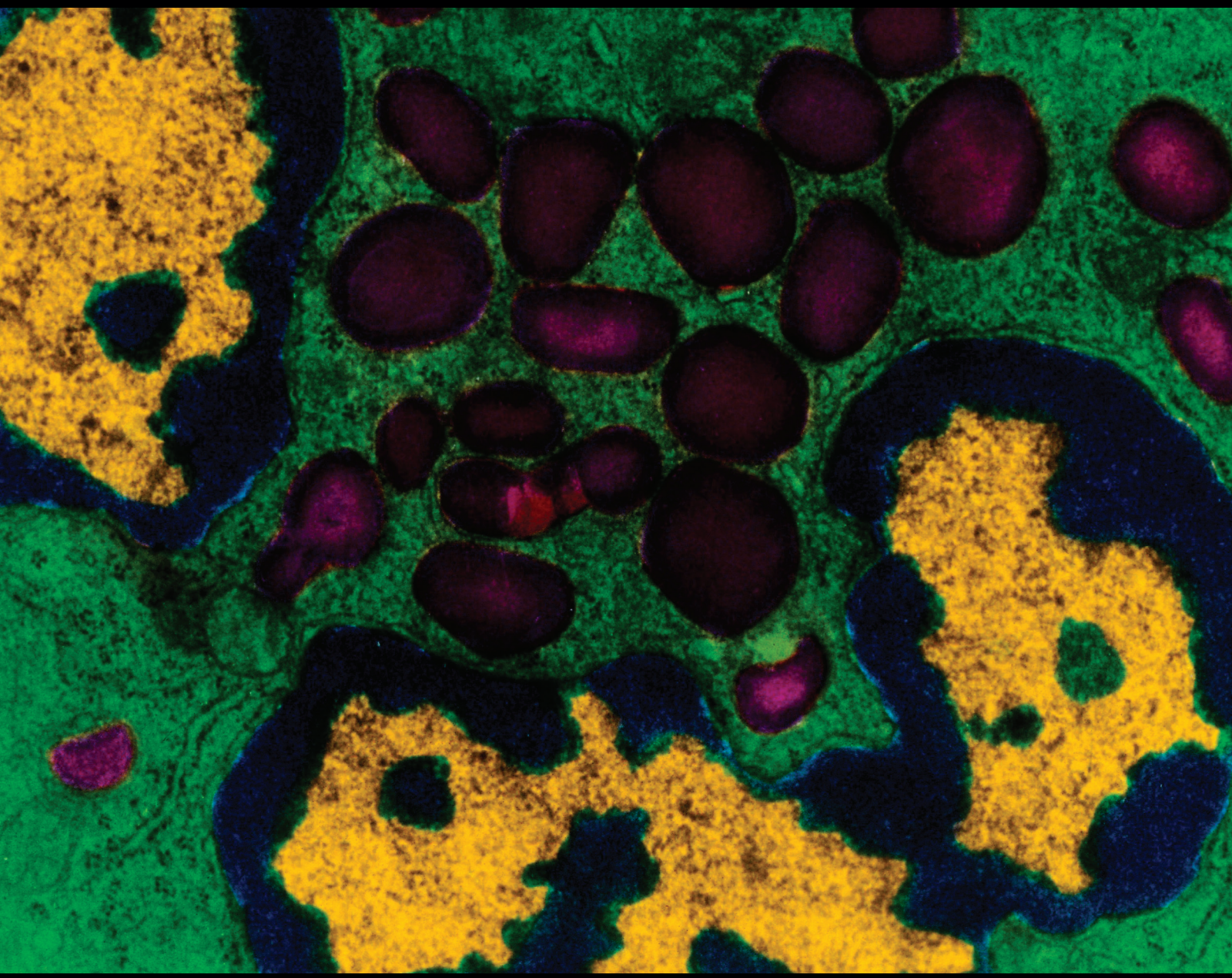
Mediators of Inflammation

Immunometabolism: Molecular Mechanisms, Diseases, and Therapies 2016

Lead Guest Editor: Jose C. Rosa
Guest Editors: Fabio S. Lira, William Festuccia, and Soumen Roy



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Editorial Board

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Editorial

Immunometabolism: Molecular Mechanisms, Diseases, and Therapies 2016

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In the second edition of the special issue titled "Immunometabolism: Molecular Mechanisms, Diseases, and Therapies," a total of 37 manuscripts were received, and 16 of these were accepted. This issue demonstrated that nonresolving, chronic low-intensity inflammation not only is involved in the development and maintenance of several metabolic diseases such as visceral obesity, type 2 diabetes, dyslipidemias, atherosclerosis, hypertension, and cancer but also acts as an important linking factor between those conditions. Metabolic disease-associated inflammation is characterized by the recruitment of cells from both the innate and adaptive immune system to metabolic tissues followed by their polarization to a proinflammatory profile, resulting in the exacerbated local production of inflammatory mediators. These processes are mainly activated by the canonical Toll-like receptor- (TLR-) NFκB signaling pathway, which can be triggered by several molecules such as the lipopolysaccharide from gut microbiota and saturated fatty acids, among others.

This special issue successfully attracted several interesting original and review articles addressing different aspects of the intricate relationships between metabolism and inflammation in health and disease. E. Nishida et al., for example, exploring the association between cardiovascular diseases and periodontitis, a chronic inflammatory disease that affects the periodontium, demonstrated that the acute-phase protein serum amyloid A, which is elevated in the liver and blood of apolipoprotein E-deficient, atherosclerosis-prone mice, promotes the expression of adhesion molecules in human aortic endothelial cells via TLR2, being therefore a candidate linking factor between periodontal disease and atherosclerosis. Subsequently, in an interesting, mechanistic study, Z.-Z. Guo et al. established a role for the c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family which is activated by inflammatory signals and other stress stimuli, as an important mediator involved in the formation of abdominal aortic aneurysm induced by angiotensin II plus nicotine, a major chemical component of cigarettes. These findings indicate that JNK inhibition may hold promise as a pharmacological target to attenuate smoking-induced abdominal aortic aneurysm formation. In an elegant study exploring the mechanisms underlying the immunomodulatory actions of bilirubin, K. F. Corral-Jara et al. found strong evidence indicating that, during hepatitis A virus infection, conjugated bilirubin differentially regulates CD4+ T lymphocytes and Tregs function by modulating intracellular pathways and by inducing changes in the proportion of Tregs expressing hepatitis A virus cellular receptor (HAVCR1/TIM-1). These findings may help in the elucidation of the mechanisms involved in hepatitis virus infection.

This special issue also gathered several studies that investigated the underlying mechanisms by which some nutrients

and modulation of tissue oxygen levels may attenuate the chronic low-grade inflammation associated with metabolic diseases. Indeed, S. I. Pærregaard et al. investigated the involvement of membrane-free fatty acid receptor-4 (FFAR4), also known as GPR120, as mediator of the beneficial actions of $\omega 3$ fatty acids on metabolic health. By feeding *Ffar4* knockouts and heterozygous mice with either a control or an $\omega 3$-rich diet for 36 weeks, S. I. Pærregaard et al. show that FFAR4 signaling is not required for the anti-inflammatory and insulin-sensitizing effects mediated by $\omega 3$ fatty acids. G. Guan et al., on the other hand, presented interesting findings indicating that intake of a diet containing chitosan, polysaccharides found in insects, fungi, squid, oysters, krill, clams, and shellfish changes the composition of mice intestinal microflora by suppressing NF-κB, TNF-α, and IL-6 and inducing a better control of inflammation and resolution of infection with *C. rodentium*. Moreover, V. Oliveira et al. showed that oligofructose supplementation in pregnancy and lactation induces the proinflammatory markers in dams and 90-day-old offspring and reduction in adiponectin pathway. In addition, F. Elbers et al. reported an impairment in the immunomodulatory and antimicrobial actions of the hepatic enzyme tryptophan 2,3-dioxygenase, which converts tryptophan, the essential amino acid for hosts and pathogens, to N-formylkynurenine, the precursor of the immune-relevant kynurenines, upon hypoxic conditions mimicking those occurring in vivo during infection and cancer, for example. These findings indicate that hypoxia might be detrimental for the appropriate host immune response towards relevant pathogens. In contrast to the deleterious effects of hypoxia, in an interesting study, S. Novak et al. reported that hyperbaric oxygenation treatment, which increases tissue oxygen content, oxidative metabolism, and production of reactive oxygen species (ROS), significantly reduces the severity of dextran sodium sulphate-induced colitis improving the inflammatory microenvironment in the gut mucosa, such an effect that seems to be mediated in part by the transcriptional factor hypoxic inducible factor 1 α (HIF-1α). Finally, regarding alternative treatments to inflammatory disease, W. Ren et al. reported that supplementation of drinking water of rodents with interferon tau, a type I interferon produced by trophectoderm cells of conceptuses of ruminant species, increased microbial diversity in the jejunum and ileum and decreased the expression of IL-17 in the intestines of normal and pathogen-infected mice, being therefore a candidate therapy strategy to treat the inflammatory intestinal diseases.

Two studies in this special issue were performed in humans aiming to further characterize obesity-associated inflammation. F.-I. Corona-Meraz et al., by evaluating the inflammatory and metabolic phenotype of subjects with obesity and insulin resistance, found that chemerin, an adipokine related to adiposity levels and fat distribution, is associated with obesity, dyslipidemia, and insulin resistance, whereas its receptor chemerin chemokine-like receptor 1 (CMKLR1) is associated only with obesity. C. Wang et al., on the other hand, by evaluating the gene expression profile of the visceral adipose tissue from lean and obese subjects from the Uyghur population, found a correlation between reduced expression of A2b adenosine receptor and the transcriptional factors Kruppel-like factors 4 and 5 with obesity-associated dyslipidemia and inflammation.

This special issue also brings some interesting review articles addressing several aspects of metabolic disease-associated inflammation. D. Ortuño-Sahagún et al., for example, reviewed recent evidence supporting the link between obesity and the pathogenesis of multiple sclerosis, a chronic autoimmune and inflammatory disease. P.-F. Bryan et al., on the other hand, reviewed the mechanisms involved in the regulation of cytokine profile by sphingolipids, the role of gut microbiota in providing signaling molecules that favor the communication between resident bacteria and intestinal cells, and the potential of sphingolipids and gut microbiota as targets or therapeutic agents for inflammatory bowel disease. H. J. Coelho Junior et al. reviewed the possible molecular mechanisms associated with muscle atrophy in stroke patients, as well as the modulatory effect of inflammation in this condition. G. van Niekerk et al. reviewed evidence suggesting that sickness-associated anorexia may be a mechanism by which autophagic flux is upregulated systemically and claim that some patients might benefit from permissive underfeeding. Finally, U. Nydegger et al. reviewed some approaches to sort out from big data the relevant results for patient care in precision medicine.

Therefore, in our opinion, this special issue brings new insights into the intricate mechanisms driving the inflammatory processes associated with metabolic diseases. We hope that these information will help to pave the way for the development of efficient strategies to prevent and treat these diseases.

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Research Article

FFAR4 (GPR120) Signaling Is Not Required for Anti-Inflammatory and Insulin-Sensitizing Effects of Omega-3 Fatty Acids

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Free fatty acid receptor-4 (FFAR4), also known as GPR120, has been reported to mediate the beneficial effects of omega-3 polyunsaturated fatty acids (ω3-PUFAs) by inducing an anti-inflammatory immune response. Thus, activation of FFAR4 has been reported to ameliorate chronic low-grade inflammation and insulin resistance accompanying obesity. However, conflicting reports on the role of FFAR4 in mediating the effects of ω3-PUFAs are emerging, suggesting that FFAR4 may not be the sole effector. Hence analyses of the importance of this receptor in relation to other signaling pathways and prominent effects of ω3-PUFAs remain to be elucidated. In the present study, we used *Ffar4* knockouts (KO) and heterozygous (HET) mice fed either low fat, low sucrose reference diet; high fat, high sucrose ω3-PUFA; or high fat, high sucrose ω6-PUFA diet for 36 weeks. We demonstrate that both KO and HET mice fed ω3-PUFAs were protected against obesity, hepatic triacylglycerol accumulation, and whole-body insulin resistance. Moreover, ω3-PUFA fed mice had increased circulating protein levels of the anti-inflammatory adipokine, adiponectin, decreased fasting insulin levels, and decreased mRNA expression of several proinflammatory molecules within visceral adipose tissue. In conclusion, we find that FFAR4 signaling is not required for the reported anti-inflammatory and insulin-sensitizing effects mediated by ω3-PUFAs.

1. Introduction

Obesity is associated with chronic low-grade inflammation causing inflammation-induced insulin resistance in various tissues [1, 2]. Overnutrition increases the need for storage of excess energy, resulting initially in adipocyte hypertrophy, later accompanied by hyperplasia, and recruitment of classically activated, proinflammatory M1 macrophages into adipose tissue [3, 4]. M1 macrophages secrete proinflammatory cytokines, recruiting additional M1 macrophages, but they also promote resident alternatively activated, anti-inflammatory M2 macrophages to differentiate towards the M1 phenotype, thereby propagating a self-amplifying vicious inflammatory cycle [4–6]. The ability of inflammation to interfere with insulin signaling was first described for tumor necrosis factor-α (TNFα) in 1993 by Hotamisligil et al. [7].

Upon binding of TNFα, IκB kinase-β (IKKβ) and c-jun N-terminal amino kinase-1 (JNK1) are activated. These serine kinases initiate proinflammatory gene transcription through activation of nuclear factor κB (NFκB) and activating protein-1 (AP-1), but they also have the potential to phosphorylate the insulin receptor substrates 1 and 2 (IRS1 and IRS2), inhibiting their association with the insulin receptor [1, 8, 9]. Additionally, TNFα-signaling upregulates expression of Suppressor of Cytokine Signaling (SOCS) protein family members, which are able to directly bind and antagonize the insulin receptor [10, 11]. Interleukin-6 (IL-6) mediated signaling similarly induces expression of proinflammatory genes and increases the expression of SOCS3 [11, 12]. Besides decreased glucose uptake into adipocytes, insulin resistance also leads to enhanced lipolysis augmenting the amount of circulating nonesterified fatty acids (NEFAs) [13]. This increases the risk for ectopic lipid accumulation in liver and muscle, further exacerbating insulin resistance, providing a link between dysfunctional adipose tissue and fatty liver [13, 14]. Yet the metabolic impact of adipose tissue inflammation varies between depots, where inflammation in visceral adipose tissue exerts a greater negative metabolic impact than inflammation in subcutaneous adipose tissue [15, 16]. Augmented secretion of cytokines, adipokines, and NEFAs, especially from visceral adipose tissue, impacts the liver, thus affecting this key metabolic organ and consequently whole-body metabolism [6, 15]. Therefore, modalities to decrease obesity-associated inflammation are of great importance. In this regard, the discovery that FFAR4 seemed to be the main receptor mediating the anti-inflammatory and insulin-sensitizing effects of ω3-polyunsaturated fatty acids (ω3-PUFAs) in adipocytes and macrophages spurred considerable interest in this receptor [17]. In recent years, the anti-inflammatory potential of FFAR4 signaling in other cell types and tissues, that is, Kupffer cells [18], colonic Caco-2 cells [19], and hypothalamus [20], has been investigated.

The anti-inflammatory effect of FFAR4 depends on the scaffold protein, β-arrestin 2, which upon ligand-binding of FFAR4 is recruited to the C-terminal, leading to internalization of the complex [17]. The complex is able to interfere with inflammatory signaling pathways, such as TNFα and toll-like receptor-4 (TLR4) mediated signaling, thereby decreasing inflammation [17]. This anti-inflammatory effect of FFAR4 was found to be responsible for the increased insulin sensitivity in a high fat diet (HFD) fed mouse model supplemented with ω3-PUFAs [17]. The authors ascribed the insulin-sensitizing effect of FFAR4 activation to derive from macrophages [17]. Interestingly, another group found FFAR4-deficient HFD fed mice to be more metabolically impaired, steatotic, and insulin resistant compared to their wild type (WT) counterparts independent of ω3-PUFA supplementation, suggesting that FFAR4 *per se* has an important role in energy homeostasis [21].

Contradicting the majority of existing literature [17, 21, 22], recent evidence suggests that FFAR4 is dispensable for the beneficial effects of ω3-PUFAs on HFD-induced obesity [23], whereas the anti-inflammatory nature of FFAR4 remains largely unchallenged. Here we show that feeding mice a high dose of ω3-PUFAs protects against HFD-induced obesity, steatosis, insulin resistance, and visceral adipose tissue inflammation independent of FFAR4 status.

Table 1: Diet composition of the three different diets. A detailed description can be found in supplementary Table S1.

	Diet composition		
	Low fat	Fish oil	Soy oil
kcal/g	3.82	4.54	4.54
Protein (kcal%)	19	15	15
Fat (kcal%)	17	42	42
ω3-PUFA	*0.82*	*14.9*	*2.5*
(% of total fat)	*(4.82)*	*(35.48)*	*(5.95)*
ω6-PUFA	*7.63*	*1.0*	*22.75*
(% of total fat)	*(44.88)*	*(2.38)*	*(54.17)*
ω6 : ω3 ratio	*9.31 : 1*	*0.07 : 1*	*9.10 : 1*
Carbohydrates (kcal%)	64	43	43
Sucrose	*13.5*	*29.5*	*29.5*
(% of total carbohydrate)	*(21,10)*	*(60.60)*	*(60.60)*

2. Materials and Methods

2.1. Animal Care and Use. The *Ffar4* KO mouse strain was generated by Lexicon Pharmaceuticals Inc. on a mixed background of 129SVE and C57BL/6J mice. The deleted sequence of *Ffar4* (gene accession NM_181748.2) corresponds to exon 1. Mice were bred at Taconic Laboratories and used under license (2014-15-2934-01027). All animal experiments were conducted in accordance with Danish national guidelines (Amendment #1306 of November 23, 2007) approved by the Danish Animal Inspectorate, Ministry of Justice.

Male mice were kept as mixed genotypes in cages (n = 3–5 per cage) under specific pathogen-free conditions at a 12 h light/dark cycle, 22-23°C, and a humidity of 30%. Mice were scaled once a week from 6 weeks of age and MR-scanned prior to the insulin tolerance test (ITT) (week 32 after diet initiation) using EchoMRI 4 in 1 (Texas, USA).

2.2. Diets. Diets were obtained from Ssniff Spezialdiäten GmBH, Germany, with catalog numbers: low fat, low sucrose reference diet (S8672-E050 EF AIN93G); high fat, high sucrose fish oil diet/ω3-PUFA (S8672-E409 EF D12079B); and high fat, high sucrose soy oil diet/ω6-PUFA (S8672-E408 EF D12079B). Diets were kept at −20°C when not in use. Diet composition is shown in Table 1, with a detailed description in Table S1, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1536047. Feed intake (Figures 1(f) and 1(l)) was measured in parallel in single-housed mice fed the same diets. All other data were derived from group-housed mice. Mice were given free access to feed and water and fed fresh experimental diets twice a week from 11 weeks of age.

2.3. Insulin Tolerance Test (ITT). After 33 weeks on experimental diets mice were feed-deprived for two hours prior to insulin injections (Actrapid Penfill, Novo Nordisk, Denmark). Insulin was diluted in succinylated gelatin (gelofusine,

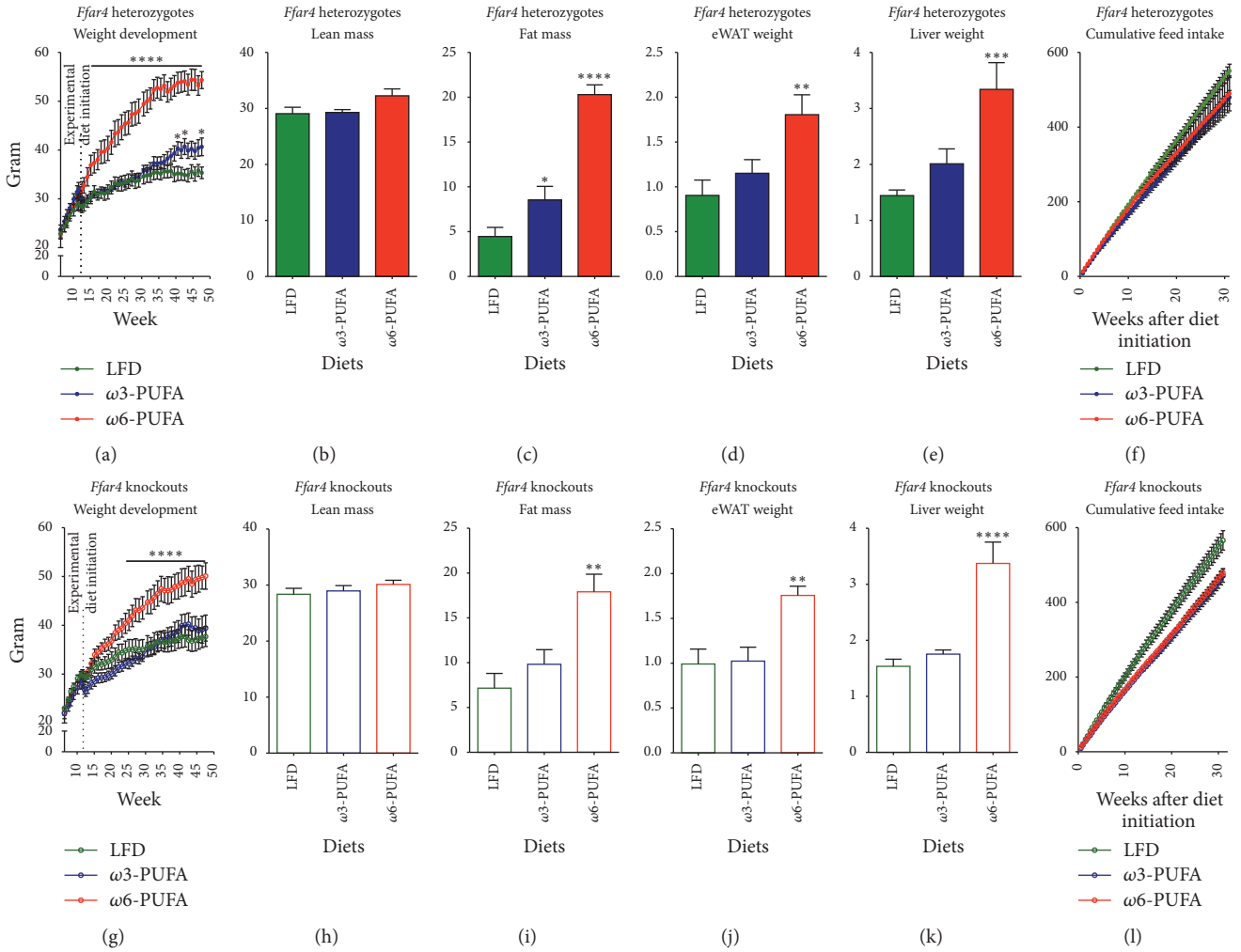

Figure 1: *Ffar4* HET and KO mice are protected against obesity development on a high fat ω3-PUFA diet. Mice were scaled weekly from 6 weeks of age; experimental diets were initiated when mice were 11 weeks old. ((a)–(f)) Heterozygotes. ((g)–(l)) Knockouts. ((a) and (g)) Weight development, $n = 6$–8. ((b) and (h)) Lean mass from MR scans 32 weeks after diet initiation (43 weeks of age), $n = 6$–8. ((c) and (i)) Fat mass from MR scans 32 weeks after diet initiation, $n = 6$–8. ((d) and (j)) eWAT weights 36 weeks after diet initiation, $n = 6$–8. ((e) and (k)) Liver weights 36 weeks after diet initiation, $n = 6$–8. ((f) and (l)) Feed intake measured two times a week in single-housed mice till 32 weeks after diet initiation, $n = 5$–7. All data are presented as means ± SEM. Both HFDs are compared to the LFD. For (a), (f), (g), and (l) data were Ln-transformed and 2-way RM ANOVA with Bonferroni correction was performed. All data shown in bar graphs were Ln-transformed and subjected to 1-way ANOVA with Bonferroni correction. Only statistical significant differences are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

B. Braun Melsungen AG, Germany) to increase accuracy of insulin delivery. 1 U insulin per kg lean body mass was injected intraperitoneally (i.p.) after measurements of initial blood glucose concentrations. Mice were bled from the tail vein, and blood glucose was measured using the Bayer Contour Glucometer (Bayer Health Care, Germany) at indicated time points (15, 30, 45, 60, 90, and 120 minutes after injection).

2.4. Measurement of Insulin and Adiponectin Levels. Plasma was collected from 5-hour feed-deprived mice by tail vein bleeding and diluted two times prior to insulin measurements. For adiponectin measurements, plasma was collected from nonfasted mice by bleeding from the submandibular vein. For all plasma samples, blood was drawn in EDTA coated tubes kept on ice and centrifuged at 4°C for 10 minutes at 1000 ×g before storage at −20°C until further use. Insulin and adiponectin measurements were carried out using an electrochemiluminescence assay (Mesoscale Diagnostics, USA) following the manufacturer's instructions.

2.5. RNA Extraction and Quantitative RT-PCR. Liver and epididymal white adipose tissue (eWAT) were homogenized in TRIreagent (Sigma-Aldrich) using the Precellys homogenizer (Bertin Technologies). Chloroform (Sigma-Aldrich) was added for phase-separation, and RNA was precipitated by addition of isopropanol (Sigma-Aldrich) and washed with 75% ethanol (CCS Healthcare) before resuspension in autoclaved Milli-Q water. 1 μg of total RNA was used for reverse transcription following manufacturer's instructions (ThermoFisher K1621). Samples were diluted in Milli-Q

Table 2: Primer sequences and annealing temperatures. Primer sequences and annealing temperature are depicted for relevant primer pairs.

Primers	Primer (forward, reverse)	Annealing temp.
Acox1	5′GGGTCATGGAACTCATCTTCGA	58°C
	5′GAATGAACTCTTGGGTCTTGGG	
Cd68	5′CTTCCCACAGGCAGCACAG	61°C
	5′AATGATGAGAGGCAGCAAGAGG	
Fas	5′ATTGGTGGTGTGGACATGGTC	61°C
	5′CCCAGCCTTCCATCTCCTG	
Il-6	5′CTCTGCAAGAGACTTCCATCCAGT	60°C
	5′GAAGTGGTATAGACAGGTCTGTTGG	
Irs2	5′TCTGCCAGCACCTATGCAA	60°C
	5′GCTTCACTCTTTCACGACTGTG	
Mcad	5′AGTATGCCCTGGATAGGAAGACAT	60°C
	5′CTTGGTGCTCCACTAGCAGCT	
Mcp-1	5′GTGTTGGCTCAGCCAGATGC	62°C
	5′GCTTGGTGACAAAAACTACAGC	
Nrf-1	5′CAGCACCTTTGGAGAATGTG	55°C
	5′CCTGGGTCATTTTGTCCACA	
Pparγ2	5′ACAGCAAATCTCTGTTTTATGC	60°C
	5′TGCTGGAGAAATCAACTGTGG	
Scd1	5′ACACCTGCCTCTTCGGGATT	61°C
	5′TGATGCCCAGAGCGCTG	
Socs3	5′GCCTTTCAGTGCAGAGTAGTG	63°C
	5′AAGAGCAGGCGAGTGTAGAG	
Tbp	5′ACCCTTCACCAATGACTCCTATG	60°C
	5′ATGATGACTGCAGCAAATCGC	
Tnfα	5′CCCTCACACTCAGATCATCTTCT	63°C
	5′GCTACGACGTGGGCTACAG	

water and 4 μL of the cDNA solution was added to a 96-well plate prior to addition of a mix consisting of 4.8 μL autoclaved Milli-Q, 0.6 μL forward primer (Tag Copenhagen A/S), 0.6 μL reverse primer (Tag Copenhagen A/S), and 10 μL SYBR Green containing ROX as reference dye (Bioline). RT-PCR was carried out on the Stratagene Mx3000P qPCR system, where samples were denatured by heating at 95°C for 5 minutes followed by 40 cycles of melting at 95°C for 15 seconds, annealing at differing temperatures as noted in Table 2 for 15 seconds, and elongation at 72°C for 20 seconds. Gene expression was normalized to that of *Tata-binding protein (Tbp)* mRNA. Primer sequences are given in Table 2.

2.6. Western Blot Analyses. Protein lysates were prepared from approximately 10 mg of liver tissue using standard protocols [24]. Western blot analyses were performed as previously described [24] and protein abundance was detected by immunoblotting using the following antibody: NFκB p65 (Santa Cruz #sc-109). Protein concentration was measured by BCA (#23223 and #23224, Thermo Scientific, USA) according to the manufacturer's instructions. Loading consistencies were verified by Ponceau staining.

2.7. Thin-Layer Chromatography. Triacylglycerol (TAG) was measured by thin-layer chromatography (TLC) using 7.5 mg liver sample. Lipids were extracted in chloroform-methanol (2 : 1) using the method of Folch et al. [25] and dissolved in chloroform as previously described [26]. Lipids were separated on silica-gel coated plates using two different separate mobile phases consisting of chloroform-methanol-acetic acid-water (50 : 50 : 5 : 5) followed by petroleum ether-diethyl ether-acetic acid (120 : 25 : 1.5). Butylated hydroxytoluene (50 mg/L) was added to both of the mobile phases. The lipids were developed by a 10% copper sulfate pentahydrate and 8% phosphoric acid solution at 120°C for 15 min. Lipids were visualized on a Typhoon FLA 7000 IP fluorescent scanner and analyzed according to weight using ImageQuant TL (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). TAG was identified with a specific glyceryl tripalmitate (#T5888, Sigma-Aldrich).

