal rabbit antisera were used: anti-M1 (1 : 200), anti-M2 (1 : 100), anti-M3 (1 : 100), and anti-β-actin (1 : 400) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin protein expression was used as an internal standard for relative quantification of muscarinic receptors expression levels. Western blots were quantified by densitometry using the ImageJ software (NIH, Washington DC, USA).

4.9. RNA Isolation, Reverse Transcription, Real-Time PCR, and Conventional-PCR. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA)) from lungs of nonmanipulated AIRmax and AIRmin mice. Integrity of the isolated RNA was verified by separation of an aliquot of the extracted RNA on a 2% ethidium bromide-stained agarose gel. DNA was removed from RNA samples by incubation with DNase I (Ambion Inc., Austin, TX, USA).

Total RNA (3 μg) was reversely transcribed to cDNA with 200U of RevertAid H Minus M-MuLV-reverse transcriptase (Fermentas Inc., Hanover, MD, USA). DNA templates were amplified by real-time PCR on the 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA,USA) using the Sybr green method [54, 56] or were amplified by PCR and analyzed as described previously [54, 56]. Variations in cDNA concentrations were normalized with β-actin as an internal control, which is a constitutively expressed gene. Experiments were performed in triplicate for each data point. Primer sequences for reverse transcription and quantitative PCR amplification (qRT-PCR) of mRNA used in this study were β-actin FWD ctg gcc tca ctg tcc acc tt, REV cgg act cat cgt act cct gct t; M2 mAChR FWD gct gcg tgg gtt ctt tcc t, REV ccc cta cga tga act gcc ag; M3 mAChR FWD cca tct ggc aag tgg tct tc, REV tgc cac aat gac aag gat gtt g. Negative controls were conducted on water and on total

RNA. All PCR reactions were quantitative reactions, made by real-time PCR.

4.10. Statistical Analysis. Statistical analyses were performed employing JMP 9.0 (JMP SAS Institute Inc). Results are expressed as mean ± SEM. Continuous variables were log-transformed for the analyses when the normality of the distribution was rejected by the Shapiro-Wilk W test. For comparisons between two groups, normality was tested and then data were submitted to Student's t-test. For comparisons amongst 3 or more groups it was used ANOVA following In the case of absence of normality, even in log-transformed variables, Mann-Whitney was used for comparisons between 2 groups and or Kruskal-Wallis tests for three or more groups.

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