2.8. Statistics. All statistical analyses were conducted using GraphPad Prism version 6 software (GraphPad Software, San Diego, USA). Data are presented as mean ± standard error of the mean (SEM).

Due to small and variable sample sizes data could not be assumed to follow a Gaussian distribution and were consequently lognormal- (Ln-) transformed prior to any test except for the ITT where data were normalized to initial blood glucose (Figures 2(a) and 2(d)). Unless otherwise noted 2-way repeated measures (RM) ANOVA with Bonferroni *post hoc* multiple comparison test was conducted for all time-dependent analyses, that is, weight development, ITT, and feed intake. For comparison between genotypes 2-way ANOVA with Bonferroni *post hoc* multiple comparison test was conducted. All other data were analyzed by 1-way ANOVA with Bonferroni *post hoc* multiple comparison test. Both HFD groups (ω3-PUFA and ω6-PUFA) were compared to the LFD group. Data were considered statistically significant with $p < 0.05$ and the different levels of significance were set to be $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Only statistically significant differences are shown on graphs.

3. Results

FFAR4 is currently believed to be the key receptor for the polyunsaturated long chain fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [17], mediating the beneficial effects of fish oil. Yet, reports challenging this view are emerging, suggesting that FFAR4 might not serve as the sole effector of the health beneficial effects of ω3-PUFAs [23], indicating that fish oil acts through multiple pathways to exert its beneficial effects on health. We examined if fish oil on a HFD background would exert a favorable metabolic effect independent of FFAR4. To address this, we acquired *Ffar4* HET and *Ffar4* KO mice from Taconic Laboratories, Denmark. Importantly, the levels of *Ffar4* mRNA were similar in WT and HET mice, showing that the expression of *Ffar4*

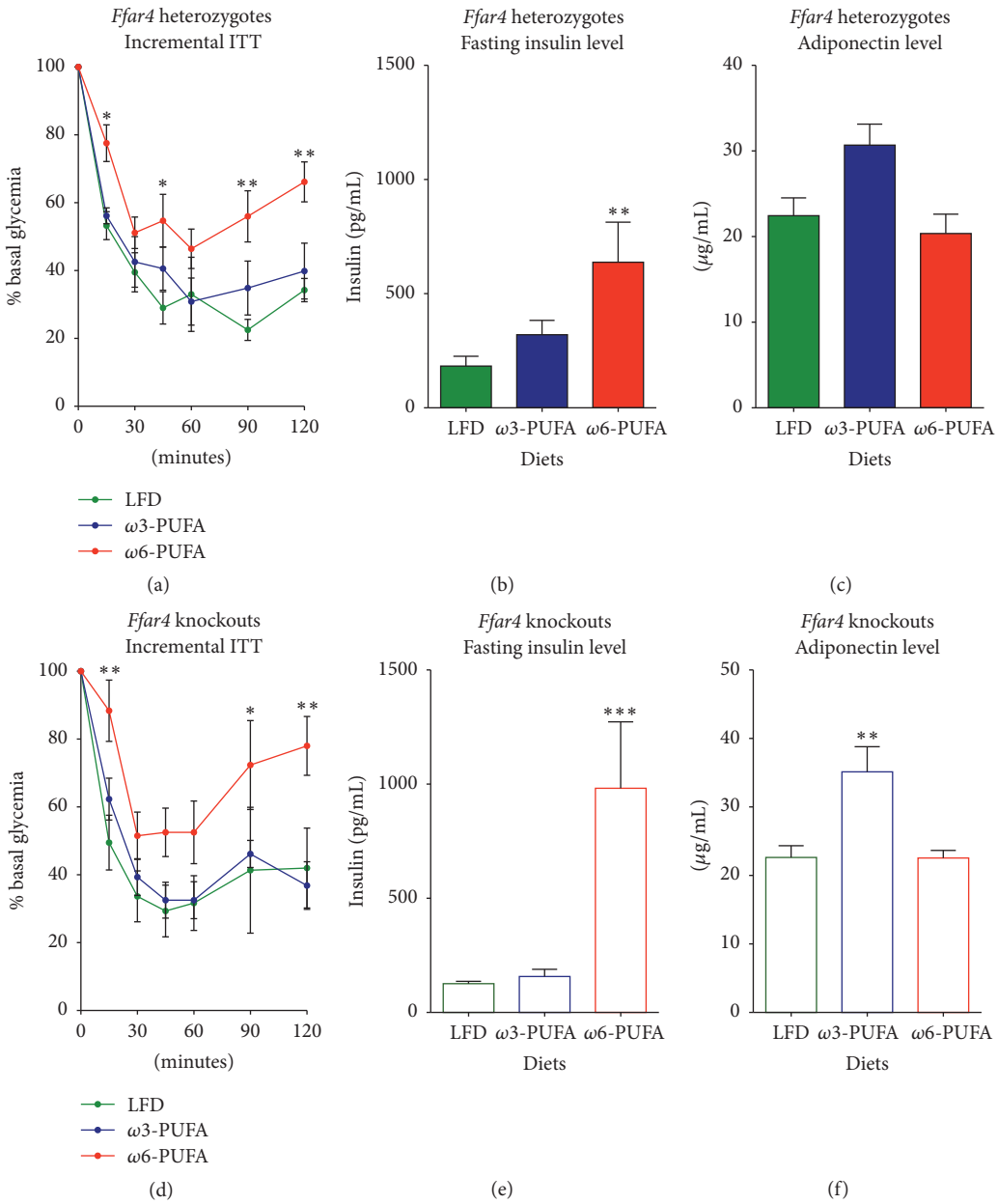

Figure 2: *Ffar4* HET and KO mice are protected against insulin resistance on a high fat ω3-PUFA diet. Insulin tolerance tests were performed at week 33 after diet initiation in 2-hour food-deprived mice. An insulin bolus of 1 U/kg lean mass was injected i.p. ((a)–(c)) Heterozygotes. ((d)–(f)) Knockouts. ((a) and (d)) ITT, $n = 4$–8; ((b) and (e)) 5-hour fasting insulin levels 32 weeks after diet initiation, $n = 6$–8. ((c) and (f)) Plasma adiponectin levels 36 weeks after diet initiation, $n = 4$–7. All data are presented as means ± SEM. Both HFDs have been compared to the LFD. For (a) and (d), 2-way RM ANOVA with Bonferroni correction was performed. All bar graph data were Ln-transformed and subjected to 1-way ANOVA with Bonferroni correction. Only statistical significant differences are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

mRNA was not gene dose dependent. No *Ffar4* mRNA was detected in KO mice (Figure S1).

We fed mice an isocaloric high fat, high sucrose diet rich in either ω3-PUFAs or ω6-PUFAs using fish oil or soy oil, respectively, as fat sources and compared the results to a low fat diet (LFD) reference group. Diet composition is shown in Table 1; a detailed fatty acid composition is given in Table S1.

3.1. The Antiobesogenic Effect of ω3-PUFAs Is Independent of FFAR4. ω3-PUFAs protected mice against weight gain irrespective of FFAR4 status for the first 28 weeks of HFD feeding. At this time, HET mice, but not KO mice, tended to gain more weight than their LFD fed counterparts (Figures 1(a) and 1(g)). The weight gain protection mediated by ω3-PUFAs is in sharp contrast to the obesogenic potential of ω6-PUFAs. Thus, mice, fed the latter, gained substantially more weight than LFD reference mice (Figures 1(a) and 1(g)). As evidenced by MR scans, weight gain was not confounded by increased lean mass (Figures 1(b) and 1(h)) but rather restricted to increased fat mass (Figures 1(c) and 1(i)), which

was further supported by increased tissue weights of liver and eWAT (Figures 1(d)-1(e) and 1(j)-1(k)). Since FFAR4 has been shown to be implicated in fat preference [27], an effect on feed intake could be suspected. However, there was no difference in feed intake between genotypes (2-way ANOVA $p = 0.9486$) nor fat sources (Figures 1(f) and 1(l)).

3.2. FFAR4 Status Does Not Affect Insulin Sensitivity. The lean phenotype promoted by *ω*3-PUFAs is generally associated with increased insulin sensitivity [28]. However, in contrast to WT mice, *Ffar4* KO mice have been shown to display attenuated insulin sensitivity upon chow [17] and HFD [21] feeding. We therefore asked if *Ffar4* KO mice in the current study would develop insulin resistance in the absence of obesity. After 33 weeks on experimental diets, mice were subjected to an insulin tolerance test. Independent of genotypes (AUC, 2-way ANOVA, multiple comparison $p > 0.9999$ for all diets), *ω*3-PUFA fed mice remained equally insulin sensitive as LFD reference mice (Figures 2(a) and 2(d)). In contrast, both HET and KO mice displayed decreased insulin sensitivity (2-way ANOVA RM, main effect $p = 0.0325$ and 0.0324, resp.) and increased fasting plasma insulin concentration when fed *ω*6-PUFAs (Figures 2(a)-2(b) and 2(d)-2(e)). The protection against insulin resistance in *ω*3-PUFA fed mice was independent of FFAR4 status and correlated with increased levels of the anti-inflammatory, insulin-sensitizing adipokine, adiponectin [29] (Figures 2(c) and 2(f)), suggesting a potential mechanism for the observed protection against insulin resistance.

3.3. ω3-PUFA Fed Mice Are Less Steatotic than ω6-PUFA Fed Counterparts. To disentangle the insulin-sensitizing, FFAR4 independent effects of *ω*3-PUFAs, we investigated hepatic expressions of lipogenic enzymes. Notably, expression of *Stearoyl coenzyme A desaturase-1 (Scd1)*, encoding an enzyme required for diet-induced hepatic insulin resistance [30], was substantially downregulated in *ω*3-PUFA fed mice compared to LFD reference mice (Figures 3(a) and 3(g)). Similarly, expression of *fatty acid synthase (Fas)* was significantly decreased in *ω*3-PUFA fed mice (Figures 3(b) and 3(h)) with a concomitant increased expression of genes involved in fatty acid oxidation, *acyl-CoA oxidase 1 (Acox1)* and *medium-chain acyl-CoA dehydrogenase (Mcad)* (Figures 3(c)-3(d), 3(i)-3(j)). This suggests that hepatic *de novo* lipogenesis was diminished by *ω*3-PUFA feeding, regardless of FFAR4 status. Moreover, expression of the adipogenic marker, *peroxisome proliferator-activated receptor-γ2 (Pparγ2)*, was significantly increased in *ω*6-PUFA fed mice compared to LFD reference mice (Figures 3(e) and 3(k)), possibly reflecting increased fat storage capacity [31]. In keeping with this notion, we observed increased levels of hepatic TAG accumulation (Figures 3(f) and 3(l)). In line with the observed decreased *de novo* lipogenesis, improved hepatic insulin resistance, and reduced TAG accumulation in *ω*3 compared with *ω*6-PUFA fed mice, hepatic *Il-6* expression as well as the protein level of the proinflammatory nuclear factor, NF*κ*B, was diminished in *ω*3-PUFA fed mice. Further, expressions of *insulin receptor substrate 2 (Irs2)* and *NF-E2-related factor 1 (Nrf1)* were lower in *ω*6-PUFA fed mice concurrent with augmented expression of *macrophage chemoattractant protein-1 (MCP-1)* (Figures 4(a)–4(j)). Again, no differences between genotypes on either diet were observed suggesting an independency of FFAR4 in both the protection and progression of hepatic steatosis.

3.4. ω3-PUFA Fed Mice Show Signs of Decreased Inflammation in the Visceral Adipose Tissue. Immunometabolism, hallmarked by tissue cross talk, has attracted considerable attention over the past decade. Adipose tissue harbors multiple immune cells [32] while adipocytes themselves have substantial immunomodulatory capacity [6]. FFAR4 has been shown to promote its positive insulin-sensitizing effect by inhibiting macrophage-mediated inflammation in adipose tissue [17], and since visceral adipose tissue exerts a larger impact on whole-body metabolism than subcutaneous fat [15, 16] we focused our immunological analyses on the former. To investigate whether inflammation and macrophage recruitment were altered between diets and genotypes, we measured gene expression levels of *Tnfα*, *Il-6*, *Mcp-1*, *Cd68,* and *Socs3* in eWAT of *Ffar4* HET and KO mice (Figure 5). The proinflammatory cytokine, *Tnfα*, was significantly increased in *ω*6-PUFA fed mice compared to LFD reference mice (Figures 5(a) and 5(f)). Interestingly, the expression levels of *Il-6* were substantially reduced in *ω*3-PUFA fed mice of both genotypes compared to their LFD fed counterparts (Figures 5(b) and 5(g)), suggesting a FFAR4 independent mechanism for at least some anti-inflammatory effects of *ω*3-PUFAs. Obesity-associated low-grade inflammation is characterized by increased macrophage accumulation in adipose tissue [5] and increased expression of *Mcp-1* is hypothesized to account for this increase [4]. While others have found that *ω*3-PUFA supplementation suppressed the expression of *Mcp-1* in adipose tissue of *Ffar4* WT mice but not *Ffar4* KO mice [17], we found no differences in *Mcp-1* expression between genotypes (2-way ANOVA $p = 0.8274$). Further, the expression of *Mcp-1* was indistinguishable between *ω*3-PUFA fed mice and LFD reference mice, whereas *ω*6-PUFA fed mice had an augmented *Mcp-1* expression (Figures 5(c) and 5(h)) concomitant with increased expression of the global macrophage marker, *Cd68* (Figures 5(d) and 5(i)), indicating an augmented infiltration of macrophages in adipose tissue of *ω*6-PUFA fed mice. Lastly, as a marker of general tissue inflammation, we analyzed the expression levels of *Socs3*. Strikingly, the finding mirrored the expression levels of *Il-6* with a selective reduction of *Socs3* expression in *ω*3-PUFA fed mice (Figures 5(e) and 5(j)) independent of FFAR4 status, further supporting the notion of diminished inflammation and implying that FFAR4-independent pathways may confer beneficial effects of *ω*3-PUFAs.

4. Discussion

The potential of fish oil to protect against cardiovascular diseases is well-established [33]. In recent years, however, there has been an increasing interest in the ability of fish oil to relieve other lifestyle diseases such as obesity and type 2 diabetes. Although human studies are inconclusive, the antiobesogenic potential of fish oil in rodents is well-documented [34, 35]. Still, the molecular mechanisms by

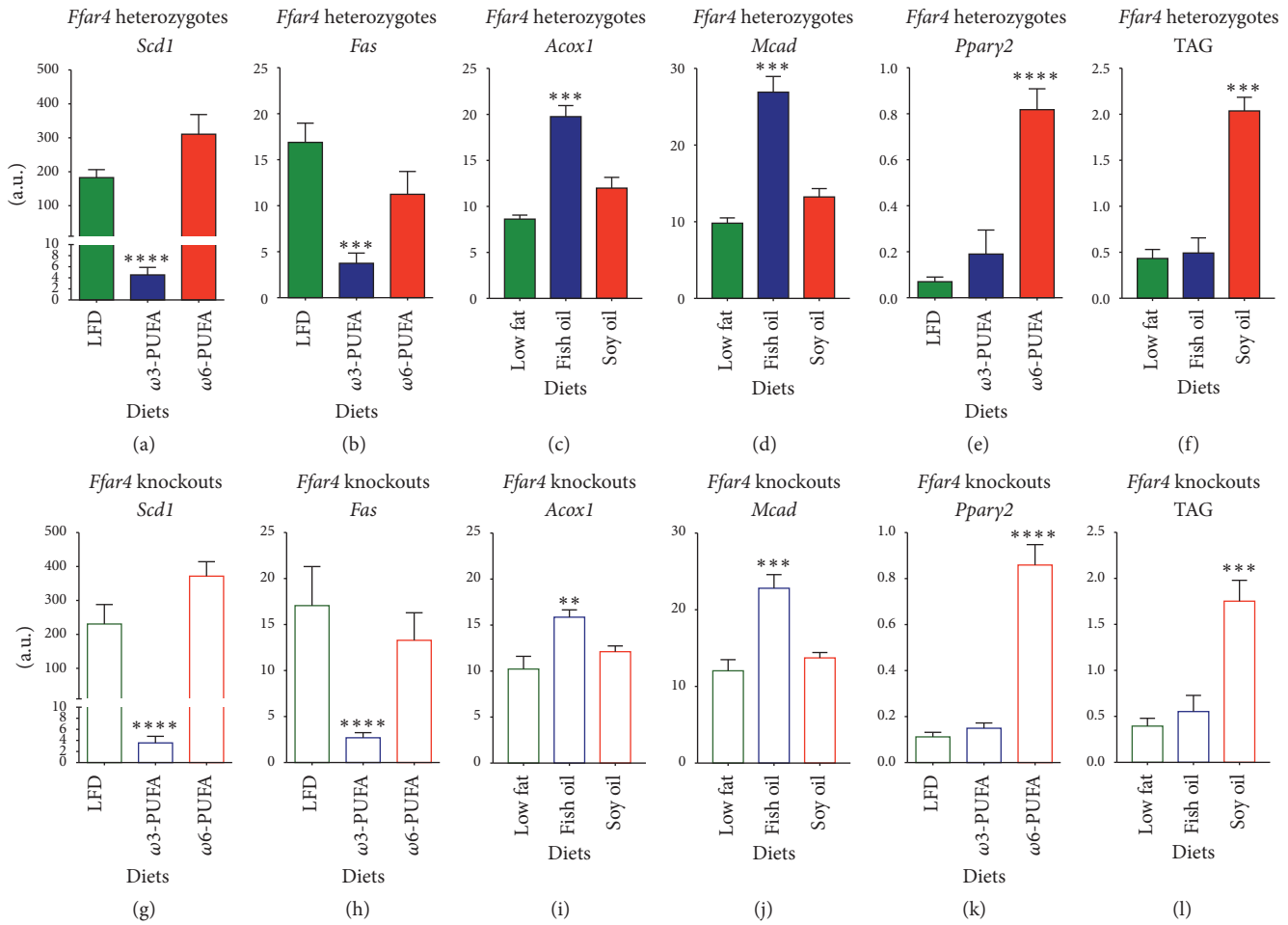

FIGURE 3: Intake of a high fat ω3-PUFA diet alleviates hepatic lipid accumulation in *Ffar4* HET and KO mice. Mice were euthanized in nonfasting state 36 weeks after diet initiation. Gene expression levels were analyzed by RT-qPCR and lipid levels were evaluated by thin-layer chromatography. ((a)–(f)) Heterozygotes. ((g)–(l)) Knockouts. ((a) and (g)) mRNA level of *Scd1*, $n = 5$–8. ((b) and (h)) mRNA level of *Fas*, $n = 6$–8. ((c) and (i)) mRNA level of *Acox1* $n = 6$–8. ((d) and (j)) mRNA level of *Mcad*, $n = 6$–8. ((e) and (k)) mRNA level of *Pparγ2*, $n = 6$–8. ((f) and (l)) Triacylglycerol content, $n = 6$–8. Data are presented as means ± SEM. Both HFDs have been compared to the LFD. All data have been Ln-transformed and 1-way ANOVA with Bonferroni correction was performed. Only statistical significant differences are shown. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

which ω3-PUFAs mediate their actions are intensely debated [17, 28, 36–41]. Several mechanisms have been proposed to explain the beneficial effects of ω3-PUFA supplementation, comprising increased fatty acid oxidation [28, 38] and anti-inflammatory actions [17, 40, 42], alleviating insulin resistance and metabolic syndrome [43]. On this note, FFAR4 was recently reported to be responsible for the anti-inflammatory and insulin-sensitizing effects of ω3-PUFAs [17]. This finding led us to investigate whether fish oil on a background of high fat, high sucrose diet would improve metabolic parameters in *Ffar4* KO mice to the same extent as observed in WT mice. We compared the results to LFD reference group and further employed an obesogenic HFD control where the fat source was based on soy oil, rich in ω6-PUFAs. Importantly, the ω6-PUFA in soy oil is linoleic acid (LA), which parallels ω3-PUFAs in the ability to agonize FFAR4 [44].

We found that the decreased liver weights of ω3-PUFA fed mice were paralleled by decreased expressions of genes encoding the lipogenic enzymes, *Fas* and *Scd1*, possibly due to suppression of processing or activity of SREBP1c [37, 45], and an increased expression of genes involved in fatty acid oxidation, *Acox1* and *Mcad*. Moreover, expression of *Nrf1*, a transcription factor protecting against hepatic steatosis [46], was selectively decreased in ω6-PUFA fed mice, while *Pparγ2* was increased; the latter is possibly reflecting a requirement for increased fat storage [31], which was further supported by increased TAG accumulation in the livers of these mice. The protection against weight gain and liver lipogenesis and adipogenesis in ω3-PUFA fed mice was associated with improved insulin sensitivity as determined by an ITT. The improved insulin sensitivity was further supported by lowered fasting plasma insulin and augmented plasma adiponectin. Importantly, both the beneficial effects of ω3-PUFA feeding and the detrimental effects of ω6-PUFA feeding were independent of genotype. These findings reflect a recent study focusing on energy metabolism and

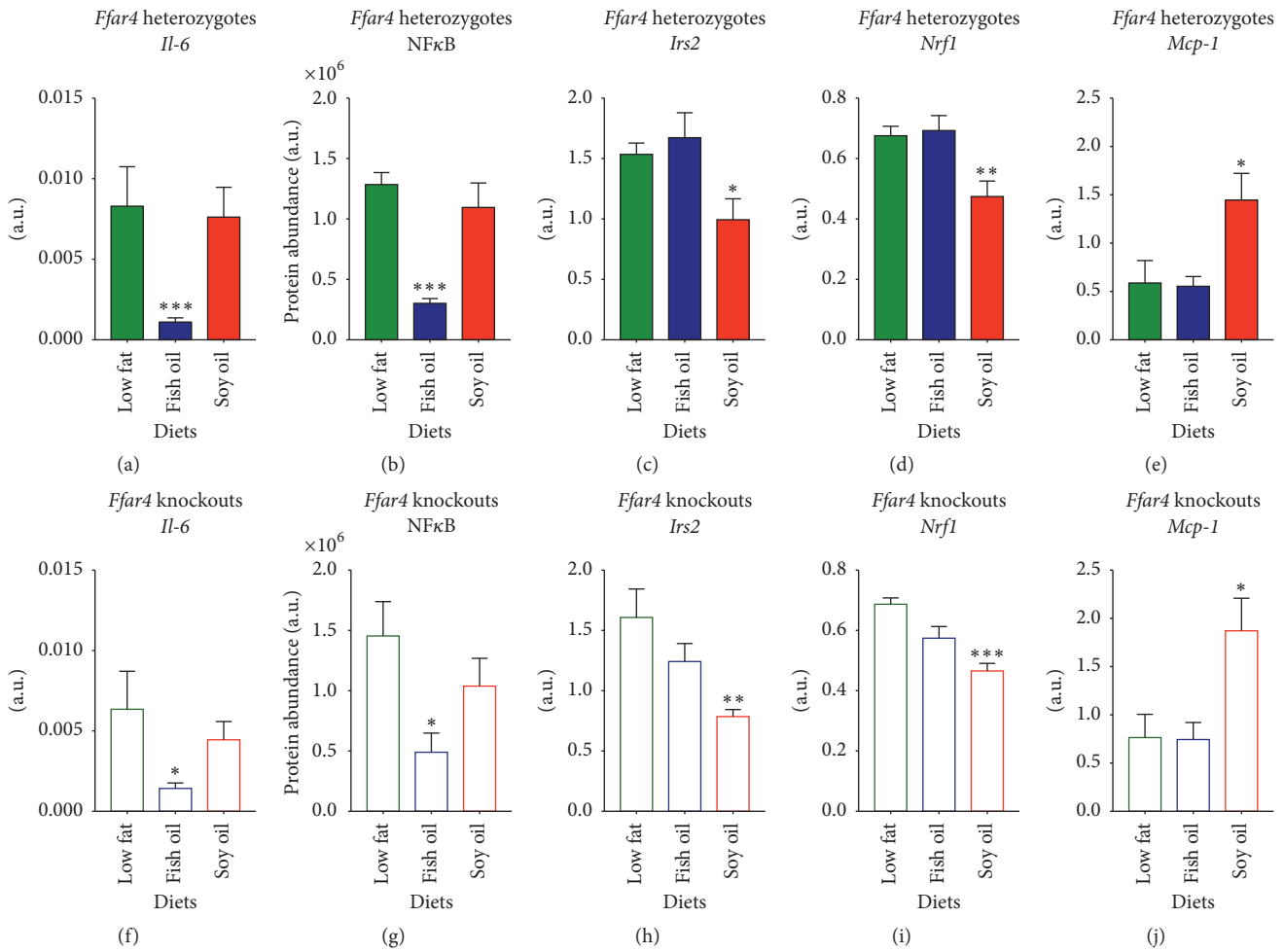

FIGURE 4: Intake of a high fat ω3-PUFA diet alleviates hepatic steatosis in *Ffar4* HET and KO mice. Mice were euthanized in nonfasting state 36 weeks after diet initiation. Gene expression levels were analyzed by RT-qPCR and protein levels were evaluated by western blot analysis. ((a)–(e)) Heterozygotes. ((f)–(j)) Knockouts. ((a) and (f)) mRNA level of *Il-6*, $n = 5$–7. ((b) and (g)) Protein level of NFκB, $n = 6$–8. ((c) and (h)) mRNA level of *Irs2*, $n = 6$–8. ((d) and (i)) mRNA level of *Nrf1*, $n = 6$–8. ((e) and (j)) mRNA level of *Mcp-1*, $n = 6$–8. Data are presented as means ± SEM. Both HFDs have been compared to the LFD. All data have been Ln-transformed and 1-way ANOVA with Bonferroni correction was performed. Only statistical significant differences are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

energy expenditure which questioned the necessity of FFAR4 signaling in fish oil-mediated health benefits [23]. Yet, the anti-inflammatory action of FFAR4-mediated signaling, as reported by Oh et al. [17], has so far remained unchallenged. Accordingly, we investigated the inflammatory status of liver and visceral fat (eWAT), where a potential anti-inflammatory effect may exert a major impact on whole-body metabolism. Surprisingly, we found decreased expression of inflammatory genes and proteins in ω3-PUFA fed mice irrespective of genotypes suggesting that FFAR4 is dispensable for the immunometabolic effects of ω3-PUFAs. This is in sharp contrast to the findings of Oh et al., who found decreased expression of *Il-6* and *Mcp-1* solely in WT mice, but not in *Ffar4* KO mice, fed a ω3-PUFA enriched HFD [17]. The different outcomes of the studies performed by Oh et al. [17] and those reported here may relate to subtle differences in the experimental setup. Both studies were performed on mice of mixed 129SVE and C57BL/6J backgrounds, but it is unclear to what extent the mice of the Oh et al. study had been backcrossed to the C57BL/6J background [17]. This could have a vast impact on the immunological outcomes of these studies, since C57BL/6J and 129SVE mice have different inflammatory responses [47]. Furthermore, our HET and KO mice were cocaged throughout the study. It has been shown that the microbiota in some instances might exert a larger impact on phenotype compared to genotype [48]. Accordingly, it is indeed possible that the effect of cocaging, hence exposing *Ffar4* KO mice to microbiota from HET mice, had masked the effect of *Ffar4* ablation.

Collectively, our findings demonstrate that ω3-PUFAs may exert positive effects independently of FFAR4 or at least that the effect of FFAR4 is minor in the setting of a high fat fish oil-based diet. This is not to question the well-described anti-inflammatory and insulin-enhancing potential of FFAR4 [17, 22] but merely an indication of the fact that there might be a certain level of redundancy of the said receptor and

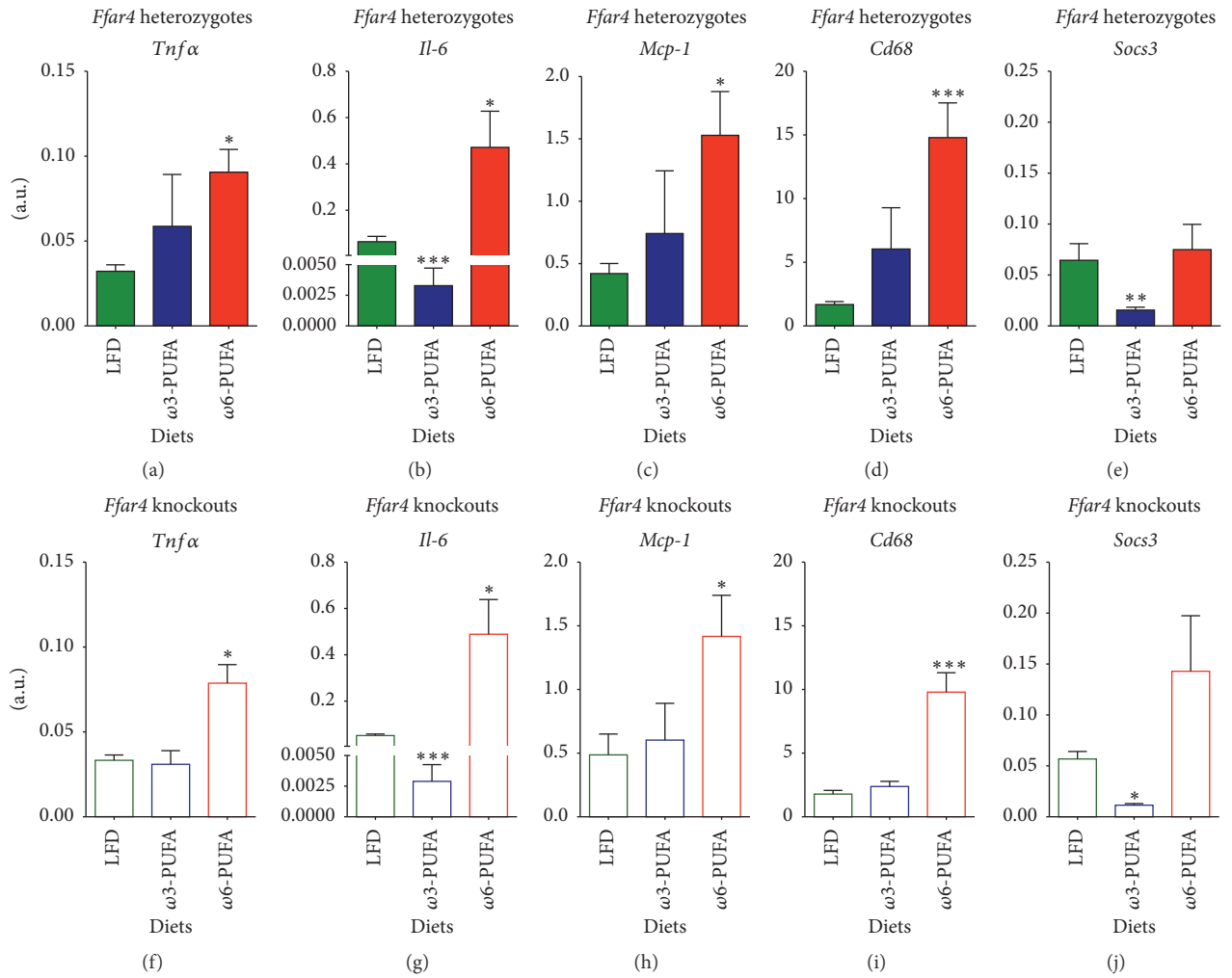

Figure 5: Inflammatory status of eWAT is indistinguishable between *Ffar4* HET and KO mice. Mice were euthanized in nonfasting state 36 weeks after diet initiation. Gene expression levels were analyzed by RT-qPCR. ((a)–(e)) Heterozygotes. ((f)–(j)) Knockouts. ((a) and (f)) mRNA level of *Tnfα*, $n = 4$–8. ((b) and (g)) mRNA level of *Il-6*, $n = 4$–8. ((c) and (h)) mRNA level of *Mcp-1*, $n = 4$–8. ((d) and (i)) mRNA level of *Cd68*, $n = 4$–8. ((e) and (j)) mRNA level of *Socs3*, $n = 4$–8. Data are presented as means ± SEM. Both HFDs have been compared to the LFD. All data have been Ln-transformed and subjected to 1-way ANOVA with Bonferroni correction. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

that ω3-PUFAs may have multiple undiscovered receptors through which they exert their beneficial actions. Indeed, ω3-PUFAs impact a myriad of metabolic processes, and the extent to which FFAR4 signaling is involved remains to be elucidated. Besides increasing adiponectin secretion [36], reported here to be independent of FFAR4, EPA and DHA serve as precursors for bioactive lipid mediators such as eicosanoids/docosanoids [49], resolvins [40], maresins [50], and protectins [51]. These compounds have anti-inflammatory effects and may potentially curb HFD-mediated low-grade inflammation, thereby relieving insulin resistance. Moreover, EPA and DHA-derived prostanoids are considered less proinflammatory than those derived from arachidonic acid (AA) [52]. Competition between ω3-PUFAs and AA for incorporation into phospholipids furthermore reduces substrate availability for synthesis of a number of oxylipins [53] as well as the two major endocannabinoids [54, 55]. The importance of such lipid mediators in relation to FFAR4-dependent signaling remains to be established. It has been shown that the beneficial effects on hepatic steatosis and adipose tissue insulin sensitivity by supplementing ob/ob mice with ω3-PUFAs were due to increased levels of protectin D1 and resolvin D1 [40]. These mediators have not been investigated in the present study, and future studies are needed to explore to what extent such lipid mediators contribute to the beneficial effects associated with fish oil intake. Additional candidates involved in ω3-PUFA signaling may comprise other G-protein coupled receptors, for example, FFAR1 and GPR119. Indeed, FFAR1 has been shown to partly mediate the anti-inflammatory effects of DHA by inhibition of inflammasome activation, where only *Ffar4*/*Ffar1* double KO abrogated this effect [56]. Hence, the beneficial effects of high dose ω3-PUFAs may also in part be mediated by FFAR1. Moreover, DHA, EPA, and their derivatives can activate

PPARs, which collectively have been found to be able to inhibit inflammation through repression of NFκB activation [57]. Furthermore, adiponectin secretion has been shown to be PPARγ-dependent [36], while PPARα activation leads to increased fatty acid oxidation [57], thus establishing PPARs as important mediators and possible effectors of the ω3-PUFA-mediated FFAR4 independent effects described in the present study.

In conclusion, our data provide evidence for alternative routes, not dependent on FFAR4, involved in mediating the beneficial effect of ω3-PUFAs, and emphasize the importance of ω3-PUFAs in relation to adequate immune regulation in curtailing the metabolic syndrome.

Disclosure

The current address for Tao Ma is The Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Metabolic Receptology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

Competing Interests

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

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Research Article

Serum Amyloid A Promotes E-Selectin Expression via Toll-Like Receptor 2 in Human Aortic Endothelial Cells

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Periodontitis is a chronic inflammatory disease that affects the periodontium. Recent studies suggest an association between periodontal and cardiovascular diseases. However, the detailed molecular mechanism is unknown. A previous study has demonstrated that experimental periodontitis induces serum amyloid A (SAA) in the liver and peripheral blood of ApoE-deficient mice as an atherosclerosis model. SAA is an acute-phase protein that affects systemic inflammation. The aim of this study is to investigate the atherosclerosis-onset mechanism using human aortic endothelial cells (HAECs) stimulated by SAA *in vitro*. Atherosclerosis PCR array and qPCR analyses showed upregulation of adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in HAECs upon SAA stimulation. In addition, the results demonstrated that Toll-like receptor, TLR2, could serve as an important receptor of SAA in HAECs. Furthermore, small interfering RNA (siRNA) against TLR2 inhibited the upregulation of adhesion molecules in HAECs stimulated by SAA. Our results suggest that SAA stimulates the expression of adhesion molecules via TLR2. SAA could be an important molecule for atherosclerosis induced by periodontal disease.

1. Introduction

Periodontitis, one of the most common diseases in humans and is an infectious disease that can result in inflammatory destruction of the periodontium (alveolar bone, cementum, periodontal ligament, and gingiva) [1]. Periodontal disease has been recognized as a risk or contributing factor for systemic diseases including atherosclerotic vascular disease, diabetes mellitus, rheumatoid arthritis, Alzheimer's disease, and cancer [2–9].

The prevalence and incidence of coronary heart disease are significantly increased in patients with periodontitis, indicating that periodontal disease independently predicts coronary heart disease [10]. Recent systematic reviews and meta-analysis of observational studies support an association between periodontal and atherosclerotic vascular diseases, which is independent of known confounders, but a causal relationship is not yet established [11, 12].

Serum amyloid A (SAA), an acute-phase protein, is markedly upregulated in response to infection and during chronic inflammation [13–15]. SAA stimulates vascular cells to express cytokines, chemokines, adhesion molecules, and matrix metalloproteinases [16], which are linked to the development of atherosclerosis. In addition, high levels of SAA in peripheral blood are significantly associated with periodontitis, and SAA levels are decreased in patients with periodontitis after periodontal therapy [17, 18].

Recently, evidence of the possible link between periodontitis and atherosclerosis has increased, and the possible association and causality are being investigated [19, 20]. Hujoel

Table 1: Primers used for screening SAA candidate receptors.

Gene symbol	Primer	
SELS	Sense	5′-CCC TCG ATT CAA TTG CCT TA-3′
	Antisense	5′-TGT GAC CAA TGA CCT CAT GC-3′
ABCA1	Sense	5′-TTT GCT GTA TGG GTG GTC AA-3′
	Antisense	5′-AAC AGC TCC AGC ACA AAG GT-3′
ABCA7	Sense	5′-ATG TGG TGC TCA CCT GCA TA-3′
	Antisense	5′-AAG CAG AAG TGG GGG AAG AT-3′
SCARB1	Sense	5′-CTC CCA TCC TCA CTT CCT CA-3′
	Antisense	5′-GCT CAG CTG CAG TTT CAC AG-3′
CD36	Sense	5′-GAT GTG CAA AAT CCA CAG GA-3′
	Antisense	5′-GGC TGC AGG AAA GAG ACT GT-3′
TLR2	Sense	5′-AAA TTT TGT CTG GGG TGC TG-3′
	Antisense	5′-GCA ACC AAT TCC CTT GGA TA-3′
TLR4	Sense	5′-AAT AAA CCC GGA GGC CAT TA-3′
	Antisense	5′-TCC CTT CCT CCT TTT CCC TA-3′
CST3	Sense	5′-ACC AGC CAC ATC TGA AAA GG-3′
	Antisense	5′-GGG AGG TGT GCA TAA GAG GT-3′
FPR2	Sense	5′-CTG CCA ATT CTG CTT CAC CT-3′
	Antisense	5′-GCA TCC TTG AAT GCC TCA AT-3′
AGER	Sense	5′-CTG AGG CAG GCG AGA GTA GT-3′
	Antisense	5′-TTG GCA AGG TGG GGT TAT AC-3′
GAPDH	Sense	5′-GTC AGT GGT GGA CCT GAC CT-3′
	Antisense	5′-TCG CTG TTG AAG TCA GAG GA-3′

et al. reported an important general trend towards periodontal treatment-induced inhibition of systemic inflammation and improvement in noninvasive markers of atherosclerosis and endothelial function [19]. However, the detailed molecular mechanism for periodontitis-induced atherosclerosis is unknown.

The aim of this study is to investigate the atherosclerosis-onset mechanism using human aortic endothelial cells (HAECs) stimulated by SAA *in vitro*. Here, we demonstrate that stimulation of HAECs with SAA results in the induction of adhesion molecules, which may be caused via Toll-like receptor, TLR2.

2. Materials and Methods

2.1. Cell Culture and SAA Treatment. HAECs were purchased from Lonza (CC-2535; Tokyo, Japan) and used in all experiments. The HAECs were cultured in Endothelial Cell Growth Medium 2 (EBM-2) medium supplied with the EBM-2 bullet kit (Lonza, Tokyu, Japan) at 37°C with 5% CO_2. Subconfluent passage 6 cells were used in all experiments. HAECs were plated at 1.5×10^5 cells/well in 6-well plates and cultured to subconfluence. The cells were then treated with 1.5 μg/mL recombinant human SAA (PeproTech, Rocky Hill, NJ) for 0, 1, 3 and 6 h.

2.2. PCR Array Analysis. Total RNA was extracted from HAECs using NucleoSpin RNA II (Macherey-Nagel, Diiren, Germany). First-strand cDNA synthesis was performed using RT^2 First Strand Kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. The Human Atherosclerosis RT^2 Profiler™ PCR Array (PAHS-038Z) (Qiagen, Tokyo, Japan) was applied to an ABI 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). The RT^2 Profiler™ PCR Array for Human Atherosclerosis contains 84 genes for responses to stress, apoptosis, blood coagulation and circulation, adhesion molecules, extracellular molecules, lipid transport and metabolism, and cell growth and proliferation. In addition, the array contains five wells for various housekeeping genes, a genomic DNA contamination control, three replicate reverse transcription controls, and three replicate positive PCR controls. Data analyses were performed using web-based analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

2.3. qPCR Analysis. cDNAs were synthesized from 1 μg total RNA using Revetra Ace qPCR Master Mix (Toyobo, Osaka, Japan). qPCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA) using the ABI 7000 Real-Time PCR System. SYBR Green primers (SELS, ABCA1, ABCA7, SCARB1, CD36, TLR2, TLR4, CST3, FPR2, AGER, and GAPDH) were designed by Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 1). The expression of each gene was normalized to the GAPDH level (SYBR Green). TaqMan primers (TLR2, Hs01872448_s1; MYD88, Hs01573837_g1; NFKB1m Hs00765730_m1; tumor necrosis factor-α (TNF-α), Hs01113624_g1; E-selectin (SELE), Hs00950401_m1) were purchased from Life Technology (Tokyo, Japan). The expression of each gene was normalized

to the 18S rRNA (4319413E, Applied Biosystems, Foster City, CA) level (TaqMan). The fold change between mRNA expression levels was determined as follows: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{18S\,rRNA})$ treated group − $(Ct_{target} - Ct_{18S\,rRNA})$ control group (Ct, cycle threshold).

2.4. RNA Interference. HAECs were transfected with TLR2 Silencer Select Pre-Designed Small Interfering RNA (siRNA, ASO0ZS41; Life Technology, Tokyo, Japan) using Lipofectamine 2000 transfection reagent (Life Technology, Tokyo, Japan) according to the manufacturer's instructions. HAECs (1.5×10^5 cells/well) were seeded in 6-well plates at 24 h before transfection. Then, the cells were transfected with siRNA against TLR2 at a final concentration of 10 nM. Cells were incubated with siRNA in EBM-2 medium for 24 h. Then, the medium was replaced with fresh medium, and the cells were incubated for another 48 h. HAECs were then stimulated with SAA before harvesting. TLR2 knockdown was verified by qPCR. Control siRNA was purchased from Life Technology.

2.5. Western Blotting. HAECs were lysed for 30 min on ice in lysis buffer (Bio-Rad, Richmond, CA) with 1 μg/mL protease inhibitors. Cell debris was removed by centrifugation (14,000 ×g, 4°C, 15 min), and supernatants were collected and stored at −80°C until use. Total protein samples (30 μg each) were boiled for 5 min, resolved by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), and blotted onto transfer membranes (Bio-Rad, Richmond, CA) (100 V, 1.5 h, 4°C). After blocking for 2 h in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) containing 5% dry nonfat milk, the membranes were washed three times in TBST and probed for 18 h at 4°C with the anti-TLR2 monoclonal antibody TL2.1 (final concentration: 1 μg/mL; Abcam, Cambridge, MA) and anti-β actin monoclonal antibody (1 : 1000 dilution; Cell Signaling Technology, Beverly, MA) in TBST. After three washes in TBST, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG (1 : 2000 dilution; Cell Signaling Technology, Beverly, MA) and then washed five times in TBST. Protein bands were detected using ECL reagents (GE Healthcare, Waukesha, WI) according to the manufacturer's instructions.

2.6. Statistical Analysis. Statistical analyses were performed using SPSS software v. 15.0 J for Windows (SPSS Inc., Chicago, IL). Data are expressed as the mean ± standard deviation. Student's t-test was used for comparisons. Significance was accepted at $p < 0.05$.

3. Results

3.1. SAA Induces Adhesion Molecules in HAECs. To explore atherosclerosis-related genes in SAA-stimulated HAECs, we used a Human Atherosclerosis RT^2 Profiler™ PCR Array (Figure 1). The comparison between HAECs at 0 h and 6 h after stimulation with SAA indicated specific up-regulation (>5-fold) of 13 genes including BIRC3 (baculoviral IAP repeat containing 3), CCL2 (chemokine (C-C motif) ligand 2), CCL5 [chemokine (C-C motif) ligand 5], CCR2 [chemokine (C-C motif) receptor 2], CSF2 [colony-stimulating factor 2 (granulocyte-macrophage)], FGA (fibrinogen alpha chain), ICAM1 (intercellular adhesion molecule-1), IL1A (interleukin 1, alpha), LIF [leukemia inhibitory factor (cholinergic differentiation factor)], NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), SELE, TNFAIP3 (tumor necrosis factor, alpha-induced protein 3), and VCAM1 (vascular cell adhesion molecule-1) (Figure 1 and Table 2). Thus, adhesion molecules such as ICAM1, VCAM1, and SELE may be upregulated in HAECs under inflammatory

Table 2: Upregulation (>5-fold) of 13 genes in HAECs after stimulation with SAA.

Gene symbol	Fold regulation
BIRC3	8.3977
CCL2	37.2715
CCL5	27.4741
CCR2	8.8766
CSF2	46.5271
FGA	51.2685
ICAM1	19.0273
IL1A	7.5685
LIF	14.3204
NFKB1	5.3147
SELE	232.3249
TNFAIP3	9.9866
VCAM1	37.2715

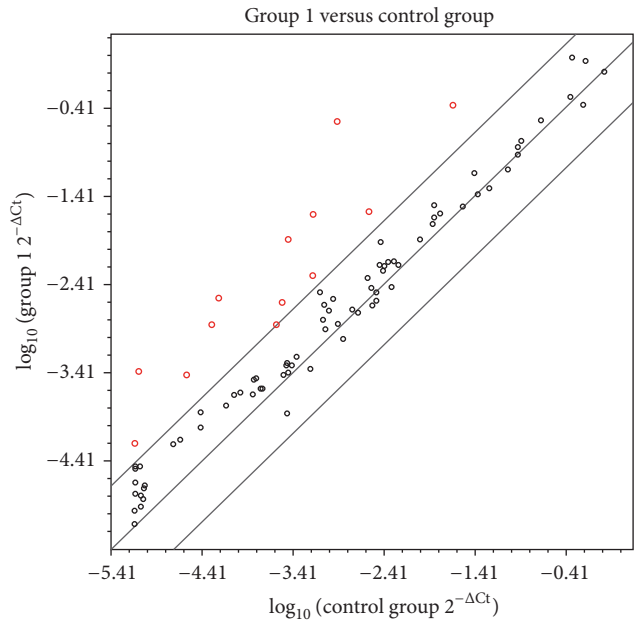

Figure 1: Gene screening by the RT^2 Profiler™ PCR Array for Human Atherosclerosis in SAA-stimulated HAECs. A total of 84 atherosclerosis-related genes were analyzed using the RT^2 Profiler™ PCR Array (n = 1 per group). Thirteen genes were identified with a more than a 5-fold change in expression between human aortic endothelial cells (HAECs) and HAECs treated with SAA for 6 h (red circles). x-axis: Control Group, HAECs; y-axis: Group 1, HAECs treated with SAA.

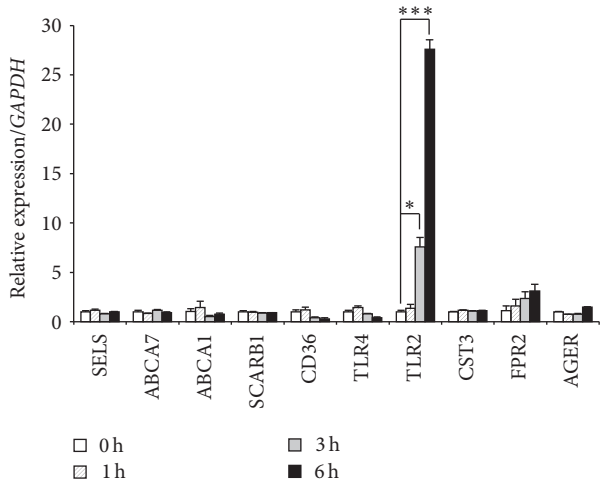

Figure 2: Screening of SAA receptors in HAECs. qPCR analysis of 10 genes that encode known SAA receptors was conducted. HAECs were treated with recombinant human SAA, and total RNA was extracted at 0, 1, 3, and 6 h. Among the expression levels of SAA receptors, TLR2 mRNA expression induced by SAA was the highest and significantly higher than that in unstimulated HAECs. Each experiment was performed in triplicate. Values were the mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

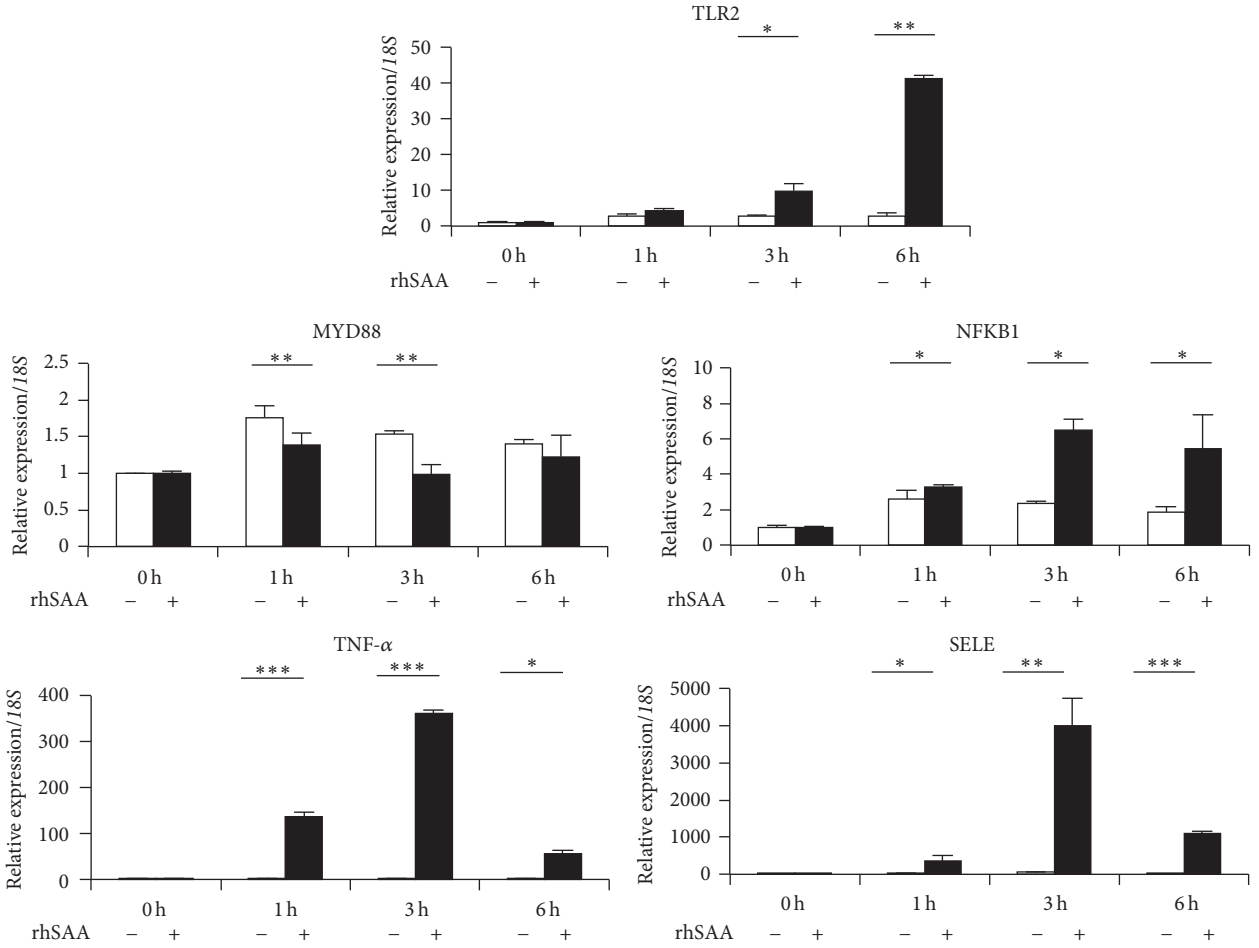

Figure 3: Expression pattern of TLR and selectin cascade genes in HAECs stimulated by SAA. Expression levels of TLR2, MYD88, NFKB1, TNF-α, and SELE mRNAs were examined at 0, 1, 3, and 6 h in HAECs stimulated by SAA. The mRNA expression of TLR2 was induced by SAA (closed column) in a time-dependent manner. Furthermore, the mRNA expression of NFKB1, TNF-α, and SELE was significantly upregulated by SAA (closed column) compared with the control (open column). Data represent the mean ± SD of four independent experiments. Each experiment was performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. rhSAA: recombinant human SAA.

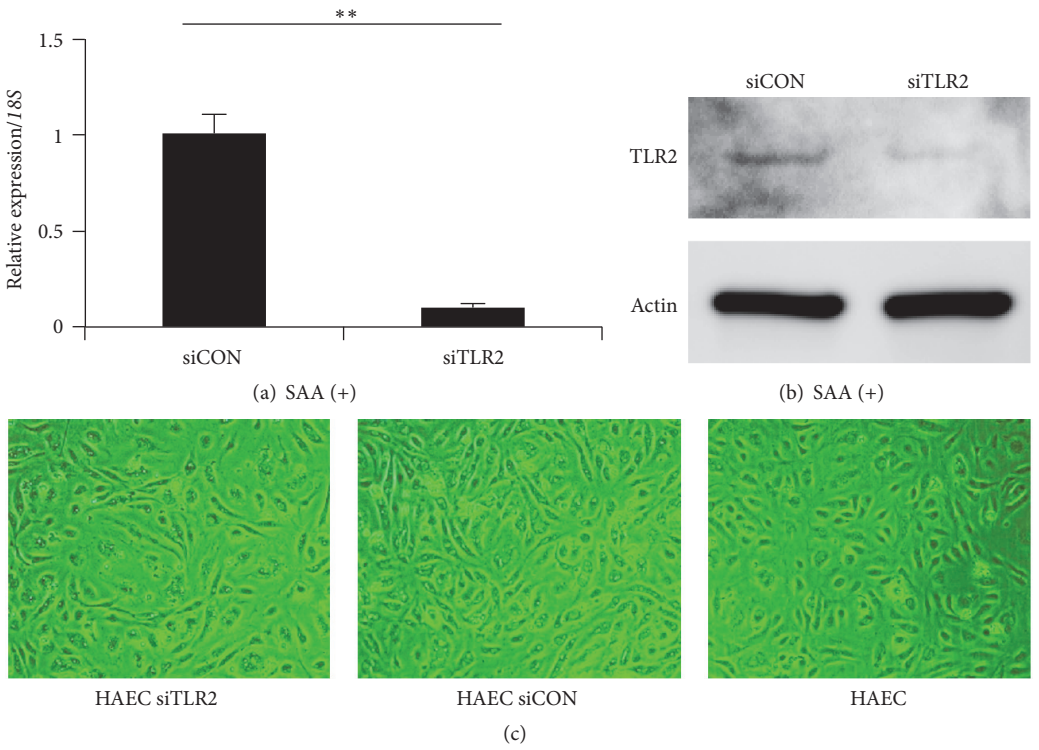

Figure 4: Effect of TLR2 siRNA on TLR2 expression and morphology in HAECs. The effect of TLR2 siRNA in HAECs was determined by qPCR analysis (a), western blotting (b), and observations of cell morphology (c). (a, b) mRNA and protein levels of TLR2 in HAECs were dramatically decreased by siRNA treatment for 6 h. (c) siTLR2 had no significant effect on the cell morphology of HAECs. Representative data of three independent mRNA experiments are shown. Each mRNA experiment was performed in triplicate. Values are the mean ± SD. $^{**}p < 0.01$. siCON: control siRNA; siTLR2: TLR2 siRNA.

conditions. Among these molecules, expression of the SELE gene was remarkable (232-fold). Therefore, SAA might have an important role in the leukocyte adhesion cascade.

3.2. TLR2 Is Upregulated by SAA among Receptor Molecules in HAECs. To identify genes related to the leukocyte adhesion cascade, we screened SAA receptors that were highly expressed in HAECs during SAA stimulation (Figure 2). SAA receptors, such as SELS (glucose homeostasis and ER stress), ABCA1, ABCA7, SCARB1 (cholesterol efflux), CD36, TLR2, TLR4, CST3 (inflammatory signaling), FPR2 (chemotaxis and immune cell activation), and AGER (amyloidosis), have been reported previously [21]. Among the candidate receptors, TLR2 mRNA expression was significantly induced by SAA in HAECs, indicating that TLR2 could serve as an important receptor for SAA. Thus, SAA may stimulate the expression of adhesion molecules via TLR2.

3.3. SAA Induces TLR2 and Its Related Genes following the Leukocyte Adhesion Cascade. To investigate the leukocyte adhesion cascade induced by SAA, mRNA expression was examined at 0, 1, 3, and 6 h after SAA stimulation in HAECs (Figure 3). TLR2 mRNA expression was upregulated in a time-dependent manner. Furthermore, the mRNA expression of NFKB1, TNF-α, and SELE was significantly upregulated at 3 h after SAA stimulation. The mRNA expression of these genes was significantly higher in SAA-stimulated HAECs compared with unstimulated HAECs. However, only the mRNA expression of MYD88 was lower in SAA-stimulated HAECs compared with unstimulated HAECs. These results indicate that SAA affects downstream of TLR signaling and induces leukocyte adhesion cascade-related genes.

3.4. Knockdown of TLR2 Affects the Expression of SELE through the Leukocyte Adhesion Cascade. To determine the contribution of TLR2 as a SAA receptor for SELE expression, TLR2 was knocked down by transfection of siRNA. We confirmed specific knockdown of TLR2 by qPCR and western blotting (Figures 4(a) and 4(b)). No difference in cell morphologies was found in HAECs transfected with TLR2 siRNA (Figure 4(c)). Silencing of TLR2 using siRNA resulted in a significant reduction in the expression of SELE mRNA compared with cells transfected with control siRNA under SAA stimulation (Figure 5). In addition, mRNA expression of NFKB1 and TNF-α under SAA stimulation was abolished by silencing TLR2.

4. Discussion

In this study, we found that SAA induced adhesion molecules, especially SELE, and TLR2 may be a critical receptor for this process in HAECs. To our knowledge, this is the first report indicating that TLR2 plays a role in HAECs as a SAA receptor

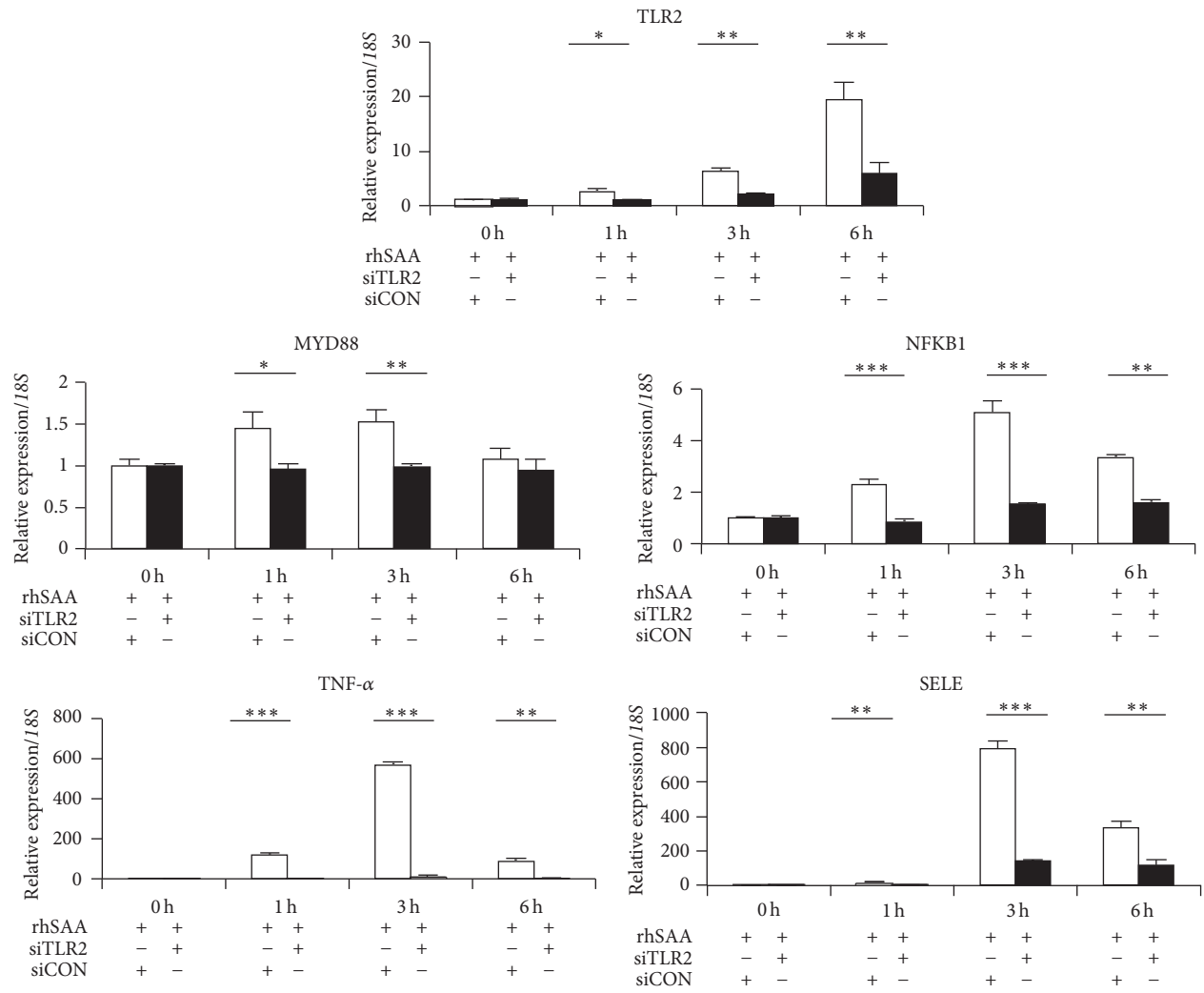

Figure 5: Effect of TLR2 siRNA on the expression of TLR and selectin cascade genes in HAECs. The mRNA expression levels of TLR2, MYD88, NFKB1, TNF-α, and SELE were examined in HAECs treated with TLR2 siRNA (closed column). After SAA stimulation for 0, 1, 3, and 6 h, the mRNA expression of all genes was dramatically decreased compared with the control (open column). Data represent the mean ± SD of four independent experiments. Each experiment was performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. rhSAA: recombinant human SAA; siCON: control siRNA; siTLR2: TLR2 siRNA.

to induce adhesion molecules, which might be involved in the onset of atherosclerosis.

The mRNA expression of adhesion molecules, including ICAM1, VCAM1, and SELE, was dramatically upregulated (>5-fold) by SAA stimulation in HAECs (Figure 1). E-selectin is important for the initial rolling interactions of neutrophils, monocytes, natural killer cells, and a subset of memory T cells in the inflamed endothelium [22, 23]. Leukocyte arrest during rolling is mediated by binding of leukocyte integrins to immunoglobulin superfamily members, such as VCAM-1 and ICAM-1, which are expressed by endothelial cells [24].

The initial step of atherosclerosis includes adhesion of peripheral blood leukocytes to activate the endothelial monolayer, directed migration of the bound leukocytes into the intima, and maturation of monocytes into macrophages and their uptake of lipids, yielding foam cells [25]. Multiple members of selectin, integrin, and immunoglobulin gene families in the process of initial attachment (rolling), stable adhesion (arrest), spreading, and ultimately diapedesis are sequentially involved in the onset of atherosclerosis [25]. Based on the PCR array results, SAA induces adhesion molecules and thus may trigger arteriosclerosis onset.

Several SAA receptors have been reported previously [21]. Among them, the expression of TLR2 was dramatically increased (about 30-fold) during SAA stimulation (Figure 2). We confirmed upregulation of TLR2 at both mRNA and protein levels by SAA stimulation, and SAA increased the expression of SELE in conjunction with the leukocyte adhesion cascade in HAECs. Endothelial cells normally express TLR2 at a very low level [26]. Thus, our results suggest that TLR2 may be an important receptor for SAA to induce adhesion molecules in HAECs.

Based on qPCR analysis of downstream molecules in TLR2 signaling (Figure 3), SAA stimulation had a remarkable

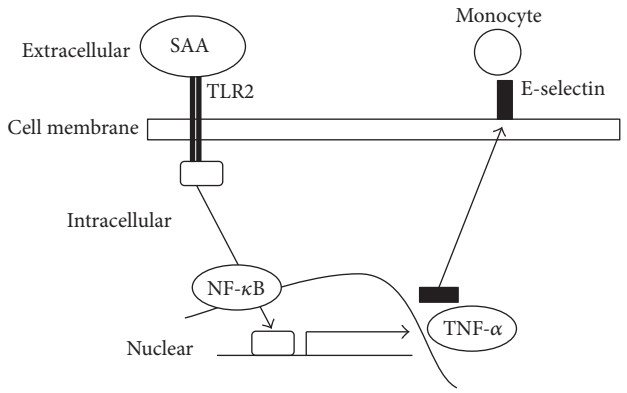

Figure 6: Summary of the possible mechanism for induction of E-selectin by SAA via TLR2. SAA may stimulate the expression of adhesion molecules including E-selectin through downstream signaling pathways such as NF-κB and TNF-α via TLR2.

influence on their expression. In particular, expression of the leukocyte cascade (i.e., NFKB1 and TNF-α) was upregulated following the induction of TLR2 by SAA stimulation. In addition, analysis of HAECs transfected with siRNA against TLR2 showed downregulation of the expression of the leukocyte cascade (NFKB1 and TNF-α) and SELE in a time-dependent manner (Figure 5).

MyD88 has a critical role in signaling via TLR2. After stimulation, TLR recruits IL-1R-associated kinase via adaptor MyD88 and induces activation of mitogen-activated protein kinases and nuclear factor-κB (NF-κB) [27]. NF-κB activation induces proinflammatory cytokines, including TNF-α and chemokines [28]. Furthermore, in human endothelial cells, adhesion molecules such as E-selectin are induced by NF-κB and TNF-α [29]. Nevertheless, in the current study, mRNA expression of MYD88 was decreased in SAA-stimulated HAECs, and the mRNA expression of NFKB1, TNF-α, and SELE was upregulated. We speculate that SAA may induce NF-κB, TNF-α, and E-selectin, in part, through a MyD88-independent pathway via TLR2. Indeed, Nilsen et al. have recently reported a novel function of TRAM/TRIF in TLR2-mediated signal transduction [30]. However, further studies are needed to investigate the detailed mechanism of MyD88-dependent or MyD88-independent signaling pathways in HAECs treated with SAA.

5. Conclusions

Our results suggest that TLR2 may have a critical role to induce adhesion molecules following TLR2 signaling and the leukocyte cascade in SAA-stimulated HAECs (Figure 6). And SAA might be a predictive risk marker for atherosclerosis onset in patients with periodontitis.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Dietary Chitosan Supplementation Increases Microbial Diversity and Attenuates the Severity of *Citrobacter rodentium* Infection in Mice

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C57BL/6 mice were tested in order to investigate the effects of dietary chitosan (COS) supplements on intestinal microflora and resistance to *Citrobacter rodentium* infection. The findings reveal that, after consuming a 300 mg/kg COS diet for 14 days, microflora became more diverse as a result of the supplement. Mice receiving COS exhibited an increase in the percentage of Bacteroidetes phylum and a decrease in the percentage of Firmicutes phylum. After *Citrobacter rodentium* infection, the histopathology scores indicated that COS feeding resulted in less severe colitis. IL-6 and TNF-α were significantly lower in colon from COS-feeding mice than those in the control group. Furthermore, mice in COS group were also found to experience inhibited activation of nuclear factor-kappa B (NF-κB) in the colonic tissue. Overall, the findings revealed that adding 300 mg/kg COS to the diet changed the composition of the intestinal microflora of mice, resulting in suppressed NF-κB activation and less production of TNF-α and IL-6; and these changes led to better control of inflammation and resolution of infection with *C. rodentium*.

1. Introduction

Citrobacter rodentium, a mucosal pathogen found in mice, and enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), enteric pathogens found in humans, create attaching and effacing (A/E) lesions that result in the colonization of mucosa within the intestinal tract [1, 2]. The intestinal epithelium becomes infiltrated by bacterial attachments, the brush border microvilli become depleted, and pedestal-shaped structures begin to form beneath the adherent bacterium [3]. Colonic tissue changes comparable to EHEC and EPEC infection, along with a rise in the production of inflammatory cytokine and leukocyte infiltration, are prompted by A/E pathogens following the colonization of the colonic epithelium [4]. *C. rodentium* amongst mice populations has been used by many researchers to represent intestinal *E. coli* since intestinal EPEC or EHEC does not infect mice.

A number of significant intestinal disorders found in humans are also frequently modelled by *C. rodentium* in mice, such as colon tumorigenesis, ulcerative colitis, and Crohn's disease [5]. Colitis develops in mice that have been infected with *C. rodentium*, which results in the bacteria becoming overabundant and the mice's natural microbiota reducing in variety and quantity [6]. When infected, 1–3% of the mice's intestinal microbiota becomes *C. rodentium* [7], whilst the

colon comes under attack by 10^9 colony forming units (CFUs) per g [8]. The genetic makeup of the mouse impacts the formation of *C. rodentium*-induced colitis, with mice such as C3H/HeJ and FVB/N being prone to develop colitis as a result of the infection and mice such as CD-1 and C57BL/6 being considered amongst those that are not prone to develop colitis and are only prone to subclinical symptoms [9]. Vulnerability to contracting *C. rodentium*, as well as tendency to show a certain immune response, has been found to be highly related to intestinal microbiota composition [10].

The human intestinal tract houses 500–1,000 species of microbiota at a total quantity of almost 100 trillion [11]. Various factors, including location and diet, have a significant impact on the metagenome, although these factors do not have the same effect on a single organism's genome. Pathogen displacement, the extraction of energy such as SCFAs from nondigestible dietary substrates, and the development of the immune system all depend on intestinal microbiota. Significant changes in the natural microbiota homeostasis have been linked to various illnesses in humans [12].

COS is among the most plentiful polysaccharides found in insects, fungi, squid, oysters, krill, clams, and shellfish. It is a natural N-deacetylated derivative of chitin [13]. Many researchers have explored the ways in which the expression of Th1 and Th2 cytokines is impacted by COS [14]. In pigs [14], mice [15], rats [16], and fishes [17], COS serves as a regulator of the immune response. However, little is known about how intestinal microbiota is impacted by dietary COS despite a small number of studies attempting to study this in pigs and chickens [18]. It would be useful to gain insight into whether it is via the intestinal microbiota that COS is able to perform its advantageous biological functions, since it is already known that a number of biological functions are impacted by the intestinal microbiota [19, 20]. Nonetheless, at present, few findings exist on the topic of *C. rodentium*, intestinal microbiota, and the effects of COS supplements on them. Thus, the objective of this study is to evaluate the effects of COS supplementation on microbial flora composition changes, proinflammatory molecule reduction (and/or anti-inflammatory molecule increase), and *C. rodentium* infection.

2. Materials and Methods

2.1. Mice and Diet. This study used male C57BL/6 mice aged 6–8 weeks old, bred and reared at Hunan Agricultural University by breeders from China's Changsha-based SLAC Laboratory Animal Center. The reason for using only male mice was that sex and maternal factors have been shown to impact microflora composition. Hunan Agricultural University's Animal Care and Use Committees provided approval for the experiments. The mice were provided plentiful water and a normal diet [21], and they were kept individually in animal colonies that were free from pathogens. The colonies were kept at a temperature of 25°C, relative humidity of 53%, and an equal balance of 12 hours of dark/light. The mice were assigned at random to the COS and control groups after being housed in the colonies for three days, with 20 mice in each group. A normal rodent diet [21] was given to the control group. 300 mg/kg COS was added to the standard diet given to the COS group, which was consumed for 14 consecutive days. COS has a 6-sugar unit of N-acetyl glucosamine with β-(1–4)-linkages [8], an average molecular weight of less than 1,000 Da, and a degree of deacetylation over 95% and is free of endotoxins. The COS provided to the mice in this study was provided by the Chinese Academy of Sciences' Dalian Chemical and Physical Institute. The findings of an earlier research paper [14] were used as a guideline for the duration and amount of COS given. Throughout the experiment, the mice's weight gain, water consumption, and food consumption were recorded. Until being ready for analysis, samples were kept at −80°C.

2.2. C. rodentium Infection and Monitoring. After giving either the basal or COS diet for a period of 14 days, 10 mice in each group were infected with *C. rodentium*. CO_2 asphyxiation was administered to all mice on D7 after infection. Samples of the mice's feces, tissue, and colonic content were gathered. Bacteria were grown in Luria Bertani broth (0.05 g/L nalidixic acid/mL) overnight. Centrifugation was used on the cultures and the pellet produced was resuspended in sterile phosphate-buffered saline (PBS). This resulted in a concentration of 5×10^9 CFU/mL. Mice were then challenged at 9 am and 5×10^9 CFU *C. rodentium* were orally administered to the mice using the gavage process. In order to prevent shedding mice from spreading infection, all mice were kept separately when infected with *C. rodentium*. The mice were fed with the same basal or COS diet for 7 days, respectively. Feces collection was conducted on D7 after infection, at which point they were also weighed and suspended in PBS. Serial dilutions were plated onto plates containing nalidixic acid. After a period of 24 hours, bacterial colonies were counted. Each dilution was grown at 37°C overnight using the pour plate method in MacConkey agar supplemented with the antibiotic kanamycin (40 μg/mL) in order to compute only *C. rodentium*.

2.3. Colon Tissue: Histological Analysis. The colon of each mouse was removed and fixed in 10% formalin. Hematoxylin and eosin were used to stain and prepare paraffin-embedded sections. Six criteria (edema, ulcers, erosion, inflammation, goblet cell hyperplasia, and cryptitis) were outlined in order to histologically grade the colitis. A scale of 0–4 was used to score the lesions as follows: no epithelial thickening/colitis (0); minor epithelial cell hyperplasia/greater quantity of mucosa leukocytes (1); inflammation at numerous loci, submucosa, and mucosa infiltrated by leukocytes and/or significant epithelial cell hyperplasia (with 2-3 times' rise in crypts) (2); greater epithelial cell hyperplasia (3–10 times higher crypt count), reduction of goblet cells secreting mucin, ulceration, and/or significant leukocytic infiltration of the submucosa and mucosa (3); extremely high epithelial cell hyperplasia (10 or more times higher crypt count), crypt abscesses, and/or serious infiltration of transmural leukocytes (4).

2.4. 16S rDNA and Illumina MiSeq Sequencing. After giving either the basal or COS diet for a period of 14 days, feces were collected and 10 mice in each group were killed to collect colon contents. Then, feces and colon contents were used

Table 1: Comparative results of 16S rRNA gene libraries' phylotype coverage and diversity at 97% similarity based on pyrosequencing analysis ($n = 6$).

	Number of reads	Number of OUT	Coverage	Richness estimator		Diversity index	
				Ace (95% Cl)	Chao (95% Cl)	Shannon (95% Cl)	Simpson (95% Cl)
Colon							
Control	12,568	37	99.88%	53 (43–86)	58 (43–112)	0.72 (0.69–0.74)	0.68 (0.66–0.70)
COS	12,794	46	99.88%	62 (52–87)	56 (50–81)	0.88 (0.85–0.90)	0.59 (0.58–0.60)
Feces							
Control	11,521	314	99.90%	324 (312–344)	329 (314–357)	4.17 (4.23–4.39)	0.025 (0.024–0.026)
COS	10,956	271	99.43%	304 (289–333)	302 (281–337)	4.05 (4.01–4.08)	0.033 (0.032–0.034)

for 16S rDNA sequencing. As per the guidelines for DNA isolation, the QiagenQIAamp DNA Stool Mini Kit was used to extract DNA from luminal colon and feces contents. In order to create a baseline sample for each sample type, equal quantities of DNA were gathered from six individual mice. Primers 515F 5′-GTGCCAGCMGCCGCGG-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′ (the barcode being an eight-base sequence individual to each specific sample) were used to amplify the V4-V5 region of the bacteria 16S ribosomal RNA gene by PCR. Biotree, a commercial firm based in Shanghai, carried out the general data analyses and Illumina MiSeq sequencing. Previous findings were used to guide the MiSeq PE Libraries, MiSeq sequencing, and additional analysis [22]. The corresponding authors can be contacted to request further information on references, raw data, and the sequencing run.

2.5. Analysis of Colonic mRNA. *IL-6* and *TNF-α* expression were incorporated in the analyses, which occurred after the collection and weighing of the proximal colon. TRIZOL regent (Invitrogen, USA) was used for the extraction of mRNA. Reverse transcription of the cDNA was conducted, and an ABI 7500 Fast RT-PCR machine (Applied Biosystems), Superscript II reverse transcriptase (Invitrogen), and oligo (dT) 20 were used to carry out real-time PCR.

2.6. Cytokine Measurement. A 50 mM Tris-HCl with 10 μg/mL protease inhibitor solution (Sigma-Aldrich Co., USA) was used to homogenise the colonic samples over ice. Centrifugation of 20 minutes was used on the homogenates at 30,000 ×g (4°C). Following centrifugation, a Sandwich ELISA Kit (ELISA Ready-SET-GO, eBioscience, CA, USA) was used to test the supernatants for TNF-α and IL-6. Normalization of cytokine was carried out to match the colonic samples' protein levels.

2.7. NF-κB (p65) Immunoblotting. The NF-κB (p65) transcription factor assay kit (Cayman Chemical Company, MI, USA) was used to measure NF-κB (p65) binding activity in the nuclear extracts. The proteins extracted from nuclear or cytoplasmic fractions were taken in equal quantities and then (1) divided using SDS-PAGE, (2) relocated to PVDF membranes (Millipore, MA, USA), and obstructed using 5% nonfat milk in a Tris-Tween buffered saline buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) over a 3-hour period. Prior to conducting the analysis with Alpha Imager 2200 (Alpha Innotech Corporation, CA, USA), overnight incubation at 4°C was carried out with the primary antibodies, whilst 60-minute incubation at room temperature was carried out with the HRP-conjugated secondary antibodies. Finally, electronic quantification and normalization of signal intensity to Lamin B protein abundance were performed.

2.8. Statistical Data Analysis. SPSS 22.0 (Chicago, IL, USA) was used to carry out all statistical analysis. The data illustrated in this section are the means ± the standard error of the mean (SEM). Student's t-test was used to analyze the data between two groups. In this study, statistical significance is shown in values of $P < 0.05$.

3. Results

According to the weight measurements, there was no postinfection change in the weight of each mouse in either of the two groups. Additionally, as shown in Figure 1, at D7 after infection, no change was noted in the quantity of *C. rodentium* in the feces or colon contents of each group. However, as shown in Figure 2, a significant increase in colitis severity was observed at D7 after infection amongst the control group of mice compared to the mice provided with COS.

As shown in Table 1, 16S rDNA sequencing was used to analyze the intestinal microbiota once the experiment was completed. The results of 16S rDNA sequencing were used to investigate the effects of COS supplements on histopathology scores. It is clear that the COS-fed mice showed reduced microbiota diversity (as per their feces samples), according to the Shannon and Simpson indices, compared to the control group of mice. The indices also showed that the COS-fed mice showed higher microbiota diversity than the control group of mice based on the colon analysis. Interestingly, it was indicated by the Ace and Chao richness indices that both groups of mice showed similar microbiota community richness in their faecal and colonic samples (Table 1).

The RDP classifier was used to perform a taxon-dependent analysis in order to identify the intestinal microbiota's taxonomy. For both groups of mice, the faecal microbiota showed nine phyla, including one candidate division (TM7), whilst the colon microbiota showed seven phyla, including one candidate division (TM7). Additionally, six

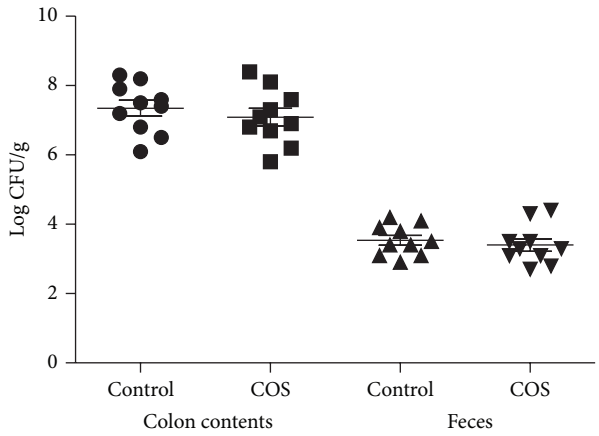

Figure 1: D7 postinfection *C. rodentium* levels in colon contents and feces of infected C57BL/6 mice. Feces and colon contents were gathered before undergoing homogenisation and being plated in serial dilution on LB agar ($n = 10$).

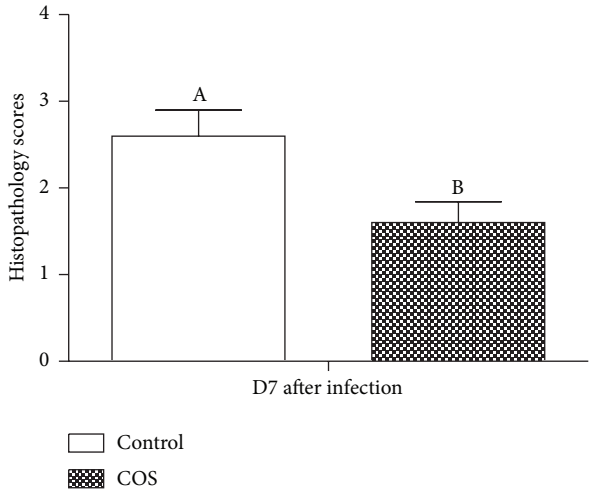

Figure 2: D7 postinfection histopathology scores of each mouse ($n = 10$).

phyla were found in each of the mice groups. As illustrated in Figure 3, in colon contents, Firmicutes (96.8%) and Proteobacteria (1.3%) were the phyla with the highest percentages in the colon microbiota of the COS-fed mice, whilst in feces Firmicutes (96.1%) and Proteobacteria (1.8%) were the phyla with the highest percentages in the control mice. The percentages of Bacteroidetes (61.4%), Firmicutes (27.1%), and Proteobacteria (4.5%) were the three most abundant in the faecal microbiota of the COS-fed mice, whilst the percentages of Bacteroidetes (53.8%), Firmicutes (39.6%), and Proteobacteria (3.5%) were the most abundant in the control mice.

As shown in Figure 4(a), colon analysis of control mice showed significantly higher *IL-6* and *TNF-α* mRNA than the COS-fed mice at D7 after infection. This finding is supported through the ELISA results (Figure 4(b)).

Nuclear NF-κB (p65) measurements were taken to identify the activation of NF-κB in the colons of the mice.

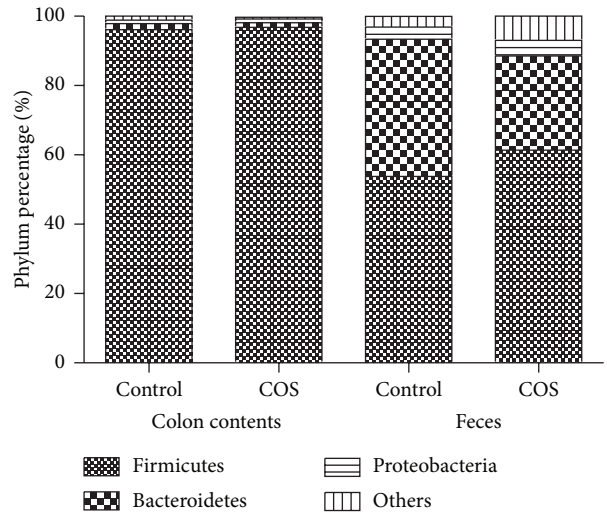

Figure 3: D7 postinfection composition of the intestinal microbiota. Microbial composition in the colon and feces of both groups ($n = 6$). Control mice were given standard drinking water and a basal rodent diet, whilst COS mice were given the same water and diet with the addition of COS at 300 mg/kg.

The purpose of this was to identify whether the beneficial outcomes of COS (i.e., in reducing infection) are supported by the suppression of NF-κB activation pathways. The results showed that the control group of mice had significantly higher nuclear NF-κB (p65) in their colonic samples than the COS-fed group of mice, which suggests that COS suppresses NF-κB activation (see Figure 5).

4. Discussion

C. rodentium, which is similar to the human enteropathogenic *E. coli* infection, is an extracellular enteric pathogen that infects mice. It has also been noted that inflammatory bowel disease (IBD) is often modelled using *C. rodentium* in mice. IBD results in intestinal inflammation. Thus, intestinal pathology is regulated by inflammatory regulators such as inducible TNF*α* and IL-6 [6]. Intestinal bacterial communities influence mice's susceptibility and ability to overcome the *C. rodentium* infection, as does the immune response of the mice [23]. *E. coli* is commonly modelled by the infection of mice with *C. rodentium* since mice are not affected by EPEC or EHEC. *C. rodentium* has been found in all mice strains but it is rare in human disease. Depending on the strain, an infected mouse can suffer subclinical disease or death after infection [9]. For instance, strains believed to be resistant to developing colitis as a result of *C. rodentium* infection include CD-1 and C57BL/6 mice. On the other hand, it is believed that susceptibility is present amongst mice strains C3H/HeJ and FVB/N [9]. Based on this information, human *E. coli* infection was modelled in this study by infecting C57BL/6 mice with *C. rodentium*.

The results showed that, after infecting the mice with *C. rodentium*, inflammation was resolved more quickly amongst mice receiving 300 mg/kg COS than the control mice.

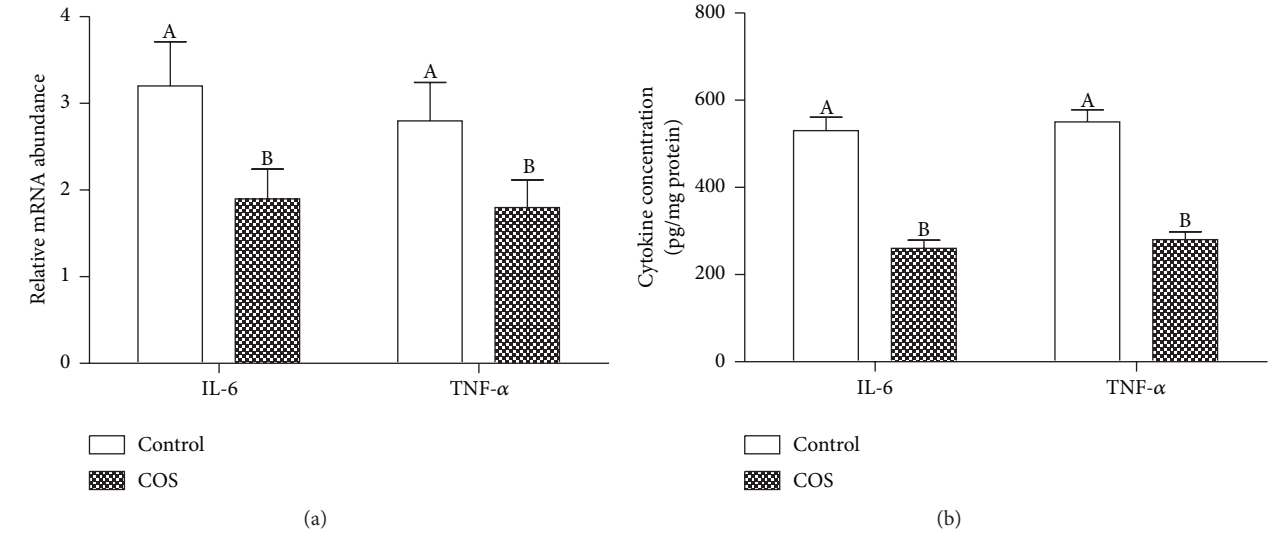

Figure 4: Control and COS group ($n = 6$) mucosal inflammatory responses. (a) RT-PRC evaluation of *IL-6* and *TNF-α* mRNA level. (b) ELISA evaluation of IL-6 and TNF-α protein level.

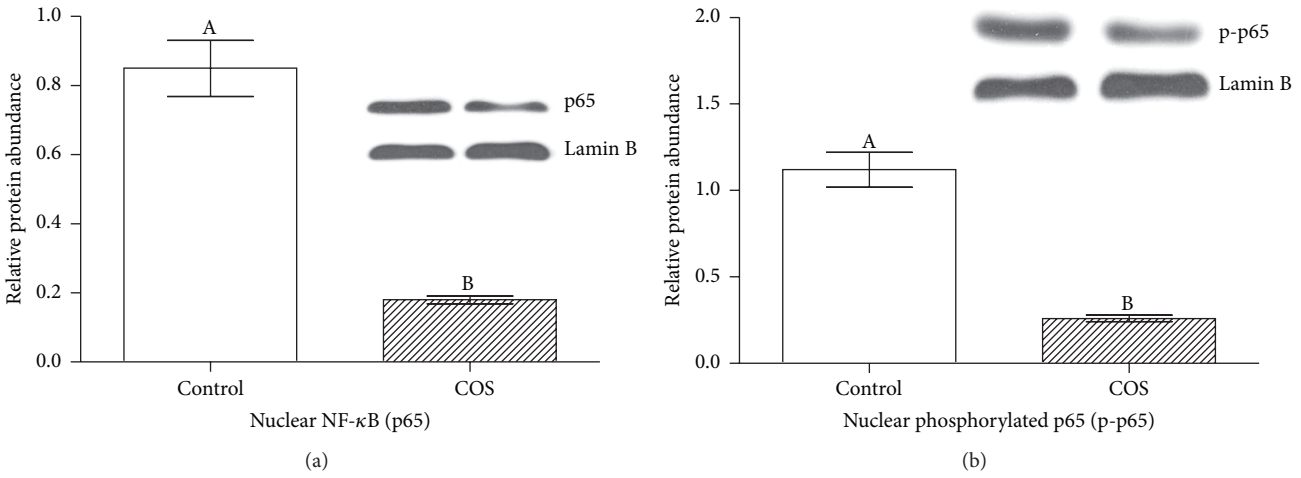

Figure 5: Immunoblotting of (a) nuclear NF-κB (p65) and (b) phosphorylated nuclear NF-κB (p65) in control and COS group ($n = 6$).

The results noted no impact on the quantity of *C. rodentium* taken from faecal samples, which suggests that the regulating impact of COS is not due to swift pathogenic eradication but rather due to an improvement in microflora diversity. Various biological functions are said to be influenced by such microbiota [19, 20], and it has been associated with cancer [24, 25], cirrhosis of the liver [26], and other diseases. Since Firmicutes has the ability to offer extra energy to the host by fermenting plant polysaccharide to SCFA, this is the reason for obesity being linked to higher concentrations of Firmicutes: Bacteroidetes [27]. At present, the reason behind COS's minimising impact on Firmicutes quantities is unclear. Some studies have also shown that a reduction in intestinal Firmicutes can be achieved through acidic oligosaccharides, a galactooligosaccharides/long-chain fructan solution (GOS/lcF, 9/1), fructooligosaccharides, and other oligosaccharides [28]. It may be that the N-acetyl glucosamine of COS may function as a binding agent that allows COS to influence the attachment of bacteria to the intestine. Given this, research on pigs and chickens indicates that intestinal microbial communities can be impacted by the introduction of COS supplements [18]. Furthermore, intestinal microbiota may change as a result of COS's role as a fermentable substrate for certain bacteria, as this could lower the pH level of the gut and activate natural acid production [26].

Various studies have indicated *in vitro* and *in vivo* anti-inflammatory effects of COS. In the current study, it has been found that IL-6 and TNF-α expression in the colon are reduced by COS, whilst microbial flora is impacted by the inflammation [23]. Thus, microbiome changes may be influenced by increased inflammation. Since the faecal samples showed no relationship between *C. rodentium* quantities and the introduction of COS, this finding is contradictory. Consequently, the speed at which *C. rodentium* resolution

occurs is related to microflora changes and not inflammation [29]. The results of this study indicate that the effects of COS on the intestines only occur in specific areas of the gut and only take on a specific form. Mice fed with COS were found to have a lower expression of inflammatory cytokines, which is linked to bacterial flora shifts that lead to faster infection recovery.

COS is a d-glucosamine oligomer that cannot be eroded by enzymes in the gut. Thus, COS directly protects the host's intestinal epithelial cells (IEC). Research indicates that IEC expresses various chemokine and cytokine receptors such as TLR4 and other Toll-like receptors [30]. In the IEC, NF-κB-regulated proinflammatory cytokine production is promoted by the interaction between TNF-α and their respective receptors, TLR4, TNFR1, and TNFR2 [31]. Studies also show that dysfunction of the intestinal epithelial barrier can occur as a result of pronounced IEC-regulated mucosal inflammatory responses, which are associated with greater TLR4 and TNFR expression [32]. In the current study, it was revealed that a significant reduction in the activation of NF-κB along with colon production of TNF-α and IL-6 was achieved through the administration of COS through the basal diet. Since recent studies have found that mice can develop disease sharing similar characteristics to Crohn's as a result of IEC-derived TNF-α [32], this indicates that the anti-inflammatory impact of COS could be the result of COS' suppressive impact on the activation of NF-κB and production of proinflammatory cytokines in the IEC.

Overall, it is clear that *in vivo C. rodentium* infection can be attenuated by the introduction of COS into mice's diet. The findings suggest that this is due to the prevention of IL-6 and TNF-α expression and NF-κB activation as well as a shift in intestinal microflora. Innovative and successful methods for protecting hosts from *C. rodentium* infection could therefore be achieved through further investigation of the organic carbohydrate oligomer COS.

Competing Interests

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

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Review Article

Multiple Sclerosis and Obesity: Possible Roles of Adipokines

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Multiple Sclerosis (MS) is an autoimmune disorder of the Central Nervous System that has been associated with several environmental factors, such as diet and obesity. The possible link between MS and obesity has become more interesting in recent years since the discovery of the remarkable properties of adipose tissue. Once MS is initiated, obesity can contribute to increased disease severity by negatively influencing disease progress and treatment response, but, also, obesity in early life is highly relevant as a susceptibility factor and causally related risk for late MS development. The aim of this review was to discuss recent evidence about the link between obesity, as a chronic inflammatory state, and the pathogenesis of MS as a chronic autoimmune and inflammatory disease. First, we describe the main cells involved in MS pathogenesis, both from neural tissue and from the immune system, and including a new participant, the adipocyte, focusing on their roles in MS. Second, we concentrate on the role of several adipokines that are able to participate in the mediation of the immune response in MS and on the possible cross talk between the latter. Finally, we explore recent therapy that involves the transplantation of adipocyte precursor cells for the treatment of MS.

1. Introduction

The prevalence of immune-mediated diseases has increased in recent years. In parallel, changes in dietary habits, which promote high-fat, high-sugar, and high-salt foods, have led to an obesity epidemic over the past years [1, 2]. According to the World Health Organization (WHO), approximately 35% of the world population is estimated to have overweight (Body Mass Index [BMI], 25–30 kg/m^2) or obesity (BMI > 30 kg/m^2) [3]. Therefore, it is reasonable to think about a possible correlation among these realities. In fact, obesity is considered a chronic state of low-grade inflammation that has been implicated as a proactive factor in several chronic autoimmune inflammatory disorders [4, 5].

Multiple sclerosis (MS) is an autoimmune disorder of the Central Nervous System (CNS), which is mainly characterized by selective and coordinated inflammatory destruction of myelin, with damage to the axon. It is a chronic and progressive inflammatory disease caused by an interaction of genetic and environmental risk factors. MS mainly affects young people with onset usually at the age of 20–50 years and a mean age-of-onset of 30 years, although the disease may also develop in childhood and after the age of 60 years [6]. Childhood and adolescence are thought to be a critical period of susceptibility to promoting factors.

This autoimmune disease has already been associated with several environmental factors, such as diet and obesity [7–10]. Obesity is a well-known risk factor for multiple conditions, including cardiovascular risk and metabolic disorders, as metabolic syndrome and insulin resistance [11], and has also been associated with a nonfavorable course of several autoimmune diseases [2]. Concomitant with the rise of MS

is the increased prevalence of children with overweight and obesity in human population over recent decades [3].

Several studies have reported an increase in the prevalence of overweight and obesity among patients with MS [12], although some reported higher frequencies of patients with MS with a lower BMI [13] and some others reported similar rates of overweight and obesity in patients with MS and in general population [14]. Numerous publications have reported an association between MS and higher BMI in youth and early life [15–19]. Obesity in female patients with MS at the time of diagnosis is associated with a relapsing course at disease onset [20] and is also associated with a greater risk of depression, lower functional capacity, and worse self-rated health status among patients with MS [21, 22]. However, a direct relationship between MS and obesity remains inconclusive [23].

A reported cooccurrence of obesity in patients with MS may simply reflect increased obesity in the population or it may be secondary to the harmful effects of the disease. However, a positive correlation between BMI and disability evaluated by the Expanded Disability Status Scale (EDSS) was reported recently [24]. To date, scarce information is available on the association of metabolic comorbidities and disability in individuals with MS. One study [25] showed that vascular comorbidities, such as diabetes, hypertension, hypercholesterolemia, and peripheral vascular disease, were independently associated with an increased risk for disability. In contrast, another study reported that worsening disability was associated with higher low-density lipoprotein cholesterol, total cholesterol, and triglycerides in patients with MS [26]. Additionally, an association exists between disability and Oxidative Stress (OS) in patients with MS [27]. What is becoming clear is that obesity influences both disease progress and treatment response. Additionally, a couple of recent studies in large populations clearly associate obesity in early life, during adolescence [28], and during childhood and/or early adulthood [29] with a higher MS risk, providing further solid evidence strongly suggesting that obesity is also relevant by means of its causal role in MS etiology.

The possible link between MS and obesity has become even more interesting in recent years since the discovery of the remarkable properties of adipose tissue. Adipose tissue has the ability to expand in response to chronic caloric excess as an adaptive response. In individuals with obesity, adipose tissue can constitute up to 50% of total body mass. Since the exciting finding of the secretory properties of adipose tissue, the relationship between obesity and autoimmunity and the understanding of the underlying mechanisms have become of major interest. Indeed, fat tissue, in addition to its function as an energy storage site, has been found to produce a wide variety of soluble mediators denominated "adipokines," which are involved in the regulation of numerous physiological functions, such as the regulation of energy balance, insulin sensitization, and the immune response [2]. Initially identified for their metabolic and appetite regulation activities, adipokines are known to be involved in various processes, including immunity and inflammation. Because of their proinflammatory action, these molecules contribute to the so-called "low-grade inflammatory state" in subjects with obesity [30].

Obesity is associated with self-directed tissue inflammation, in which local or systemic factors other than infectious agents activate the cells of the innate immune system. During the development of obesity, adipocytes undergo considerable differentiation and expansion in order to store lipids [31]. However, immune cell infiltration and activation within adipose tissue are a major source of proinflammatory cytokines, which impair adipocyte function in chronic obesity. Thus, alterations in adipose tissue and the development of chronic inflammation are the hallmarks of obesity and are at least partially responsible for the induction of insulin resistance.

On this basis, the aim of this review was to present, integrate, and discuss recent evidence on the possible link between obesity as a chronic inflammatory state and the pathogenesis of MS as a chronic autoimmune and inflammatory disease. First, we briefly describe the main cells involved in MS pathogenesis, both from neural tissue, including glial cells (such as microglial cells, oligodendrocytes, and astrocytes) and neurons, as well as cells from the immune system such as different T cell lineages, CD4+ (such as Th1, Th2, and Th17), CD8+, and Treg (CD4+CD25+FoxP3+), and also including a new participant, the adipocyte, a cell from the adipose tissue, focusing on their roles in MS. Second, we concentrate on the role of several adipokines that are able to participate in the mediation of the immune response in MS and on the possible cross talk between these. Finally, we explore recent therapy that involves the transplantation of adipocyte precursor cells for the treatment of MS.

2. Cells Involved in MS

2.1. CNS Cells

2.1.1. CNS: Microglia. Microglia constitute the resident macrophages of the CNS [40] and are involved in immune processes as such as Antigen Presenting Cells under pathological conditions [41]. Microglia express several immunological markers, such as MHC I, MHC II, CD40, CD11b, Fc receptors I–III, Complement Receptors (CR1, CR2, and CR4), β2 integrins, CD80, CD86, and IntraCellular Adhesion Molecule-1 (ICAM-1) [42]. Dual roles of macrophages have been described in human neuroimmune diseases such as MS. Whereas proinflammatory macrophages secrete harmful molecules to induce disease development, anti-inflammatory macrophages produce beneficial mediators to promote disease recovery [43].

In experimental autoimmune encephalomyelitis (EAE) in mice, an animal model for MS, the expressions of MHC II and CD86 are increased (Table 1). This suggests that microglia participate in the antigen presentation to the T cells that migrate into the CNS. In addition, in MS lesions, microglia and CNS-infiltrating macrophages interact with oligodendrocytes by their expression of VCAM-1 [44] and with phagocyte myelin and axon debris, via HLA-DR, contributing to the demyelination process [45].

The activation of microglia in MS is a dynamic process, because there is an intermediate state in all preactive and

Table 1: Cell types affected in the experimental autoimmune encephalomyelitis (EAE) mouse model or in patients with multiple sclerosis (MS).

Adipokine	EAE	Multiple sclerosis	Reference
Leptin		↓ IFN-γ- and IL-17-secreting T cells in patients treated with Metformin ↓ TNF-α- and IL-6-secreting T cells in patients treated with Pioglitazone ↑ CD4+CD25+FoxP3 Treg in patients treated with Metformin and Pioglitazone	[72]
		↓ NK cells, ↓ B cells, ↓ CD4+DR+ T cells, and ↓ CD4+CD45RA+ T cells in patients treated with GA	[73]
		↓ nTreg cells in patients with RRMS	[74]
	↓ CD4+ T cells, CD8+ T cells, and CD11b+Gr1+ granulocytes in ENdothelial leptin receptor-specific Knock-Out (ENKO)		[32]
Adiponectin	Adiponectin treatment is associated with increase in Treg		[36]

remyelinating lesions that differ from microglia profiles in actively demyelinating lesions [46]. Antigen-activated microglia produce proinflammatory cytokines, such as InterLeukin-1beta (IL-1β), Tumor Necrosis Factor alpha (TNF-α), and IL-6 [42]. A subset of CD4+ T cells that produces IL-17 or IL-17 and InterFeroN gamma (IFN-γ) migrates to the CNS prior to the onset of EAE clinical symptoms (revised forward). This event coincides with microglia activation and their production of IL-1β, TNF-α, and IL-6 in the CNS [47]. Treatment with Glatiramer Acetate (GA) prevents interaction between T cells and microglia and contributes to the amelioration of MS severity in patients [48]. These facts support that, in the pathogenesis of MS, both peripheral immune system cells and CNS resident cells are involved from disease onset.

In a recent study, Michels et al. demonstrated, in an animal model of sepsis, that costimulation of microglia through CD40-CD40L is associated with brain inflammation, oxidative damage, and Blood Brain Barrier (BBB) dysfunction [49]. In addition, soluble CD40L has been associated with the complications of obesity, such as cardiovascular disease, insulin resistance, and chronic inflammation [50]. CD40 is a transmembrane receptor expressed in a variety of cell types including microglia in the CNS [49]. The interaction between CD40 and its ligand (CD40L), expressed by T cells, induces costimulation in the presenting antigen and enhances the production of cytokines, chemokines, matrix metalloproteinases, growth factors, and adhesion molecules, mainly through NF-κB [51]. Also, the presence of CD40L plus IFN-γ induces ERK1/2-mediated Monocyte Chemoattractant Protein-1 (MCP-1) and p38-mediated IFN-Inducible Protein-10 (IP-10) production by microglia. These chemokines have been associated with CNS pathologies, including MS [52]. It is possible that CD40/CD40L interactions between microglia/macrophages and T cells are involved in MS pathogenesis; thus, future studies should be conducted on this aspect.

Regarding microglia cells participation in axonal damage, activated microglia releases glutamate, which induces excitotoxicity and contributes to damage. Blockade of glutamate release decreases neuronal death [53]. In this respect, higher levels of glutaminase, produced by macrophages and microglia, are found in MS lesions, and these levels are correlated with axonal damage in animals. In addition, GLutamate Transporter-1 (GLT-1) has low expression levels in active lesions, and Glutamine Synthetase (GS) and Glutamate DeHydrogenase (GDH) are absent in active and chronic, silent MS lesions [54]. This glutamate unbalance leads to neural death and increases CNS damage and disease severity.

Microglia can not only be involved in the inflammatory and neurodegenerative process. It is possible to induce a neuroprotective state in the microglia through a stimuli with IL-4 and IL-25 [55]. This neuroprotective state can induce oligodendrogenesis through its activation by IL-4 and IFN-γ and a later release of Insulin-Like Growth Factor-1 (IGF-1) [56]. Additionally, intraparenchymal microglia produce a Macrophage-Derived Chemokine (MDC/CCL22), which could induce Th2 cell migration into the CNS and mediate Th1-migration [57]. Furthermore, Programmed Death-Ligand-1 (PD-L1) acts as an inhibitory costimulatory molecule that is expressed by microglia after stimulation with IFN-γ [58] or IL-6 [59], and CNS-infiltrating T cells exhibit overexpression of their receptor Programmed Death-1 (PD-1) at the EAE peak and prior to remission. In this regard, microglia can inhibit CD4+ T cell proliferation and Th1 cell differentiation in a PD-L1-dependent manner [41]. Therefore, microglia can play a dual role in the pathogenesis of MS, depending on the cytokine microenvironment, where it can perform inflammatory functions that promote axonal damage and neural death or anti-inflammatory functions that inhibit cell migration into the CNS.

In vitro, Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), a regulator of adipocyte differentiation implicated in obesity [60], is strongly upregulated following demyelination mediated by antibodies directed against the Myelin Oligodendrocyte Glycoprotein (MOG) in the presence of complement [61]. Pioglitazone, an agonist of PPAR-γ that modulates the transcription of insulin-sensitive

genes [62], partially protects aggregates from anti-MOG demyelination, and it appears to be linked with an inhibition of glial cell proinflammatory activities following anti-MOG-induced demyelination [61]. Additionally, a similar effect to that of pioglitazone can be provided by nutraceutics, which can modulate PPAR-γ signaling and which can be employed as a complimentary treatment for obesity-related disorders [63]. Activation of PPAR-γ signaling regulates adiponectin production, a protein with anti-inflammatory properties trough the inhibition of proinflammatory cytokines and a protective role against insulin resistance [64]. PPAR-γ agonists can modulate EAE by the inhibition of NO, TNF-α, IL-1β, IL-6, and MCP-1 production from microglia and astrocytes [65] and also by inhibiting IL-12 production and signaling and Th1 differentiation [66]. In addition, it has been demonstrated that Steroid Receptor Coactivator-3 (SRC-3) inhibits adipocyte differentiation [67] and controls the expression of PPAR. SRC-3 deficiency attenuates EAE severity through the upregulation of PPAR-β in the CNS and the subsequent microglia-alternative activation, which modulates neuroinflammation and promotes remyelination [68]. Together, these data suggest that the PPAR agonist could modulate the demyelination process in EAE and MS.

On the other hand, microglia express estrogen receptor-β, and their signaling reduces NF-κB expression, as well as NF-κB-induced gene-inducible Nitric Oxide Synthase (NOS) in microglia and CD3+ T cells [69]. It has been demonstrated that T cells induce microglia activation through C/EBPbeta, but not NF-κB, in EAE, but only in female mice. This is one of the mechanisms that could explain why MS is more prevalent in women [70]. Also, estrogen pretreatment in EAE mice enhanced the frequency of regulatory B cells and anti-inflammatory M2 microglia [71]. These evidences suggest that estrogen treatment promotes neuroprotection through the inhibition of T cell and macrophage recruitment into the CNS and microglia activation, which may represent a benefit for MS treatment.

2.1.2. CNS: Oligodendrocytes. Oligodendrocytes are the myelinating cells of the CNS. Both neurons and oligodendrocytes are affected during brain damage in acute and chronic neuroinflammation, leading to demyelination processes [102]. In MS, oligodendrocytes are damaged by different processes and their number decreases. Oligodendrocyte Precursor Cells (OPC) are present in both normal CNS and MS lesions [103]. There is evidence indicating changes in the mechanisms of migration of OPC and myelinating oligodendrocytic activity. In this sense, Bin et al. found that netrin-1, an inhibitor of Oligodendrocyte Precursor Cell migration, is secreted by myelinating oligodendrocytes in MS lesions [104]. In addition, Sádaba et al. demonstrated the presence of IgM and IgG in demyelinating lesions localized in axons and oligodendrocytes from the autopsies of patients with MS [105]. However, the presence of Platelet-Derived Growth factor alpha Receptor (PDGRalphaR) and NG2 OPC in active MS lesions, as in remyelinated MS tissue, indicates that these cells are potential sources of remyelinating oligodendrocytes in active lesions [106]. Furthermore, the expression of CD200 in neurons, oligodendrocytes, and reactive astrocytes in MS chronic plaques and the interaction with its receptor CD200R suppress immune activity [107], and this affects neuron-microglia and glia-glia interactions.

In an attempt to prevent this damage, it has been reported that oligodendrocytes stimulated with IFN-γ, a proinflammatory cytokine, could be protected. Pancreatic Endoplasmic Reticulum Kinase (PERK) signaling can be activated in oligodendrocytes in the presence of IFN-γ in MS lesions [108]. The activation of PERK signaling pathway in oligodendrocytes attenuates EAE severity by a reduction of oligodendrocytic apoptosis and demyelination [109], indicating that activation of an integrated stress response could ameliorate MS severity.

2.1.3. CNS: Astrocytes. Astrocytes are the most abundant type of cells in the CNS. They participate in maintaining normal brain function and are in constant communication with neurons, oligodendrocytes, and Endothelial Cells. Also, astrocytes are involved in angiogenesis, neurogenesis, synaptogenesis, and axonal remodeling [110]. However, B7-1 (CD80) or B7-2 (CD86) is not expressed by astrocytes and CNS Endothelial Cells (EC) in EAE. This suggests that neither type of these cells can induce costimulatory signals via B7 molecules and that they are incapable of acting as Antigen Presenting Cells (APC) [111].

It has been demonstrated that astrocytes are involved in the progress of MS through several mechanisms, which include the following: releasing cytotoxic factors, inhibiting axonal remyelination, and contributing to axonal mitochondrial dysfunction [112]. In this regard, neuregulin production, an oligodendrocytic growth factor, is depleted in astrocytes [113], leading to the decrease of the oligodendrocytic maturation ability. Also, CD24 is expressed in both immune and CNS cells. Liu et al. demonstrated that CD24 expression enhanced costimulatory activity of astrocytes with T cells and increased EAE severity [114]. Furthermore, astrocytes and microglia can express inducible NO Synthase (iNOS) in EAE lesions [115], which contributes to axonal damage [116]. These data demonstrate the relevant contribution of astrocytes to MS pathogenesis and progression.

2.1.4. CNS: Neurons. The immune-mediated destruction of CNS myelin and oligodendrocytes has traditionally been considered the primary damage in patients with MS; however, the irreversibility of the neurological disability corresponds to cortical damage, driven by either neuronal loss induced by retrograde degeneration from white-matter lesions or as a direct consequence of the localization of demyelinated plaques within the cortex [117]. Main damage to neurons derives from the demyelination process, which affects myelin sheaths as well as the oligodendrocyte itself [118].

The profound oxidative injury observed in MS lesions appears to be related to mitochondrial impairment in damaged axons [119]. Consistently, synaptic loss comprises a characteristic feature of gray matter lesions (particularly in type I lesions) [120], and neurite density is proportionally reduced with increased meningeal inflammation [121]. Neurons can also interact with CD4+ T cells and induce its differentiation into CD25+ TGF-beta1+ CTLA-4+ FoxP3+

Treg through the interaction between the B7-CD28 and TGF-beta1-TGF-beta receptor signaling pathways. These Treg suppress encephalitogenic T cells in a CTLA-4 manner [122]. This data indicates that neurons can execute mechanisms that attempt to reverse the damage in the demyelination process.

2.2. Immune System Cells

2.2.1. Immune System: T Cells. T cells are a highly heterogeneous population of cell subtypes that mediate adaptive immunity and specific tolerance [123]. There are several subpopulations of T cells that differentiate upon encountering antigens in the peripheral lymphoid organs [124]. In MS, the role of T cells is crucial to trigger the immunopathological processes that culminate in demyelination and subsequent damage to oligodendrocytes and axons [125]. Several studies demonstrate that pathogenic T cell response against myelin antigens is followed by a neurodegenerative process [126]. However, it is difficult to establish a correlation between CNS-infiltrating and peripheral T cell subsets. In this respect, it is necessary to consider the possible existence of subgroups of patients, depending on treatment and time of disease evolution [127] to identify the correlations of these subgroups with variations in the T cell subsets present in MS pathogenesis.

2.2.2. Immune System: CD4+ T Cells. CD4+ T cells could play a central role in the pathogenesis of the EAE model. These cells cross the BBB and cause axonal damage and neuronal death [128]. Within the CNS, microglial cells interact with T cells by antigen presentation and the production of injurious or neurotrophic outcomes in their vicinity [129]. Additionally, activation of memory CD4+ T cells is associated with the exacerbation of MS; in addition, activation of memory CD8+ T cells (see later) reflects a dysregulation of the immune system in patients with MS [130]. Full activation and posterior proliferation of T cells usually require an Antigen Presenting Cell- (APC-) derived costimulatory signal, which is delivered via B7/CD28 [131]. However, the T cell proliferative response in MS is relatively independent of B7-CD28 costimulation [132–134].

Additionally, autoreactive T cells against myelin components can be detected in both the serum and CerebroSpinal Fluid (CSF) of patients with MS [135]. Burns et al. described that 84% of the MBP-reactive T cells are memory T cells in peripheral blood [136] but, according to Chou et al., only 37% of T cells isolated from patients with MS are MBP- or PLP-reactive in LCR [137]. Furthermore, CD4+ T cells in CSF are predominantly helper inducers, and their increase may contribute to local autoimmune process in the CNS [138]. Taken together, these studies prove that T cells reactive to myelin play an important role in the pathogenesis of MS.

2.2.3. Immune System: Th1 and Th2. Th1 cells secrete high levels of IFN-γ and IL-12, which are considered proinflammatory cytokines. On the other hand, Th2 cells secrete high levels of anti-inflammatory cytokines, mainly IL-4, IL-5, IL-9, and IL-13, and promote the humoral response [139].

Originally, MS was considered as an autoimmune disease, driven by Th1 cells. This was supported by three facts: (1) CD4+ T cells isolated from CNS from mice with EAE express detectable levels of IFN-γ messenger RNA (mRNA) [140]; (2) IL-12p40-defective (IL-12p40−/−) mice are resistant to EAE induction, because IL-12 is a requirement for naïve T cell differentiation in Th1 cells [141]; and (3) patients with MS exhibited exacerbation during treatment with IFN-γ [142]. However, several studies suggest that there is a dysregulation in the balance between Th1 and Th2 cytokine profiles in MS [143].

The Transforming Growth Factor-Beta (TGF-β) inhibitor Smad7 is an important negative modulator that regulates the strength of TGF-β signaling [144] and it is upregulated in peripheral CD4+ T cells from patients with MS during disease relapse, but not during remission. This Smad7 expression correlates with T-beta responses, suggesting that Smad7 drives Th1 differentiation and regulates the inflammatory cellular response [145]. Additionally, disease progression can be altered by a hormonal component that modifies T cell number and cytokine secretion. In this sense, CD4+ IFN-γ-producing cells fluctuate with MS relapses: declining during pregnancy, in women with MS, and continuing to decline after parturition, in women with relapses. In contrast, these cells rise, or remain stable, in women with nonrelapsing MS or healthy pregnant women [146].

In contrast, Th2 cells have been described as being protective in MS/EAE [147]. Th2 cells are able to suppress microglial activation via cell-to-cell contact [148]. Furthermore, treatment with GA, which shifts the cytokine profile from Th1 to Th2, is regularly utilized as immunomodulatory therapy for MS [149, 150]. This suggests that Th2 cells are activated by GA in the periphery, migrate into the CNS, and then produce the Th2 cytokine profile after local recognition of Myelin Basic Protein (MBP) [150]. In the case of other treatments, patients with MS treated with IFN-β showed downregulation of circulating T cells secreting IFN-γ and IL-4 [151].

However, despite the anti-inflammatory behavior of Th2 cells in autoimmune diseases, in a recent work, Planas et al. identified clonally expanded CD4+ T cells releasing Th2 cytokines in T cell infiltrate of pattern II brain autopsy lesions, and the authors argue that this subset possesses a pathogenic and not a protective role in MS [152].

It is noteworthy that Th1 and Th2 cells infiltrate adipose tissue which varies according to regional fat depot. Th1 cells are higher in Visceral Adipose Tissue (VAT) than in Subcutaneous Adipose Tissue (SAT) and peripheral blood in healthy individuals with overweight and obesity. However, these proinflammatory T cell frequencies in VAT are correlated with SAT and peripheral blood [153]. This suggests that peripheral T cell subsets could also be associated with adipose tissue-infiltrating T cells in patients with MS and with obesity.

2.2.4. Immune System: Th17. The T-helper 17 (Th17) cells are a subset of CD4+ effector T lymphocytes that challenges the Th1/Th2 paradigm of the immune response. Th17 cells mainly secrete IL-17 and are currently recognized for their

involvement in the pathogenesis of autoimmune diseases [154, 155].

In the CSF of patients with Relapsing-Remitting Multiple Sclerosis (RRMS), Th17 cell frequency is higher in the relapse than in the remission stage or than in other noninflammatory neurological diseases [156]. In acute lesions, the expression of Retinoic Acid-Related Orphan nuclear hormone Receptor (RORc) and the cytokine genes that participate in Th17 expansion are upregulated in acute autopsy lesions of patients with RRMS [157]. Because Th17 promotes BBB disruption and causes the increase of CD4+ T cell infiltration in the CNS [158], the presence of Th17 cells in the CNS is able to give rise to microglial activation and the increase of the proinflammatory cytokine microenvironment [47]. Furthermore, IL-17, a cytokine produced mainly by Th17 cells, induces higher production of glutamate, causing excitotoxicity [159]. This cytokine has been investigated in autoimmune diseases [160], including MS [161].

Th17 cells can be influenced by the cytokine microenvironment, being able to exhibit certain developmental plasticity by upregulating IFN-γ and T-bet with a decreasing expression of IL-17 and RORγt [162]. Additionally, Carbajal et al. demonstrated that plastic Th17 cells can also be mediators of demyelination and axonal damage, as well as of Th1, and can induce the classical features of MS independently of differentiation factors IL-23 and IL-12 [163]. These evidences indicate that there is a possible link between Th1- and Th17-mediated autoimmune demyelinating processes, which can provide a future, common therapeutic target for MS treatment.

The search for serum biomarkers that describe the behavior of MS includes the study of cytokines such as IL-17. However, it is difficult to correlate these levels with the evolution of the disease. Thus, the existence could be considered of subgroups of patients with specific characteristics, considering age, treatment, and time to disease progression [127]. In this respect, it would be possible for T cell subsets to also vary according to these subgroups in patients with MS.

Regarding treatment, IFN-β induces greater STAT1 activation in Th17 compared to Th1 because Th17 cells express higher levels of InterFeroN alpha/beta Receptor 1 (IFN-α/βR1). This result indicates that IFN-β can inhibit Th17 cell expansion [164]. Therefore, IFN-β suppresses Th17 differentiation *in vitro* by inhibiting Th17 cell-lineage markers RORc, IL-17A, IL-23, and CCR6 and by inducing IL-10 production [165, 166].

Recently, it was reported that obesity may predispose induction of Th17 cells, at least, in part, in an IL-6-dependent process, which exacerbates autoinflammatory diseases such MS in mouse models [167]. Paradoxically, IL-17 has also been shown to inhibit adipogenesis [168]. Therefore, the precise role of Th17 cells and IL-17 in the obesity-associated inflammatory conditions needs to be further clarified.

2.2.5. Immune System: CD4+CD25+FoxP3+ Treg. Regulatory T cells (Treg) comprise specialized suppressor T cell population that restrains the pathogenic immune response [169], while CD4+CD25+FoxP3+ Treg maintain tolerance to self-antigens [170]. Treg mediate the autoimmune inflammation induced in EAE through Cytotoxic T Lymphocyte Antigen- (CTLA-) 4-dependent suppression of pathogenic T cells [122]. Posterior recovery correlates well with the accumulation of Treg, that are producers of IL-10 [171]. In MS, the Treg function is defective and this contributes to the pathogenesis of the disease [170]. Also, Treg are capable of migrating across the BBB under noninflammatory conditions but, in patients with RRMS, this ability is impaired [172]. This change contributes to breaking the homeostasis in the CNS and could facilitate the initiation of CNS inflammation.

The suppressor activity of T cells in the peripheral blood of patients with patients in remission-clinical-phase was first described by Huddlestone and Oldstone [173]. Since that time, controversial results have been reported concerning the presence of Treg during the fluctuating behavior of MS. In this regard, the frequency of CD4+CD25+FoxP3+ Treg was described as lower in patients with MS than in healthy controls [174, 175], but there was no correlation with clinical variables [174]. Bach et al. previously described that these cells are depressed in patients with acute exacerbation of MS [176]. Contrariwise, Dalla Libera et al. demonstrated that Treg are restored during an acute clinical attack [177]. This suggests that Treg are not involved in causing clinical relapses, but, rather, they can react to the inflammation in an attempt to restore the balance.

Regarding treatment, Praksova et al. described that both IFN-β and GA increase naïve T cells and decrease central memory T cells [175], but only Glatiramer Acetate (GA) increases Treg [175, 178]. However, this is in contrast with other works reporting that CD4+CD25+FoxP3+ Treg are increased in patients with MS treated with IFN-β [179, 180], without any significant effect on *FoxP3* gene expression after 6 months [180]. It has been described that lFN-β induces downregulation of CTLA-4 in Treg [179]. With respect to treatment effect, it has not been possible to date to establish any relationship between obesity and the use of lFN-β or GA [181].

On the other hand, glucocorticoid treatment restores impaired Treg function and increases IL-10 production in relapsing patients with MS [182]. Further, glucocorticoids modulate T-bet and STAT1 expression in mononuclear cells from patients with RRMS [183]. The effect of glucocorticoid treatment contributes to the decrease of neuroinflammation. However, the chronic use of glucocorticoids is associated with weight gain and increased obesity and related disorders [184, 185].

With respect to sexual hormones, these appear to be involved in the increase of Treg, as well as other T cell populations. It has been described that, during gestation, there is a physiological expansion of Treg that contribute to decreasing EAE manifestations and reducing CNS demyelination [186]. In addition, E2 and P2 enhance Treg function *in vitro* [187], leading to the suggestion that the use of E2 is capable of being a therapy for MS [188, 189] but that estrogens participate in metabolic functions and their use in humans produces the accumulation of subcutaneous fat [190] which could trigger obesity and metabolic disorders.

2.2.6. Immune System: CD8+ T Cells. Because the study of the immunological basis involved in MS has focused mainly on CD4+ T cells as mediators of the disease [191, 192], the role of CD8+ T cells in MS is unclear to date. However, CD8+ T cells constitute the predominant T cell population in lesions in patients with MS and are oligoclonally expanded at the site of the pathology [192]. Furthermore, it has been recently demonstrated that CD8+ T cells engage in both roles: the pathogenic and the regulatory role in MS [192].

CD8+ T cells and CD4+ T cells exhibit different epigenetic signatures because they have distinct DNA methylation profiles [193]. It has been demonstrated that myelin-specific CD8+ T cells infiltrate the CNS and play a pathogenic role in EAE. In addition, the presence of CD4+ T cells is required for more severe disease and sustained neuroinflammation [194]. Furthermore, CD8+ T cells participate in myelin-specific CD4+ differentiation into CNS-infiltrating effector cells in secondary lymphoid organs [195]. Therefore, all of these evidences strongly suggest that disease severity depends on the cooperation between CD4+ and CD8+ T cells.

On the other hand, CD4+ T cells expressing Latency-Associated Peptides (LAP) suppress EAE depending on the TGF-β [196]. But not only CD4+ T cells can express LAP. CD8+ LAP+ Treg comprise a novel subset that has a regulatory function and that reduces the disease severity of EAE by TGF-β- and IFN-γ-dependent mechanisms [197].

In the same way that CD4+CD25+FoxP3+ does, CD8+CD28− Treg also regulate the balance between immunity and tolerance [174]. CD8+CD25+FoxP3+ T cells are IL-10 and TGF-β producers and downregulate costimulatory molecule expression in dendritic cells through a STAT3-mediated pathway, inducing a less efficient antigen presentation and inducing 2,3-dioxygenase (IDO) through STAT3 and cytotoxic T lymphocyte antigen-4-dependent mechanisms [198]. Furthermore, CD8+ T cells, with immunosuppressive functions, are induced in MS patients treated with GA, and they appear to be HLA-E restricted [192]. These latter data demonstrate how CD8+ T cells can play a regulatory role in the evolution of MS.

2.3. Adipose Tissue

2.3.1. Adipose Tissue: Adipocytes. Although clinical evidence correlating obesity and MS is growing [9, 181], very little is known to date about the possible involvement of adipose tissue in MS pathogenesis as well as in cross talk among the tissues involved. Therefore, this constitutes a whole new field to be explored. It has been hypothesized that there is a link between metabolism and MS that involves proinflammatory mediators such as leptin, which promotes environmental conditions that in turn promote the loss of immune self-tolerance [199]. Additionally, human adipocytes express the costimulatory receptor CD40, suggesting that these cells contribute to obesity-related inflammation. Also, T cells regulate adipokine production through soluble factors and contact with adipocytes, involving CD40 [200].

The apparently passive role played by adipocytes, in pathophysiological terms, has been gradually substituted with a metabolically active performance, relevant to many biochemical mechanisms that may contribute to a chronic low-grade inflammatory status, which increasingly imposes itself as a key feature of obesity [181]. As natural regulatory T cells (nTreg) do, adipocytes also could play an important role in the immunopathogenesis of MS and in the associated inflammation. In fact, very recently, cells that are able to differentiate into adipocytes, as mesenchymal stromal cells, are being utilized in transplantation as therapy to treat autoimmune diseases, in particular MS (see the last part of this review).

3. Adipokines Involved in Cellular Cross Talk in MS

Clinical and experimental data, together with epidemiological studies, have suggested that the pathogenesis of MS might involve factors that link the immune system with the metabolic status [199]. Therefore, a very exciting field in recent investigation comprises the role of adipokines in the pathogenesis of MS. Some studies have reported increased levels of leptin, resistin [32], and visfatin and decreased levels of adiponectin in patients with RRMS in comparison with healthy controls [33], a profile also observed among subjects with obesity [201–203] (Table 2).

3.1. Adipokines

3.1.1. Leptin. Leptin is an adipokine predominantly produced by adipose tissue and secreted into the circulation [204]. This adipokine is a regulator of body weight that promotes satiety and stimulates energy expenditure [205], and its levels correlate with the body adipose mass as well as with adipocyte size [206]. Leptin is upregulated by inflammatory mediators, such as TNF-α, IL-1 [207], and IL-6 [208]. In addition to its metabolic effects, leptin is also a potent modulator of immune responses [209]. Thus, patients who are congenitally leptin-deficient have a higher incidence of infection-related death due to dysfunctional immune response [210], with reduced numbers of circulating CD4+ T cells, impaired T cell proliferation, and cytokine release [211]. Leptin modulates immune response toward a proinflammatory profile and is found at the crossroads between inflammation and autoimmunity. Upsetting its balance may result in immunosuppressed condition, or, conversely, in a proinflammatory state, then facilitating the development of autoimmune diseases [2].

Leptin receptor (LepR) is expressed in CD4+, CD8+ [212, 213], Treg [214], and Natural Killer (NK) cells [215] and in monocytes/macrophages [212]. Because of the expression of their receptor, leptin induces differential effects in CD4+ T cells. Naïve T cells increased proliferation, whereas memory T cells were inhibited. Furthermore, leptin increases IFN-γ production and inhibits IL-4 production in memory T cells [216].

In patients with MS, LepR expression has been found significantly higher in CD8+ T cells and monocytes from patients in relapse phase than that observed in patients in remission (or in healthy controls). Moreover, exogenous leptin treatment sustains STAT3 phosphorylation, but only in

Table 2: Adipokines involved in the experimental autoimmune encephalomyelitis (EAE) mouse model or in patients with multiple sclerosis (MS).

Adipokine	EAE	Multiple sclerosis
Leptin	↑ In serum before clinical onset [75] Transport in hippocampus and cervical spinal cord in early stage [77] In females rather than in males in pup mice and decrease in time-dependent manner [57] ↑ IFN-γ and TNF in Histidine DeCarboxylase-deficient (HDC−/−) mice [78] In Endothelial Leptin receptor-specific Knock-Out (ELKO) [80] Microvascular leptin receptors [80]	↑ In patients [32, 39], in CSF and serum [35] during remission [76] ↑ pSTAT3 and ↓ SOCS3 in patients with RRMS on relapsing [76] ↓ sCD40L and ↓ TNF-R in patients treated with GA [73] In IFN-β-treated patients [79] In females [81] and pregnant females with MS [82] ↑ Leptin receptor in CD8+ T cells in monocytes from patients with RRMS in relapse rather than remission [76] ↑ Leptin receptor (ObR) in T cell lines after activation with human Myelin Basic Protein (hMBP) [35]
Leptin	↓ In triterpene-pretreated EAE mice [83] ↓ IL-6 in EAE mice with caloric restriction [84]	↓ In patients treated with Metformin and Pioglitazone [72] In postdelivery females with MS [82] After IFN-β treatment [85] and ↓ IL-6 in patients with SPMS treated with IFN-β [86] In untreated or methylprednisolone-treated patients [79]
Leptin	Negatively correlates with clinical score in females [57] Correlates with disease susceptibility, reduction in food intake, and decrease in body weight [75] Blockade of leptin with antileptin or soluble mouse receptor chimera (ObR:FC) improves clinical score, reduces disease relapses, inhibits T cell proliferation, and induces Th2 cytokine profile [88]	Negatively correlates with testosterone in males [87] Positively correlates with BMI in females [87] Correlates with disease duration, but not with EDSS, in females [81] ObR in plasma are similar between patients and controls [79] Correlates in serum with IFN-γ in CSF [35] Inversely correlates with Treg [35] Correlates with BMI in patients with low and intermediate, but not with high, EDSS [89] Induces IFN-γ in PBMC from patients with MS in relapse, but not in remission [90] Induces TNF-α, IL-6, and IL-10 production in PBMC from relapsing patients with MS, but not in remission [91]
Adiponectin	As treatment, ameliorates EAE in ADPKO mice [84]	↓ In patients with MS [37] In patients with RRMS [32] In patients treated with Metformin and Pioglitazone [72] ↑ In CSF from twins with MS [92] ↓ IL-6 in EAE mice with caloric restriction [84]
Resistin		↑ In patients with MS and in patients with RRMS [32] ↑ TNF-α, ↑ IL-1β, and ↑ hs-CRP in patients with MS [34] and in patients with PPMS [33]
Visfatin		↑ In patients [33]
A-FABP		↑ In patients with SPMS and in pediatric-onset patients [39]
Adipsin		↑ In CSF from twins with MS [92] In serum and CSF in individuals with overweight/obesity and in patients suffering from hypertension or diabetes mellitus [93] Correlates in serum and CSF and is gender-independent [93] In CSF does not correlate with oligoclonal-band presence [93]

Table 2: Continued.

Adipokine	EAE		Multiple sclerosis
Chemerin	↑ In CNS of mice [94] It is present in intralesional cerebrovascular endothelial cells, and its receptor is expressed in CNS-infiltrating leukocytes [95]	↑	In patients with MS with overweight/obesity [38]
Omentin-1			Correlates with BMD at femoral neck, total hip, osteopontin, and osteocalcin [96]
Vaspin			Does not correlate with age, BMI, biochemical, and BMD measurements in patients with MS [96]

monocytes from relapsing patients [76], suggesting that LepR might play a role in the modulation of clinical relapses during MS. A gene microarray analysis of Th1 lymphocytes from active MS lesions has shown elevated transcripts of many genes of the neuroimmune endocrine axis, including leptin [217]. Additionally, leptin serum levels are elevated in patients with MS before relapses and after treatment with IFN-β [85]. These data suggest that leptin and its receptor induce Th1 cell and cytokine environment and favor the induction of inflammation in MS.

Moreover, in a recent work, Yu et al. demonstrated that Th17 cell frequency is reduced in leptin-deficient (ob/ob) mice and that the administration of exogenous leptin restores the Th17 cell count. Additionally, leptin induced Th17 response through RORγt transcription in normal human T cells [218]. In the animal model of arthritis, leptin enhances Th17 cell response and exacerbates joint inflammation [219]. Thus, the findings suggest that leptin can induce an altered immune state and should contribute to neuroinflammation in MS through both Th1 and Th17 cell response.

Although there is evidence suggesting that leptin is associated with the development of CNS in mice [220, 221], it has been demonstrated in the animal model that ob/ob mice are resistant to EAE induction and that leptin-deficient (ob/ob) mice exhibit impaired cellular immune response, abnormal cytokine production, and impaired T cell proliferation [211]. Protection in ob/ob mice was associated with a progressive decline in the survival of autoreactive CD4+ T cells and reduced production of Th1 and Th17 cytokines. T cells demonstrated downregulation of Bcl-2, a survival protein, reduction in P-ERK1/2, and cell-cycle arrest associated with reduced degradation of cell-cycle inhibitor $p27^{kip1}$. Additionally, there is impairment at the level of the nutrient-energy-sensing AKT-mTOR/S6 signaling pathway, which can be restored *in vivo* with leptin administration [222]. Taken together, the evidence allows the consideration of leptin and/or its receptor as important players in the pathogenesis of MS, as well as their consideration in therapeutic targets of the disease.

Matarese et al. found that leptin is increased in CSF and in the serum of patients with RRMS. These levels are related to IFN-γ production in the CSF and, inversely, with the proportion of Treg [35]. In this respect, several studies showed that Treg accumulated in normal adipose tissue [223–225].

Recently, the relationship between caloric restriction and survival in MS has been investigated. In an animal model, Piccio et al. established that caloric restriction and reduction of leptin serum levels can increase survival and lifespan through the reduction of inflammation, demyelination, and axonal injury [84]. At the same time, Longo and Fontana demonstrated that caloric restriction reduces inflammatory parameters and suggest that nutritional intervention improves the inflammatory response [226]. Caloric restriction has also been associated with an increase of the ghrelin, NeuroPeptide Y (NPY), which can moderate EAE [227]. All of the previous evidence indicates that leptin is able to participate as a link between metabolism and MS. So, it is necessary to find out the role of neurohormonal response in the ingestion of food, which is orchestrated by the hypothalamic circuitry in patients with MS.

3.1.2. Adiponectin. Adiponectin (APN) is the most abundant circulating adipokine and is involved in metabolic diseases such as type 2 diabetes, metabolic syndrome, and related complications, especially cardiovascular diseases. Also, evidence indicates that APN is related to the severity of rheumatoid arthritis, systemic lupus erythematosus, and osteoarthritis [228]. Systemic and local levels of APN are elevated in patients with inflammatory and immune-mediated diseases [155, 168, 229].

APN exerts its effects through its receptors. AdipoR1 is expressed predominantly in skeletal muscle, AdipoR2 is found more abundantly in liver, and T-cadherin is mainly expressed in the cardiovascular system [230]. In this regard, APN exhibits anti-inflammatory activity on immune system cells [231]. Its functions include suppression of the proliferation of myelomonocytic precursor cells and the phagocytic activity of mature macrophages [232], regulation of monocyte-to-fibroblast transition [233], regulation of TNF-α and IFN-γ production in response antigen presentation [234], and the induction of IL-10 and IL-1 Receptor Antigen (IL-1RA) in monocytes, macrophages, and dendritic cells (DC) [2]. However, it has been reported that APN plays a dual role in immune system, indicating that APN possesses proinflammatory actions as a possible result of the presence of isoforms [2]. Jung et al. demonstrated that APN can promote DC activation, leading to Th1 and Th17 polarization [235].

Piccio et al. investigated the role of APN in adiponectin deficient (ADPKO) mice with EAE. These mice developed higher CNS inflammation, demyelination, and axonal injury. T cells from ADPKO mice produced higher levels of TNF-α, IFN-γ, IL-17, and IL-6 and a decreased Treg number and function, demonstrating that the absence of APN contributes to elevating the disease severity. Furthermore, the use of APN as a treatment in these animals ameliorates EAE by the increase of the number of Treg [36]. These data indicate that APN could exert beneficial effects on the treatment of MS, but information on the role of APN with the animal model of MS is insufficient and more efforts must be made in this respect.

Regarding APN serum levels in patients with MS, Kraszula et al. observed that APN is decreased in patients with RRMS compared with healthy individuals. Also, the authors found higher levels of leptin, correlating with the number of Treg; however, APN was not correlated with Treg [32]. In line with these findings, Musabak et al. also found that the APN serum levels were lower than those of healthy controls, and these levels were higher in female than in male patients with MS. Then, it is possible that APN is gender-dependent, because the same behavior is observed in healthy controls [37].

In contrast, Hietaharju et al. found higher CSF concentrations of APN and adipsin in twins with MS in remission compared with their asymptomatic twins, although these levels did not correlate with plasma levels. In this regard, the authors suggest that there is a possible intrathecal synthesis

of adipokines or increased transport across the BBB following enhanced systemic production [92]. However, the sample size of this study is small; thus, there is a need for larger studies to clarify the significance of APN levels in patients with MS.

3.1.3. Resistin. Resistin is a mediator of insulin resistance and is also known as ADSF (ADipocyte-Secreted Factor) or FIZZ3 (found in inflammatory zone 3) [168, 236, 237]. This adipokine has been implicated in obesity, diabetes [238], atherosclerosis [239], coronary heart disease, and cardiovascular disease [240]. Additionally, there is evidence that serum resistin levels are increased in patients with rheumatoid arthritis (RA) [241].

These adipokines are produced by adipocytes [242], intestinal epithelium, adrenal gland, and skeletal muscle [243]. It has been reported that resistin can be expressed in CNS by the pituitary gland and that its expression is age- and gender-dependent in mice [244]. Also, resistin can influence sympathetic nerve activity in CNS [245].

A few studies had attempted to discover the relationship between resistin and MS pathogenesis in patients with the disease [32–34]. In an endeavor to determine the possible association, Emamgholipour et al. observed an elevation of resistin, leptin, and visfatin levels, as well as a decrease in the FoxP3 mRNA expression of T cells. Additionally, the authors found that these adipokines are positively correlated with some inflammation mediators in healthy controls, suggesting that adipokines may play a role as inducers of proinflammatory mediators (TNF-α, IL-1β, and hs-CRP) [33]. Hossein-Nezhad et al. also found higher serum levels of resistin in patients with MS compared with the control group [34], which adds evidence to support the role of resistin in MS, although more studies are needed.

3.1.4. Visfatin. Visfatin was originally known as PBEF (Pre-B cell colony-Enhancing Factor). Leukocytes, adipose tissue macrophages, hepatocytes, or skeletal muscle participates in visfatin production [246]. Visfatin is related to glucose metabolism because it can bind to and activate the insulin receptor [247].

In the area of immune activities, visfatin induces the production of IL-6, TNF-α, and IL-1β in monocytes. Moreover, visfatin increases the surface expression of costimulatory molecules CD54, CD40, and CD80 [248]. In RA, visfatin is described as a proinflammatory and destructive mediator of joint inflammation in RA [249]. Emamgholipour et al. found a positive correlation of visfatin with TNF-α, IL-1β, and hs-CRP in patients with MS [33]. Almost nothing is known about the relationship between visfatin and MS.

3.1.5. Adipocyte-Fatty Acid-Binding Protein. Serum Adipocyte-Fatty Acid-Binding Protein (A-FABP) is produced by adipose tissue, monocytes, and macrophages, and its expression is enhanced by Toll-Like Receptor-2 (TLR-2) stimulation [250]. Higher levels of A-FABP have been associated with increased triglycerides, elevated fasting serum glucose, and hs-CRP in coronary artery disease [251]. Regarding MS, A-FABP levels are highest in Secondary Progressive MS (SPMS), suggesting a possible role in the pathogenesis of this disease subtype. Also, A-FABP levels are increased in patients with Pediatric-Onset MS (POMS) and may play a role in the early stages of disease [39]. Therefore, more studies are necessary on this adipokine and its relationship with MS pathogenesis in humans and the animal model to clarify the mechanisms in which A-FABP is involved.

3.1.6. Adipsin. Adipsin was described as a molecular marker of obesity in rodents [252]. The function of adipsin in relation to energy homeostasis and systemic metabolism remains unknown [253]. In patients with a specific neurological diagnosis without prior selection, serum adipsin levels are correlated with CSF levels. Also, serum and CSF levels are correlated with age and are higher in patients with diabetes mellitus or hypertension. Adipsin CSF levels are correlated with inflammation mediators, but not with the presence of oligoclonal bands [93]. In a study of twins with MS, Hietaharju et al. found higher CSF concentrations of APN and adipsin in twins with MS in remission compared with their asymptomatic twins and no correlation with its plasma levels [92]. Recently, a study conducted by Natarajan et al. has provided novel insights into the impact of adipokines on MS and suggests that adipsin exerts predictive potential as a biomarker of neurodegeneration [254]. However, very little is known about the role of adipsin in MS pathogenesis; therefore, more studies are needed.

3.1.7. Chemerin. Chemerin is an adipokine secreted by adipocytes and is associated with obesity, the metabolic syndrome, and insulin resistance [255]. Chemerin has been identified as a chemoattractant for Antigen Presenting Cells (APC), including DC and macrophages; in addition its receptor ChemR23 induces the release of calcium, inhibition of cAMP accumulation, and phosphorylation of p42–p44 MAP kinases [256]. In addition, chemerin is a proteolytically regulated leukocyte chemoattractant when it binds to CheMoKine-Like Receptor-1 (CMKLR-1) [94].

This adipokine is expressed in vascular Endothelial Cells in the meninges and in white-matter lesions of MS, whereas its receptor is expressed in infiltrating leukocytes, including plasmacytoid DC. These data suggest that chemerin is directly involved in the migration of peripheral cells into the CNS and that they contribute to the inflammatory process [95]. CMKLR-1 Knock-Out (KO) mice exhibit reduced symptoms of EAE [94]. It has been demonstrated that targeting with alpha-NETA (α-NETA), an antagonist of CMKLR1, inhibits CNS-infiltrating cells and modulates inflammation in EAE mice. However, α-NETA does not modify T cell proliferation [257]. In a recent study, it was found that chemerin plasma levels are higher in patients with MS with overweight or obesity compared with patients with MS and without obesity and controls [38]. These results suggest that obesity in patients with MS increases chemerin levels and causes an increase in CNS-infiltrating cells that may, in turn, contribute to disease severity.

3.1.8. Omentin. Omentin is produced mainly by Visceral Adipose Tissue and its expression is reduced in obesity,

insulin resistance, and type 2 diabetes. This adipokine has anti-inflammatory, antiatherogenic, anticardiovascular, and antidiabetic effects [258]. In patients with MS, omentin-1 serum levels are correlated with Bone Mineral Density (BMD) at femoral neck, total hip, osteopontin, and osteocalcin [96]. It has been reported that omentin-1 is involved with insulin activity, induces Akt phosphorylation [259], and is inversely correlated with obesity [260]. These evidences provide little knowledge; thus, more studies on these adipokines are needed to establish whether or not omentin participated in the pathogenesis of MS.

3.1.9. Vaspin. Vaspin is an adipokine that is predominantly secreted by Visceral Adipose Tissue and its serum levels are increased and associated with obesity and impaired insulin sensitivity. To the contrary, its correlation is abrogated in type 2 diabetes and its levels are higher in females as compared with males [261]. Vaspin has been poorly studied in patients with MS. In this regard, Assadi et al. did not find any correlation between vaspin and age, BMI, biochemical, and BMD measurements in patients with MS [96].

In summary, dysregulated adipokines can be involved in the pathophysiology of MS, increasing the risk of the disease development after obesity, during adolescence or the early-adult stage, as well as influencing elements that affect disease evolution and treatment response for obese adults with MS (Figure 1). Undoubtedly, there remains such research to be conducted with regard to these aspects.

4. Transplantation of Adipose Tissue-Mesenchymal Stem Cells as Therapy for MS

Mesenchymal Stem Cells (MSC) are a pleiotropic population of precursor cells that are self-renewing and capable of differentiating into canonical cells of the mesenchyme, including adipocytes, chondrocytes, and osteocytes [262]. Due to their immunomodulatory and neuroprotective effects, Adipose Tissue-Mesenchymal Stem Cells (AT-MSC) may be proper candidates for stem cell-based MS therapy. The intraperitoneal (i.p.) route exerts a more pronounced effect on maintaining splenic CD4+CD25+FOXP3+ T cell population and increasing IL-4 secretion. In addition, i.p. injection of cells resulted in lower IFN-γ secretion and reduced cell infiltration in brain more effectively as compared with the intravascular (i.v.) route [263].

In mice with established EAE, *in vivo* infusion of wild-type Adipose Stromal/Stem Cells (ASC) significantly ameliorated the disease course, autoimmune-mediated demyelination, and cell infiltration through regulation of the inflammatory responses. However, mice treated with autologous ASC exhibited no therapeutic improvement in terms of disease progression [264].

The preclinical efficacy of AT-MSC obtained from the SJL/JCrl mouse strain (SJL-AdMSC) has been assessed by autologous transplantation in RR-EAE-induced SJL mice (a well-established mouse model for the study of RRMS) by ameliorating the RR-EAE course, suggesting that these could modulate disease progression [265].

The therapeutic efficacy of ASC isolated from lean subjects (BMI < 25, lnASC) and subjects with obesity (BMI > 30 in ObASC) were determined in murine EAE. Compared with EAE disease-modifying effects of lnASC, ObASC consistently failed to alleviate clinical symptoms or to inhibit inflammation in the CNS. When activated, ObASC expressed higher mRNA levels of several proinflammatory cytokines compared with InASC. Additionally, Conditioned Media (CM) collected from ObASC markedly enhanced T cell proliferation and differentiation, whereas CM from lnASC did not. These results indicate that obesity reduces, or eliminates, the anti-inflammatory effects of human ASC; therefore, they may not be a suitable cell source for the treatment of autoimmune diseases. The data suggest that donor demographics may be particularly important when identifying suitable stem cells for treatment [266].

The majority of Mesenchymal Stem Cells (MSC) clinical trials are currently in phase 2 of development, during which safety and tolerability of treatment continue to be evaluated (Table 3). These works include patients with median or high Expanded Disability Status Score (EDSS) and who are mainly in the SPMS stage. Sample size is reduced and the protocol of administration is variable, both in number of cells transplanted and in the administration pathway (mainly intravenously or intrathecally). It appears to be that, in the majority of cases, there is a decrease in the EDSS index after transplantation; however, it is necessary to follow up patients for a longer time period (>6 months) and to be more accurate with respect to the possible improvement achieved. The results are also highly variable, as well as the adverse events presented (see summary, Table 3). Consequently, although AT-MSC transplantation can be regarded as a potential source of treatment for MS, several studies now at clinical stages need to see whether these show a real benefit in practice, particularly in MS progressive stages (PPMS and SPMS). These works will contribute to the design of future trials conducted to establish whether MSC transplantation comprises an effective therapy for patients with MS.

In conclusion, since the discovery of the remarkable properties of adipose tissue the possible link between MS and obesity has been rendered even more interesting. Several evidences on the possible link between obesity and the pathogenesis of MS were discussed. Although it is well-known that CNS and immune system cells are involved in the pathogenesis of MS, adipokines comprise the possible cross talk between them (Figure 2). Therefore, it will be also relevant to explore the role of neuropeptides, like NPY, leptin, ghrelin, and other proteins, in relation to feeding behavior, whose mechanisms are regulated by orexigenic and anorexigenic hypothalamic neurons, because an adequate regulation of this neural circuitry will be related to an improvement in the inflammatory response and survival in MS. Finally, it seems to be more clear that once MS is initiated, obesity can contribute to increasing disease severity by negatively influencing disease progress and treatment response, but, also, obesity in early life (mainly during adolescence) is highly relevant as a susceptibility factor and causally related increased risk for late MS development.

Table 3: Studies in human therapy for MS by transplants of adipose tissue-mesenchymal stem cells.

MS form	Sample size	Dosage and administration	Changes in EDSS	Adverse events	Results	Ref
SPMS	10	1.6×10^6 cells/Kg Intravenously	Initial 6.1 decrease after treatment	Macular rash, scalp pruritus, upper-respiratory tract infection, and urinary-tract infection	No changes in the posttreatment period for T cell subset counts (CD3, CD4, CD8, CD19, and CD56) Decrease of T1 hypointense lesion volume after treatment Increase in optic nerve area and reduction in visual evoked response latency	[97]
SPMS and PRMS	25 (23/2)	2.95×10^7 cells Intrathecally	Initial 5.5 to 7 decrease or stability after treatment in 68% of patients	N/A	MRI score decrease in 68% of patients Increase of IL-6 gene expression No changes in IL-4, IL-6, IL-10, IFN-γ, and TGF-β gene expression	[98]
SPMS and RRMS	10 (9/1)	(a) High 1×10^8 cells (b) Medium $3.2–5.2 \times 10^7$ cells (c) Low $1.1–1.5 \times 10^6$ cells Intrathecally and intracisternally	Initial 4.0 to 7.5 decrease after treatment	Transient encephalopathy with seizures in one patient	Clinical but not radiological efficacy Improvement in 5/6 and worsening in 1/6 patients New or enlarging lesions in 5/7 and Gadolinium (Gd+) enhancing lesions in 3/7 patients	[99]
MS	15	6.32×10^7 cells Intrathecally	Initial 6.7 decrease after treatment	Transient fever and headache	Increase of CD4+CD25+ Treg Decrease of CD40+, CD83+, CD86+, and HLA-DR on myeloid dendritic cells	[100]
MS	1	N/A	N/A	The patient development headache, nausea, vomiting, and difficulty of ambulation	Accumulation of CD-68 positive myelin-laden macrophages and T cells Extensive demyelination in a pattern typical of multiple sclerosis in brain biopsy	[101]

PRMS = progressive relapsing multiple sclerosis.

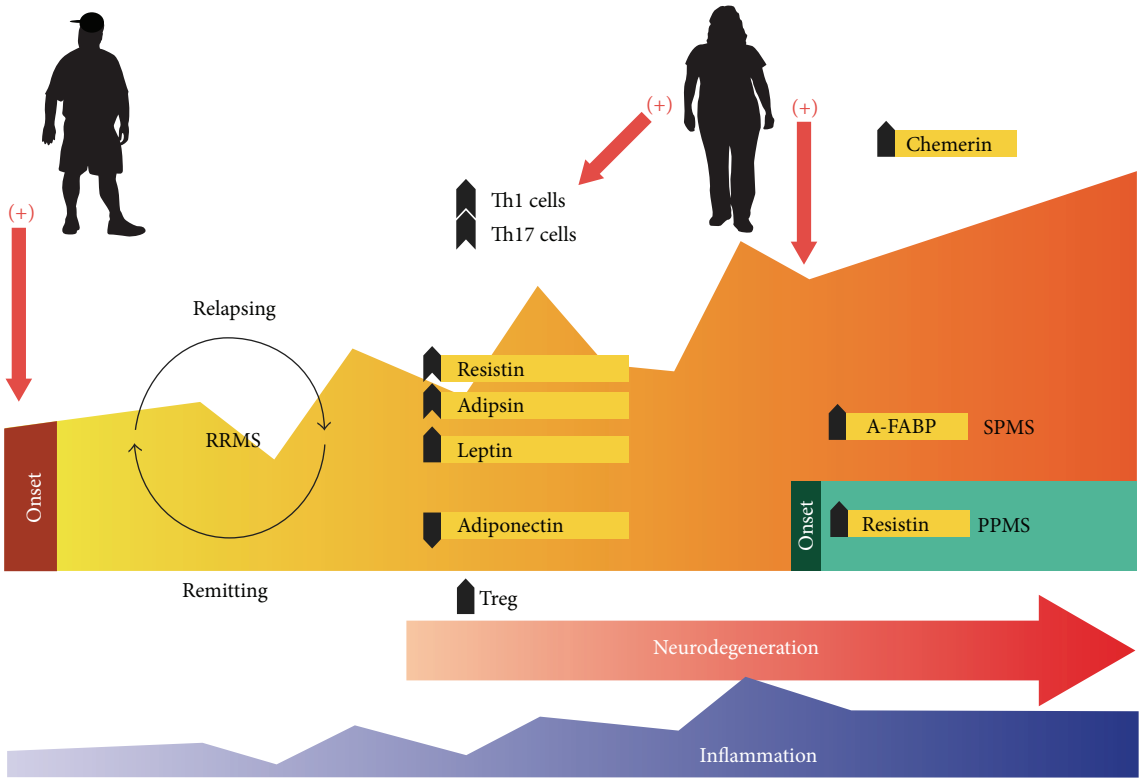

Figure 1: An integrative view of the possible involvement of some adipokines present in patients with multiple sclerosis (MS) at different disease stages. Obesity during adolescence constitutes a relevant risk factor for the later development of MS. Adult obesity negatively affects evolution of the disease and response to treatment in patients with MS. During relapsing-remitting stages, a decrease in adiponectin has been reported as well as, concomitantly, an increase in resistin, adipsin, and leptin concentrations (in serum or CSF or both). Additionally, A-FABP is increased in patients with SPMS, and resistin is also increased in patients with PPMS. Given that inflammation occurs at a variable intensity from the onset of the disease and that neurodegeneration process starts after disease initiation, in this context the adipokines produced by lipid tissue constitute an additional element in the neuroimmunomodulation complex of organisms with MS (see text for further explanation); thus the adipokines produced constitute an additional element in the neuroimmunomodulation complex of organisms with MS (see text for further explanation). RRMS, Remittent Recurrent Multiple Sclerosis. SPMS, Secondary Progressive Multiple Sclerosis. PPMS, Primary Progressive Multiple Sclerosis. CSF, CerebroSpinal Fluid. Resistin (serum) [32–34]. Adipsin (in CSF) [35]. Leptin (serum and CSF) [35]. Adiponectin (in serum) [32, 36, 37]. Chemerin (serum) [38]. A-FABP, Adipocyte-Fatty Acid-Binding Protein (in serum) [39].

Abbreviations

APN: Adiponectin
BBB: Blood Brain Barrier
CNS: Central Nervous System
CSF: CerebroSpinal Fluid
EDSS: Disability Status Scale
EC: Endothelial Cells
EAE: Experimental Autoimmune Encephalomyelitis
GA: Glatiramer Acetate
GS: Glutamine Synthetase
GDH: Glutamate DeHydrogenase
IGF-1: Insulin-Like Growth Factor-1
ICAM-1: IntraCellular Adhesion Molecule-1
MOG: Myelin Oligodendrocyte Glycoprotein
MS: Multiple Sclerosis
OPC: Oligodendrocyte Precursor Cells
PERK: Pancreatic Endoplasmic Reticulum Kinase
POMS: Pediatric-Onset MS
PBMC: Peripheral Blood Mononuclear Cells
PPAR-γ: Peroxisome Proliferator-Activated Receptor gamma
PD-L1: Programmed Death-Ligand-1
PD1: Programmed Death-1
SPMS: Secondary Progressive MS
A-FABP: Adipocyte-Fatty Acid-Binding Protein
SAT: Subcutaneous Adipose Tissue
VAT: Visceral Adipose Tissue.

Additional Points

Apologies are due to authors whose works have not been reviewed and to those whose papers have not received the emphasis that they merit. The authors also apologize to authors whose work has not been appropriately cited due to space limitations and/or to limitations of the present paper's authors' knowledge.

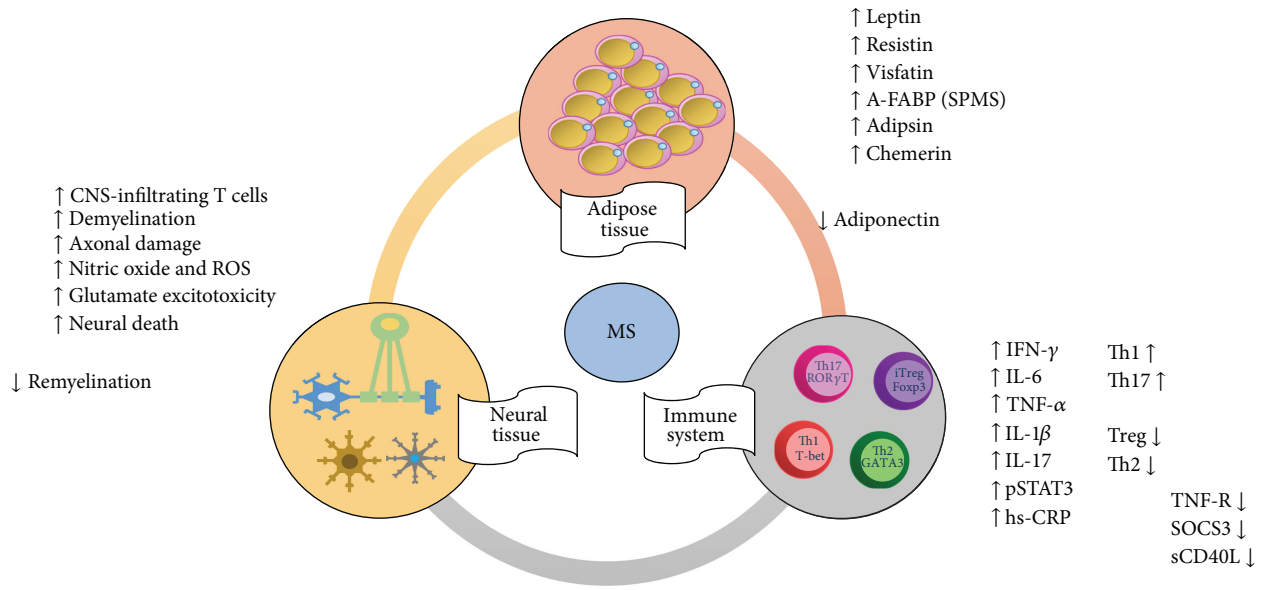

Figure 2: Cross talk among immune, neural, and adipose tissues. Adipocytes release leptin, resistin, and visfatin, which induce a low-grade inflammatory state in patients with multiple sclerosis (MS) with obesity. T cells migrate into the Central Nervous System (CNS), with Th1/Th17 cell release of proinflammatory cytokines, which promote the inflammatory status, and Th2/Treg release of anti-inflammatory cytokines, which contributes to modulating the severity of multiple sclerosis (MS). Neurons and oligodendrocytes are those mainly affected in MS. Axons are demyelinated by cell- and molecular-mediated mechanisms. Antigen presentation between microglia and CNS-infiltrating T cells induces a proinflammatory positive feedback loop. Astrocytes costimulate CNS-infiltrating T cells through CD24 expression.

Competing Interests

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

Authors' Contributions

José de Jesús Guerrero-García, Lucrecia Carrera-Quintanar, Rocío Ivette López-Roa, Ana Laura Márquez-Aguirre, Argelia Esperanza Rojas-Mayorquín, and Daniel Ortuño-Sahagún drafted the paper. All authors reviewed the paper and approved the final version.

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Research Article

SP600125 Attenuates Nicotine-Related Aortic Aneurysm Formation by Inhibiting Matrix Metalloproteinase Production and CC Chemokine-Mediated Macrophage Migration

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Nicotine, a major chemical component of cigarettes, plays a pivotal role in the development of abdominal aortic aneurysm (AAA). c-Jun N-terminal kinase (JNK) has been demonstrated to participate in elastase-induced AAA. This study aimed to elucidate whether the JNK inhibitor SP600125 can attenuate nicotine plus angiotensin II- (AngII-) induced AAA formation and to assess the underlying molecular mechanisms. SP600125 significantly attenuated nicotine plus AngII-induced AAA formation. The expression of matrix metalloproteinase- (MMP-) 2, MMP-9, monocyte chemoattractant protein- (MCP-) 1, and regulated-on-activation, normal T-cells expressed and secreted (RANTES) was significantly upregulated in aortic aneurysm lesions but inhibited by SP600125. *In vitro*, nicotine induced the expression of MCP-1 and RANTES in both RAW264.7 (mouse macrophage) and MOVAS (mouse vascular smooth muscle) cells in a dose-dependent manner; expression was upregulated by 0.5 ng/mL nicotine but strongly downregulated by 500 ng/mL nicotine. SP600125 attenuated the upregulation of MCP-1 and RANTES expression and subsequent macrophage migration. In conclusion, SP600125 attenuates nicotine plus AngII-induced AAA formation likely by inhibiting MMP-2, MMP-9, MCP-1, and RANTES. The expression of chemokines in MOVAS cells induced by nicotine has an effect on RAW264.7 migration, which is likely to contribute to the development of nicotine-related AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is an important cause of mortality in older adults. It affects approximately 5% of men and 1% of women over the age of 60, and it is the thirteenth leading cause of death in the USA [1]. AAA is a focal full-thickness dilatation of the abdominal aorta to greater than 1.5 times its normal diameter. The diameter of the aortic aneurysm is often used to assess the risk of rupture. Currently, therapeutic strategies for the treatment of AAA development and rupture are limited to surgical treatments, which are clinically suitable for large but not for small aneurysms; therefore, an effective nonsurgical therapy to attenuate the formation and development of AAA is necessary.

Smoking is the only modifiable risk factor associated with the formation, development, and rupture of AAA. Nicotine, as a major chemical component of cigarette smoke, receives increasing research attention [2, 3]. Recent studies indicated that infusion of nicotine (1.5 mg/kg/d or 5 mg/60 d) markedly increased the incidence of AAA in apolipoprotein E knockout ($ApoE^{-/-}$) mice [4, 5]. Another study has shown that AngII plays an important role in the development of aortic aneurysms, with a low dose of AngII (0.72 mg/kg/d) inducing a low incidence of AAA [6]. But the existing animal model is not ideal enough to simulate the pathological changes in the older smoker; the deficiency of apolipoprotein E may influence the formation of AAA. To identify the pathological features triggered by nicotine, in this study,

we coadministered nicotine (4 mg/kg/d, a dose producing a plasma concentration in rodents similar to that in heavy smoker [7]) and a low dose of AngII into aged C57BL/6J wild-type mice by infusion.

AAA is characterized by chronic inflammation, mainly involving mononuclear phagocytes and lymphocytes [8]. Inflammatory cells infiltrating the aortic wall are thought to be important in the destructive connective tissue remodeling that occurs before and during aneurysmal degeneration, particularly through the production of proinflammatory cytokines and enzymes capable of degrading elastin, collagen, and other structurally important matrix proteins. Despite the recognized association between chronic inflammation and aneurysm degeneration, it is notable that several chemotactic cytokines with activities towards monocytes, macrophages, and lymphocytes are produced in human AAA tissue, including the CC chemokines MCP-1 and RANTES. Expression of these chemokines has been confirmed as an early event during the progression of experimental aneurysm formation by promoting leukocyte infiltration into the outer aortic wall in an elastase-induced AAA animal model [9].

c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinases (MAPK) family, plays a role in multiple processes such as inflammation, proliferation, and apoptosis. The JNK signaling pathway has been shown to contribute to the development of aortic aneurysm. Inhibition of JNK attenuated aortic aneurysm formation in an elastase-induced AAA model and led to a reduction in the aortic diameter after the establishment of AAA [10]. Meanwhile, the activation of JNK can upregulate MMPs in smooth muscle cells and, subsequently, AAA formation [11]. While these studies have demonstrated the importance of JNK in the formation of aortic aneurysm, the relationship between JNK and nicotine-related abdominal aortic aneurysm requires further research.

In the present investigation, we focused on MCP-1 and RANTES, whose roles in vascular biology have been characterized. The purpose of this study was to investigate the potential roles of CC chemokines in aneurysmal degeneration. Accordingly, we examined whether (1) the development of nicotine plus AngII-induced AAA in the mouse is accompanied by altered local production of MCP-1 and RANTES under control of the JNK pathway *in vivo* and (2) exposure of cultured MOVAS cells to nicotine has an effect on their cellular chemokine expression, which may induce the migration of RAW264.7 cells. The results of our experiments would provide evidence that nicotine-induced production of CC chemokines within the aortic media may be an important biologic mechanism underlying experimental aneurysm degeneration (Figure 1). Meanwhile, AAA has a tight relationship with vascular remolding, in which MMPs play a pivotal role, and MMP-2 and MMP-9 are considered to be crucial to extracellular matrix (ECM) degradation (Figure 1); furthermore, MMPs may facilitate inflammation [12] and vice versa, inflammation contributions to extracellular matrix remodeling [13]. Accordingly, we evaluated the production of these two MMPs in the urine aneurysm model treated with nicotine plus AngII.

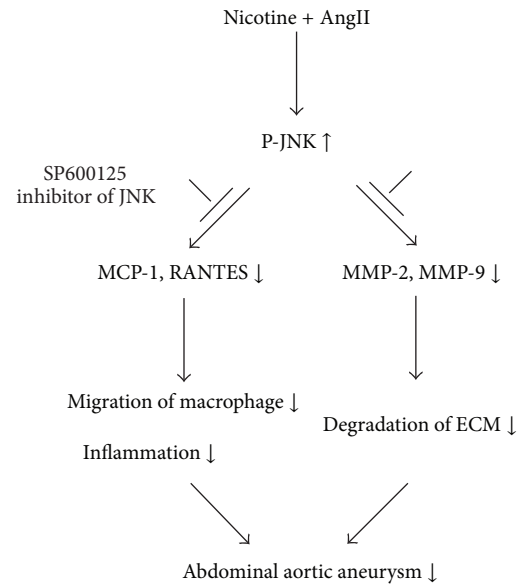

Figure 1: Proposed mechanism of SP600125-mediated suppression of nicotine-related AAA in the C57BL/6J mouse model. AngII, angiotensin II; JNK, c-Jun N-terminal kinase; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated-on-activation, normal T-cell expressed and secreted; MMP, matrix metalloproteinase; ECM, extracellular matrix.

2. Materials and Methods

2.1. Animal Experiments. This research was approved by the Animal Care and Use Committee, School of Medicine, Shanghai Jiao Tong University, and the procedures in the Principles of Laboratory Animal Care and Guidelines for the Care and Use of Laboratory Animals were followed. One-month-old male C57BL/6J wild-type mice were purchased from the Model Animal Research Center of Nanjing University. The mice were maintained in cabin-type isolators under standard environmental conditions: 22–25°C and 40–70% humidity, with a 12-h photoperiod. The mice were given free access to a sterilized NIH-31 modified mouse diet and water. Ten-to-twelve-month-old mice were used in the experiments. The chemicals used in the animal experiments were obtained from Sigma-Aldrich (St. Louis, USA).

The animal experiments comprised two parts. In the first part, 42 mice were randomly divided into 4 treatment groups: (1) a saline-infused group (control group; $n = 10$); (2) a nicotine-treated group ($n = 10$); (3) an AngII-treated group ($n = 10$); and (4) a cotreatment group (nicotine plus AngII; $n = 12$), as described previously [14]. Both nicotine and AngII were dissolved in 0.9% saline and delivered at 4 mg/kg/d and 0.72 mg/kg/d, respectively. Miniature osmotic pumps (Alzet, Model 2004; DURECT, Cupertino, CA, USA) containing 0.9% saline, AngII, nicotine, or nicotine plus AngII were implanted subcutaneously for 28 days.

On the basis of former research, in the second part, another 40 mice were randomly divided into 2 groups: (1) a cotreatment group (nicotine plus AngII; $n = 20$) and (2) a cotreatment plus SP600125 group ($n = 20$). Nicotine

and AngII were delivered as mentioned above. SP600125 was dissolved in DMSO and delivered via hypodermic injection at 30 mg/kg, twice per day. As a control, mice were injected with DMSO.

At the end of the 28-day treatment period, the mice were euthanized by cervical vertebra dislocation. The aortas of euthanized mice were observed and dissected under a surgical microscope (SXP-1C; Shanghai Medical Instruments, Shanghai, China) at the end of the 28-day treatments. Digital photographs of the abdomens were taken to measure the maximum external diameters. A segment of the suprarenal abdominal aorta (±5 mm in length) as the site with the highest risk of AAA was excised from each mouse and fixed in 10% neutral formalin for histological analysis. The remaining aortic segment was snap-frozen in liquid nitrogen and stored at −80°C for western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

2.2. Immunohistochemistry. Cross sections (4 μm thick) of the suprarenal abdominal aorta tissue were used for histological analysis. The aortic cross sections were deparaffinized and hydrated in order, then rinsed 3 times with tap water, and hyperbaric heating antigenic repair for 3–5 min, and then rinsed with several changes of PBS, followed by blocking with 5% goat serum in PBS for 30 min. Slides were incubated with rabbit anti-mouse RANTES immunoglobulin G (IgG), rabbit anti-mouse MCP-1 IgG, rabbit anti-mouse MMP-2, rabbit anti-mouse MMP-9, or reagent-grade nonimmune rat IgG (R&D Systems, Minneapolis, MN, USA) overnight at 4°C in a humidified chamber. Then, the slides were incubated with biotin-conjugated goat anti-rabbit IgG (1 : 200 in PBS; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, followed by incubation with the horseradish peroxidase-conjugated avidin/biotin complex for 10 min. Immune complexes were detected with horseradish peroxidase substrate (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin before examination with light microscopy.

2.3. Culture of Mouse Aortic Vascular Smooth Cells and Macrophages. Mouse vascular smooth muscle (MOVAS, ATCC, Manassas, VA, USA) cells and mouse macrophage cells (RAW264.7, ATCC, Manassas, VA, USA) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, Grand Island, NY, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every 2 days. The cells were plated on 6-cm diameter culture dishes at a density of 2×10^5 cells/mL. Approximately 48 h later, when most of the cells were in the logarithmic growth period, the medium was changed to serum-free medium to starve the cells overnight. Then, the medium was changed to fresh DMEM without FBS, and the cells were pretreated with the JNK inhibitor SP600125 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h prior to being supplemented with indicated concentrations of nicotine for 3 h. Both the supernatant and the cells were harvested for subsequent experiments.

2.4. RNA Extraction and qRT-PCR. Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and reverse-transcribed using a reverse transcription kit (Takara, Otsu, Japan). The MCP-1 and RANTES mRNA expression levels were detected by qRT-PCR using SYBR green dye. qRT-PCR was carried out using the ABIPRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Expression of the genes of interest was normalized to GAPDH expression. All values were expressed as fold changes relative to GAPDH expression. The cycle threshold (Ct) for each sample was calculated, with the relative expression calculated as the difference ($\Delta\Delta$Ct) between the ΔCt values of the test sample and those of the control sample. The relative expression of target genes was calculated and expressed as $2^{-\Delta\Delta Ct}$. Dissociation curves were utilized to confirm the absence of significant amounts of primer dimers.

2.5. Western Blotting. Total protein of aortic issue was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with 100 μg/mL phenylmethylsulfonyl fluoride (Roche, Molecular Biochemicals, Mannheim, Germany) added according to the manufacturer's protocol. A BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was used to quantify the total protein. Aliquots of protein were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% nonfat milk/TBST (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 7.4) for 1 h and incubated with rabbit polyclonal antibodies against MCP-1 (1 : 1000, Abcam, Cambridge, UK), RANTES (1 : 500, Abcam, Cambridge, UK), MMP-2 (1 : 500, Abcam, Cambridge, UK), or MMP-9 (1 : 500, Abcam, Cambridge, UK) in primary antibody dilution solution (Beyotime Institute of Biotechnology, Haimen, China) overnight with gentle shaking. The next day, the membranes were washed with TBST for 30 min at room temperature, followed by incubation with HRP-conjugated secondary antibodies for 1 h and three washes in TBST. Then, the membranes were incubated with rabbit monoclonal antibody against GAPDH (1 : 1000, Cell Signaling Technology, Boston, MA, USA) overnight with gentle shaking, probed with HRP-conjugated goat anti-rabbit secondary antibody (1 : 2000, Cell Signaling Technology, Boston, MA, USA) for 1 h, and washed in TBST. All membranes were visualized using the West-Pico ECL kit (Pierce, Rockford, IL, USA). Protein expression was normalized to GADPH levels.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). Cell culture supernatants and serum were collected after centrifugation at 800 ×g at 4°C for 5 min. The supernatants and serum samples were aliquoted, snap-frozen, and stored at −80°C for further analysis. Humoral-response levels of MCP-1 and RANTES levels were assessed using the Mouse MCP-1 and RANTES ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

2.7. Transwell Migration Assay. Cell migration was quantitated in duplicate by use of 24-well Transwell inserts with polycarbonate filters (8-mm pore size) (Corning Costar, Acon, MA, USA). For the migration assay, 8×10^4 MOVAS cells were transferred onto the membranes of the lower chambers containing DMEM with 10% fetal bovine serum. The cells were permitted to adhere in complete medium for 24 h after which the medium was changed to serum-free medium. Macrophages (1.5×10^5) were added to the upper chambers of the inserts containing 200 μL of high-glucose DMEM with 10% FBS. RAW264.7 cells were seeded onto the membranes of the upper chambers containing serum-free DMEM. After incubation for 8 h, the cells in the bottom chambers were quantified by light microscopy; the membrane was washed twice in phosphate-buffered saline (PBS) and fixed with methanol for 10 min. Finally, the chambers were stained with 0.5% crystal violet solution for 30 min, and the cell numbers were determined at magnifications of 10x and 400x for each well using ImageJ. The average number of cells per field of view at a magnification of 400x served as a migration index.

2.8. Statistical Analysis. Data were expressed as the means ± standard deviations (SDs). Data were analyzed by analysis of variance (ANOVA) followed by Bonferroni posttests or by using the Student t-test (for ELISA data). $P < 0.05$ was regarded as denoting statistical significance. Statistical analysis was conducted using the GraphPad InStat software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Nicotine Plus AngII Induces Aneurysm Development. In the first part of our study, 42 10–12-month-old mice were infused with saline, nicotine, AngII, or nicotine plus AngII for 28 days. No changes in body weight were observed in the experimental and control groups over the course of the experiment ($P > 0.05$, Figure 2(c)) [14]. Nicotine and AngII infusions alone failed to induce macroscopic aortic aneurysm formation (Figure 2(a)). Nicotine plus AngII cotreatment induced the formation of aortic aneurysms in 6 out of 12 mice (Figure 2(a)), two of which died on the 7th and 11th day after implantation of the pump. Autopsy indicated that the two mice had died of aneurysm rupture. While no significant difference in the external diameter of the suprarenal abdominal aorta was detected between the nicotine and AngII treatment groups ($P > 0.05$), when compared to the control group, both treatments contributed to an increased maximum external diameter (Figure 2(e)). However, the maximum external diameter of the suprarenal abdominal aorta in the cotreatment group was significantly larger than those in the three other experimental groups ($P < 0.01$ versus nicotine or AngII group; $P < 0.001$ versus control group) (Figure 2(e)) [14]. Taken together, these results indicated that nicotine plus AngII contributes to the formation and development of aortic aneurysms in aged male C57BL/6J wild-type mice and even can induce early death caused by aneurysm rupture.

3.2. SP6000125 Attenuates AAA Formation Induced by Nicotine Plus AngII. Our findings indicated that combined loading of nicotine and AngII accelerated AAA formation in aged mice and phosphorylated JNK is significantly upregulated in aneurysm tissue, although the underlying mechanism remains elusive [14]. Therefore, we investigated whether the JNK pathway plays a role in nicotine plus AngII-induced AAA. In the second phase of the present study, nicotine plus AngII produced aortic aneurysm in 8 out of 20 mice (Figure 2(b)), approximating the incidence in our previous study [14]. Cotreatment with SP600125 significantly reduced the AAA formation induced by nicotine plus AngII (Figure 2(b)); aortic aneurysm formation was not observed in any of the 20 mice. As expected, the diameter of the abdominal aorta was markedly enlarged by cotreatment. However, the aortic diameter in SP600125-supplemented mice was significantly lower than that in the cotreated group (Figure 2(f)), suggesting that the inhibition of JNK suppresses AAA formation induced by nicotine and AngII in aged mice.

3.3. SP600125 Inhibits MMP-2 and MMP-9 Expression in Abdominal Aortic Lesions. To evaluate whether SP600125 inhibits the expression of MMP-2 and MMP-9 via the JNK pathway in abdominal aortic tissue, we applied immunohistochemical staining and western blotting to abdominal aortic tissue samples. Although MMP-2 and MMP-9 could be detected in both the nicotine plus AngII-treated and the SP600125-supplemented groups, the staining signal was significantly higher in tissue samples from mice treated with nicotine plus AngII, especially in the area around the site of rupture of the aortic media (Figure 3(a)). This finding indicated that the expression of MMP-2 and MMP-9 positively relates to the incidence of AAA and was inhibited by SP600125. Western blot analysis showed that, compared to the nicotine plus AngII group, the band intensities for MMP-2 and MMP-9 in the cotreatment plus SP600125 group were obviously lower ($P < 0.05$) (Figures 3(b) and 3(c)), corroborating the immunohistochemical staining results.

3.4. SP600125 Inhibits MCP-1 and RANTES Expression in Abdominal Aortic Lesions. Although chemokine expression is negligible in normal, unperfused aorta [15], MCP-1 and RANTES expression in abdominal aortic lesions increased remarkably in the group treated with nicotine plus AngII as compared with the saline-treated control group (Figures 4(a) and 4(b)). Both proteins appeared to be localized in the medial and outer smooth muscle cells. However, SP600125 supplementation suppressed the increase in protein expression of MCP-1 and RANTES induced by nicotine plus AngII as indicated by immunohistochemistry. These findings were supported by western blotting and ELISA results (Figures 4(c), 4(d), and 4(e)).

3.5. Nicotine Induces MCP-1 and RANTES Expression in MOVAS Cells in a Dose-Dependent Manner. In our previous study, nicotine regulated the expression of MMP-2, MMP-9, and VCAM-1 in a concentration-dependent manner in MOVAS cells and RAW264.7 cells [16]. To assess whether

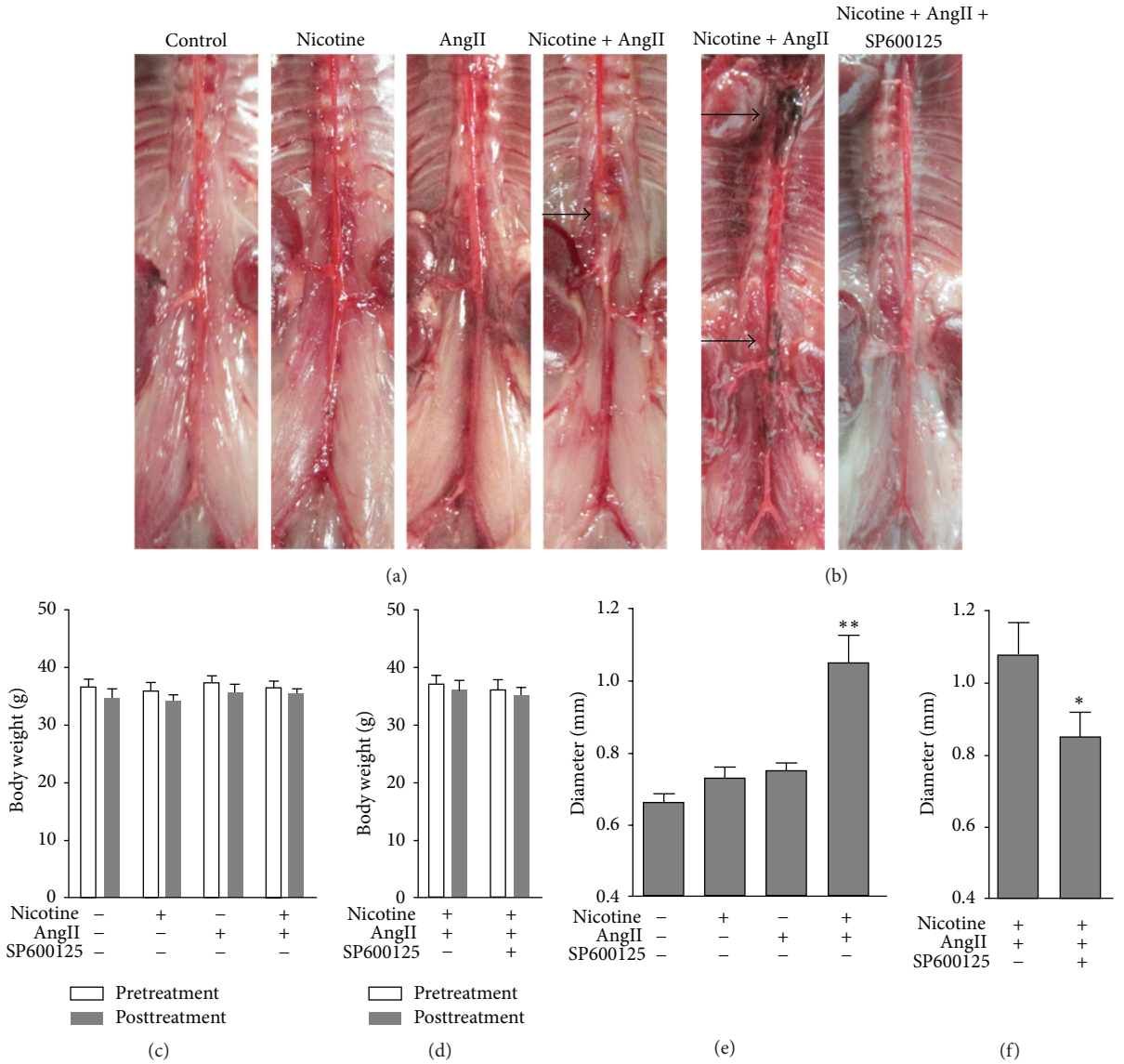

Figure 2: SP600125 attenuates the formation of aortic aneurysm induced by nicotine plus AngII. (a) Representative photomicrographs of abdomens of mice of all four experimental groups. No aortic aneurysms were detected in saline-, nicotine-, or AngII-treated groups. Nicotine plus AngII induced the formation of aortic aneurysms (published in Experimental & Clinical Cardiology [14]). (b) Representative photomicrographs of abdomens of the mice in the cotreatment and SP600125-supplemented groups. Nicotine plus AngII induced the formation of aortic aneurysms. No aortic aneurysms were detected in the SP600125 group. (c, d) In all experimental groups, the body weight did not change from initiation to the end of the experiment. (e, f) The maximum diameter of the suprarenal abdominal aorta was measured using ImageJ and normalized to the body surface area, which was calculated using the Meeh–Rubner formula (area = $K(W\hat{}(2/3))/1000$, W: weight, $K = 9.1$). Data are represented as the mean ± SEM. $^{*}P < 0.01$ versus nicotine or AngII group; ∗∗ refers to $P < 0.01$ versus control group. AAA, abdominal aortic aneurysm.

nicotine stimulates vascular smooth muscle cells to express MCP-1 and RANTES in a dose-dependent fashion, MOVAS cells were exposed to nicotine at various concentrations (0, 0.5, 5, 50, and 500 ng/mL) for 3 h. Both qRT-PCR (Figures 5(a) and 5(b)) and ELISA (Figures 5(c) and 5(d)) showed that the strongest MCP-1 and RANTES expression was induced by 0.5 and 5 ng/mL nicotine, while expression was reduced following treatment with 50 ng/mL and a significant inhibitory effect was noted at 500 ng/mL.

3.6. SP600125 Suppresses Nicotine-Induced Upregulation of MCP-1 and RANTES in MOVAS Cells. To determine whether the upregulation of MCP-1 and RANTES induced by 0.5 ng/mL nicotine is regulated by JNK, MOVAS cells were pretreated with 10 μM SP600125 for 30 min followed by exposure to 5 ng/mL nicotine for 3 h. RT-PCR showed that 10 μM SP600125 suppressed the nicotine-induced upregulation of MCP-1 and RANTES (Figures 6(a) and 6(b)). Furthermore, ELISA showed that the nicotine-induced secretion of MCP-1

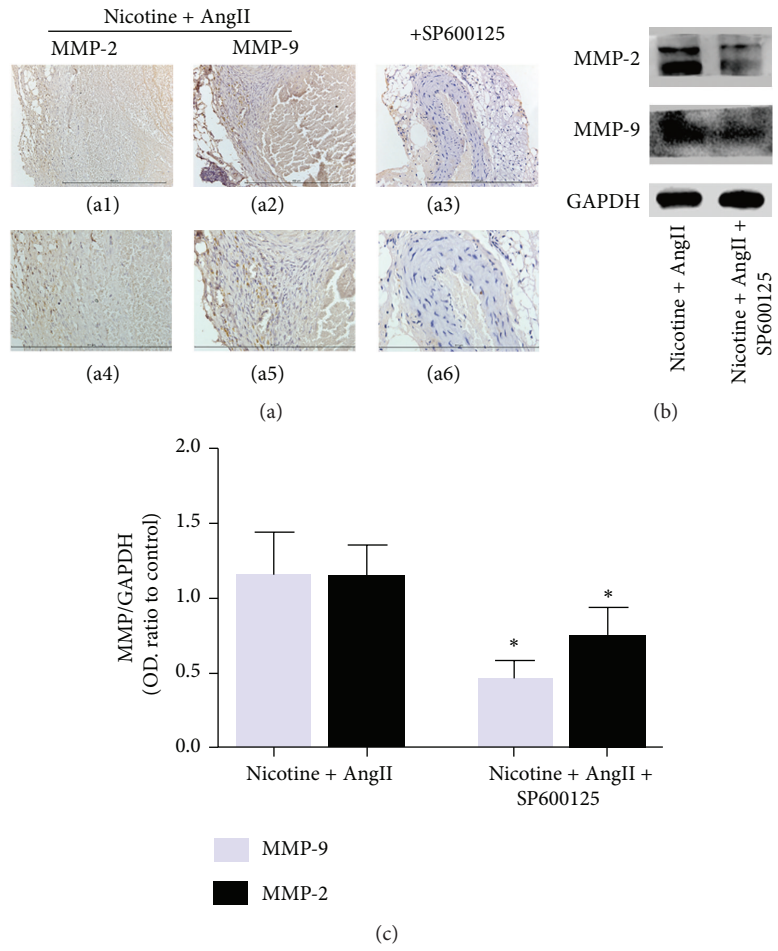

Figure 3: SP600125 suppresses the production of MMP-2 and MMP-9 in aortic tissue. (a) Representative images of immunohistochemical staining showing that MMP-2 and MMP-9 proteins are abundant in nicotine plus AngII-induced AAA tissues (a1, a2, a4, and a5), where they appear to be localized in the medial and outer smooth muscle cells. SP600125 downregulated the production of MMP-2 and MMP-9 in AAA lesions (a3 and a6). (b, c) Representative western blots for MMP-2 and MMP-9 revealing that SP600125 inhibits the expression of MMPs. The band optical density (OD) values (mean ± SD) of MMP-2 and MMP-9 were evaluated with ImageJ. GAPDH was used as an internal control and results are from independent triplicate experiments. ∗ refers to $P < 0.05$ versus coadministration.

and RANTES was suppressed following treatment with 10 μM SP600125 (Figures 6(c) and 6(d)). These results indicated that JNK is involved in the nicotine-induced expression and secretion of MCP-1 and RANTES in MOVAS cells.

3.7. SP600125 Attenuates Macrophage Migration by Inhibiting Vascular Smooth Muscle Cell Production of CC Chemokines. Nicotine stimulated the expression of MCP-1 and RANTES in MOVAS cells, which is essential for macrophage migration. To investigate whether nicotine has an effect on macrophage migration by regulating the expression of MCP-1 and RANTES in MOVAS cells, a Transwell migration assay was conducted. MOVAS cells were exposed to different concentrations of nicotine (0, 0.5, 5, 50, and 500 ng/mL). Consistent with our previous findings, qRT-PCR and ELISA showed that the strongest MCP-1 and RANTES expression was induced by 0.5 and 5 ng/mL nicotine, with lower expression at 50 ng/mL and significant inhibition at 500 ng/mL (Figures 7(a) and 7(c)). A similar dose-dependent stimulatory effect of nicotine on macrophage migration was observed. However, pretreatment of the MOVAS cells with SP600125 suppressed the nicotine-stimulated macrophage migration (Figures 7(b) and 7(d)).

4. Discussion

Although the ability of nicotine to induce the development of AAA in ApoE$^{-/-}$ mice model has been proved [4, 5] and AngII alone was able to induce the AAA formation in ApoE$^{-/-}$ mice [17, 18], a more physiologically relevant experimental model without the interference of lipid metabolism is necessary and the underlying mechanisms remain to be studied. In this study, we chose to coadminister nicotine with a low dose of AngII by infusion in aged, wild-type C57BL/6J mice for 28 days. The results showed that nicotine or AngII alone did not induce AAA formation, while nicotine plus AngII did. Moreover, coadministration significantly enlarged the aortic diameter. Further, SP600125 supplementation

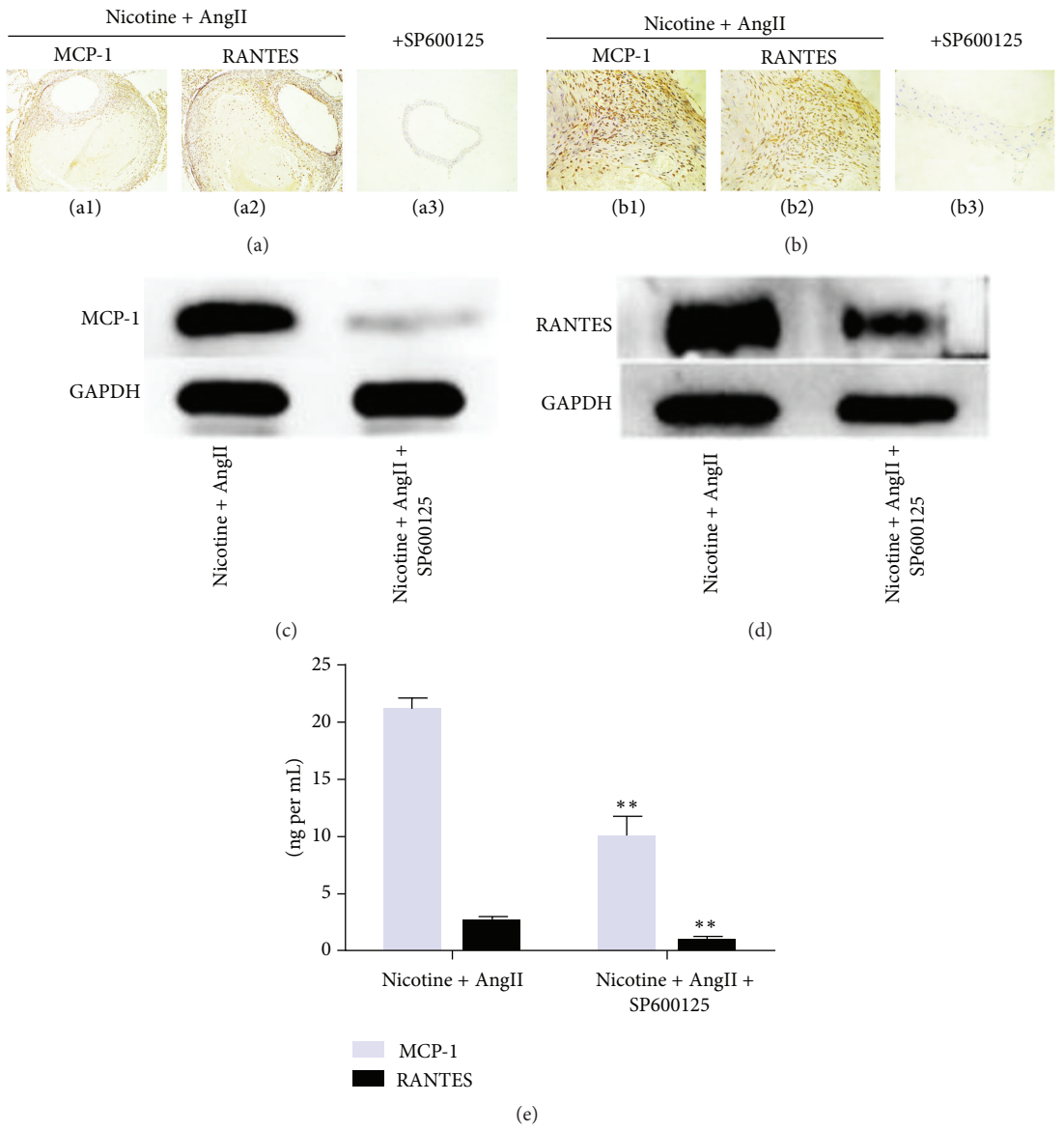

Figure 4: SP600125 suppresses the protein expression of chemokines in aortic tissue. (a) Representative transverse sections of mouse aortic tissue obtained after transient perfusion with nicotine plus AngII, with or without SP600125. MCP-1 and RANTES proteins were undetectable in normal aorta but were abundant in nicotine plus AngII-induced AAA tissues (a1 and a2), where they appear to be expressed in the medial and outer smooth muscle cells. SP600125 inhibited the protein expression of MCP-1 and RANTES in AAA lesions (a3). (c, d) Representative western blots for MCP-1 and RANTES. The band optical density (OD) values (mean ± SD) of MCP-1 and RANTES were evaluated using ImageJ. GAPDH was used as an internal control and results are from independent triplicate experiments. Chemokine expression in the serum as detected by ELISA (e). $^{**}P < 0.01$ versus coadministration.

significantly attenuated the AAA formation and aortic diameter dilation induced by nicotine plus AngII.

According to previous studies, either nicotine or AngII facilitates the formation and development of AAA, which seems to contradict our experimental results. Wang et al. demonstrated that 1 or 5 mg/kg/d of nicotine induced the development of aortic aneurysms in ApoE-deficient mice [19]; however, compared to ApoE-deficient mice, the incidence of AAA in C57BL/6J is much lower. Moreover, Zhang and Ramos found that 0.72 mg/kg/d of AngII induced AAA formation, but only in 2 out of 12 male C57BL/6J mice (6 to 8 months old) [6]; this difference may be explained by the smaller number of animals used, age distinction, or individual differences. Therefore, the different results may arise from the use of different animal models, the low dose used in this study, or individual differences.

The formation or rupture of AAA is a result of the concerted action of aortic wall inflammation and decreased medial smooth muscle cells [20]. MMPs, a family of zinc-dependent endopeptidases, are the most responsible for ECM degradation, especially in AAA [21]. The expression of MMP-2 and MMP-9 not only affects the prevalence of

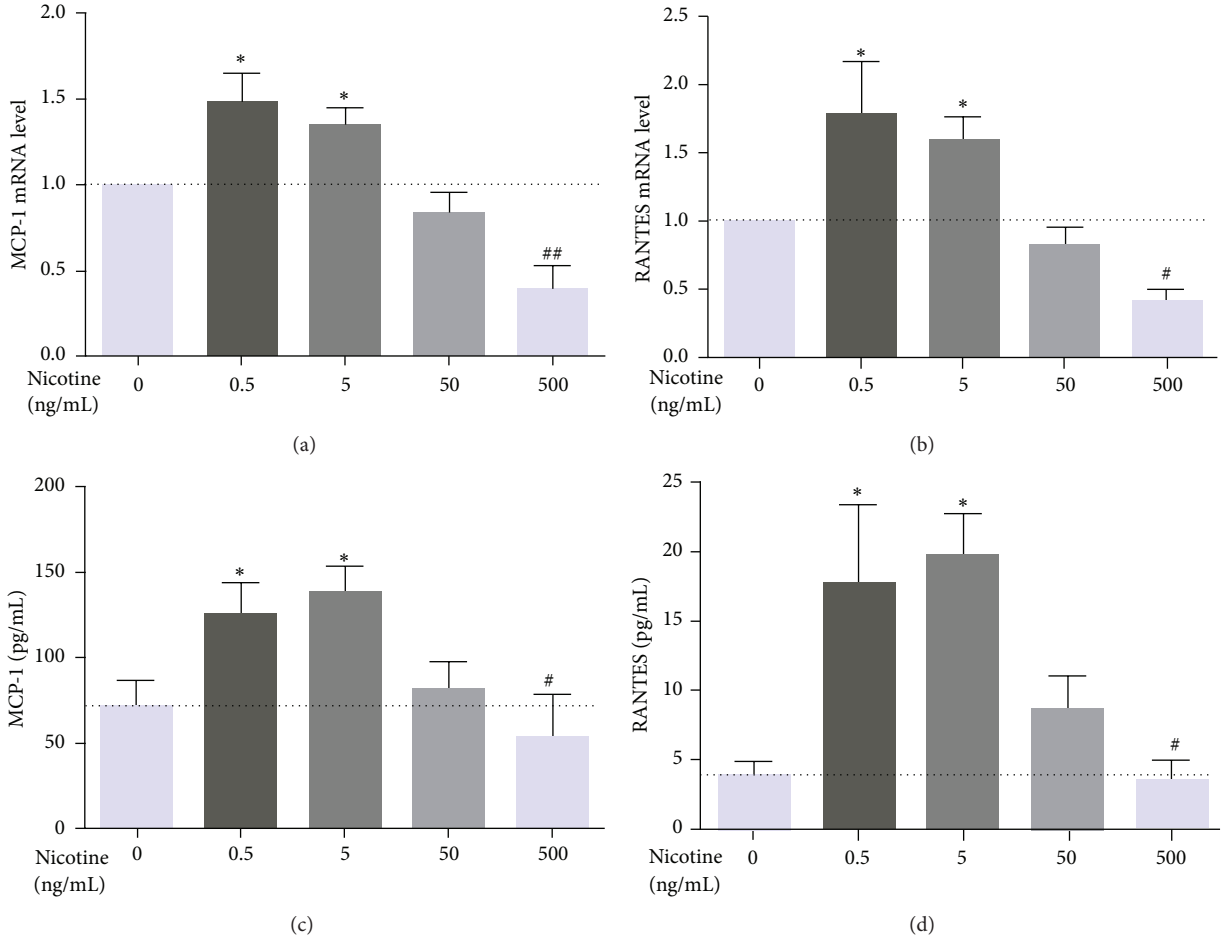

Figure 5: Expression of MCP-1 and RANTES under a concentration gradient of nicotine in MOVAS cells. Cellular mRNA expression levels of MCP-1 and RANTES (a, b) and the levels of secreted MCP-1 and RANTES in the supernatant (c, d) are shown. MCP-1 and RANTES expression as well as secretion was induced by nicotine in a dose-dependent fashion. The strongest expression was observed for 0.5 ng/mL and 5 ng/mL nicotine. Data are from independent triplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the group treated with 5 ng/mL nicotine.

AAA but also correlates positively with the diameter of the AAA [22]. Additionally, MMPs contribute to the infiltration of macrophages [23]. As mentioned before, our previous study revealed that the inhibition of JNK could suppress the production of MMP-2 and MMP-9 *in vitro* [16]; however, the relationship between JNK and MMPs in the nicotine-related aneurysm model remained unclear. The results of our present study indicated that JNK inhibition significantly decreased the expression of MMP-2 and MMP-9 as well as the incidence of AAA, implying that JNK may take part in AAA formation by regulating the MMP expression.

Chemokines are a superfamily of small secreted proteins (8–16 kDa), which are characterized by specific motifs in their N-terminal amino acid sequences [24]. MCP-1 and RANTES are two representative proteins of the CC chemokine subfamily, and both proteins exert a broad spectrum of effects in inflammatory responses [25]. A previous study in an elastase-induced AAA model demonstrated that the early events in aneurysm formation involve the accumulation of inflammatory cells in the adventitia via either the recruitment of circulating monocytes or the proliferation of resident macrophages [9]. In addition, monocytes from AAA patients show greater adhesion and transmigration [26]; however, the underlying mechanism is unclear. Our results show that SP600125 inhibits the production of MCP-1 and RANTES in aorta tissue of AAA model, indicating that the JNK pathway may contribute to nicotine plus AngII-induced AAA by inhibiting the expression of MCP-1 and RANTES.

From our *in vitro* experiment, we concluded that nicotine induced MCP-1 and RANTES expression in MOVAS cells in a dose-dependent manner, which was consistent with our previous finding of nicotine dose-dependently inducing MMP-2, MMP-9, and VCAM-1 expression [16]. Thus, nicotine might stimulate DNA synthesis in and proliferation of endothelial cells at low concentrations (10^{-8} M, 1.62 ng/mL) but inhibit this process at higher concentrations (10^{-6} M, 162 ng/mL) [26]. However, the exact mechanism is still unclear.

Our mouse model recapitulates a number of important features of AAA in older smokers, such as the aged artery and more real concentration of nicotine, thus providing a useful

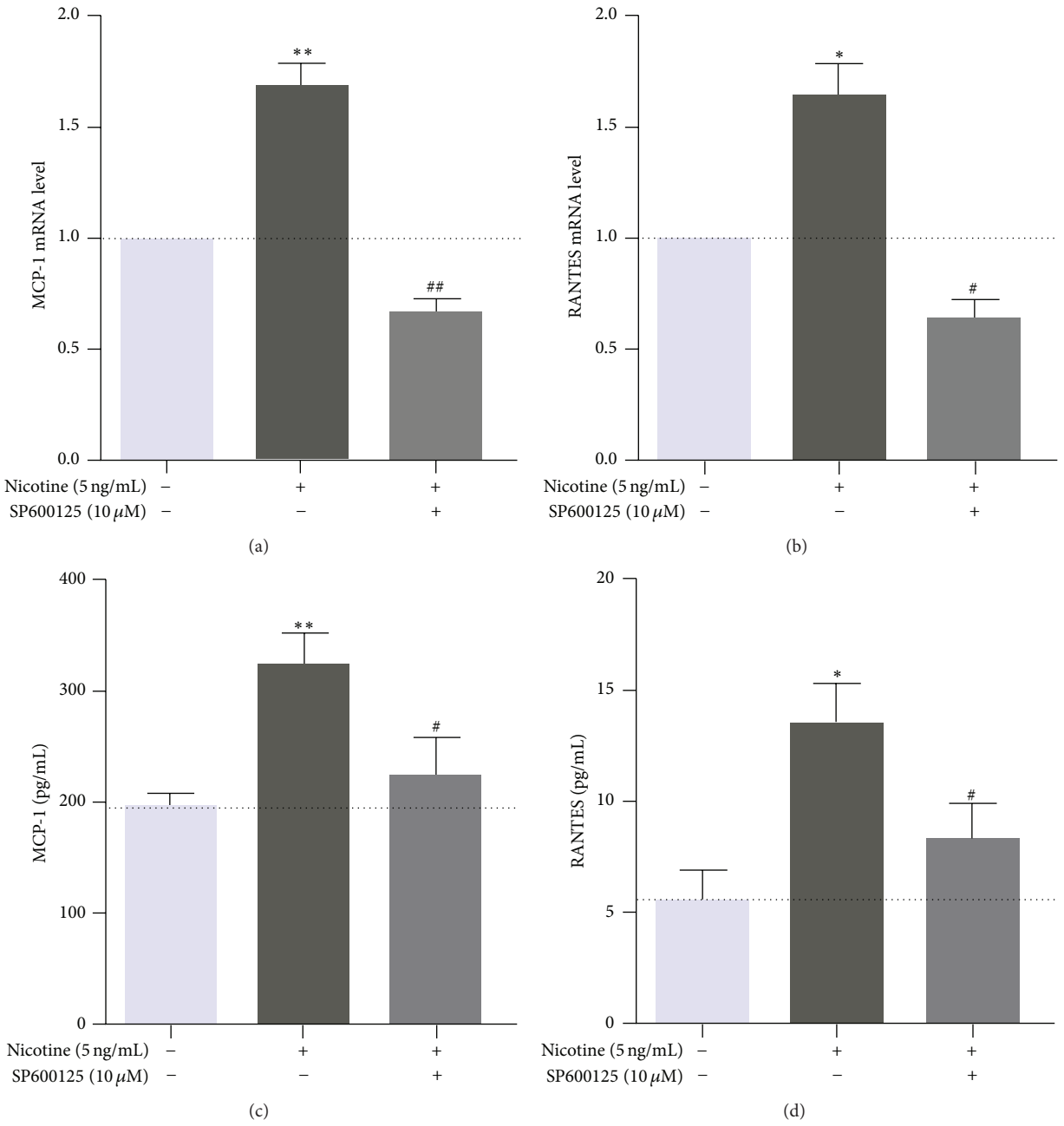

Figure 6: Influence of SP600125 on nicotine-induced expression of MCP-1 and RANTES in MOVAS cells. Cellular mRNA expression levels of MCP-1 and RANTES (a, b) and the levels of secreted MCP-1 and RANTES in the supernatant (c, d) are shown. Nicotine at 5 ng/mL could significantly upregulate the cellular mRNA expression as well as secretion of MCP-1 and RANTES, while SP600125 eliminated this effect. Data are from independent triplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the group treated with 5 ng/mL nicotine.

experimental model to investigate the molecular and cellular events underlying aneurysm degeneration. However, our study had some limitations. While coadministration of nicotine and AngII contributed to aortic rupture and aneurysm in the aged mice, we cannot make a conclusive statement on whether these results involved an addictive effect or interactions between nicotine and AngII. In addition, it is not clear whether SP600125 attenuated the development of AAA by acting on each of nicotine and AngII or through a destructive effect on the interaction between both molecules. Further research is warranted.

Taken together, the data indicate that JNK is a proximal signaling molecule in the pathogenesis of nicotine plus AngII-induced AAA, a chronic inflammatory disease characterized by disruption of the ECM. Inhibition of JNK can suppress the formation of AAA by downregulating the expression of RANTES, MCP-1, MMP-2, and MMP-9. With further research, JNK-targeted therapy may provide

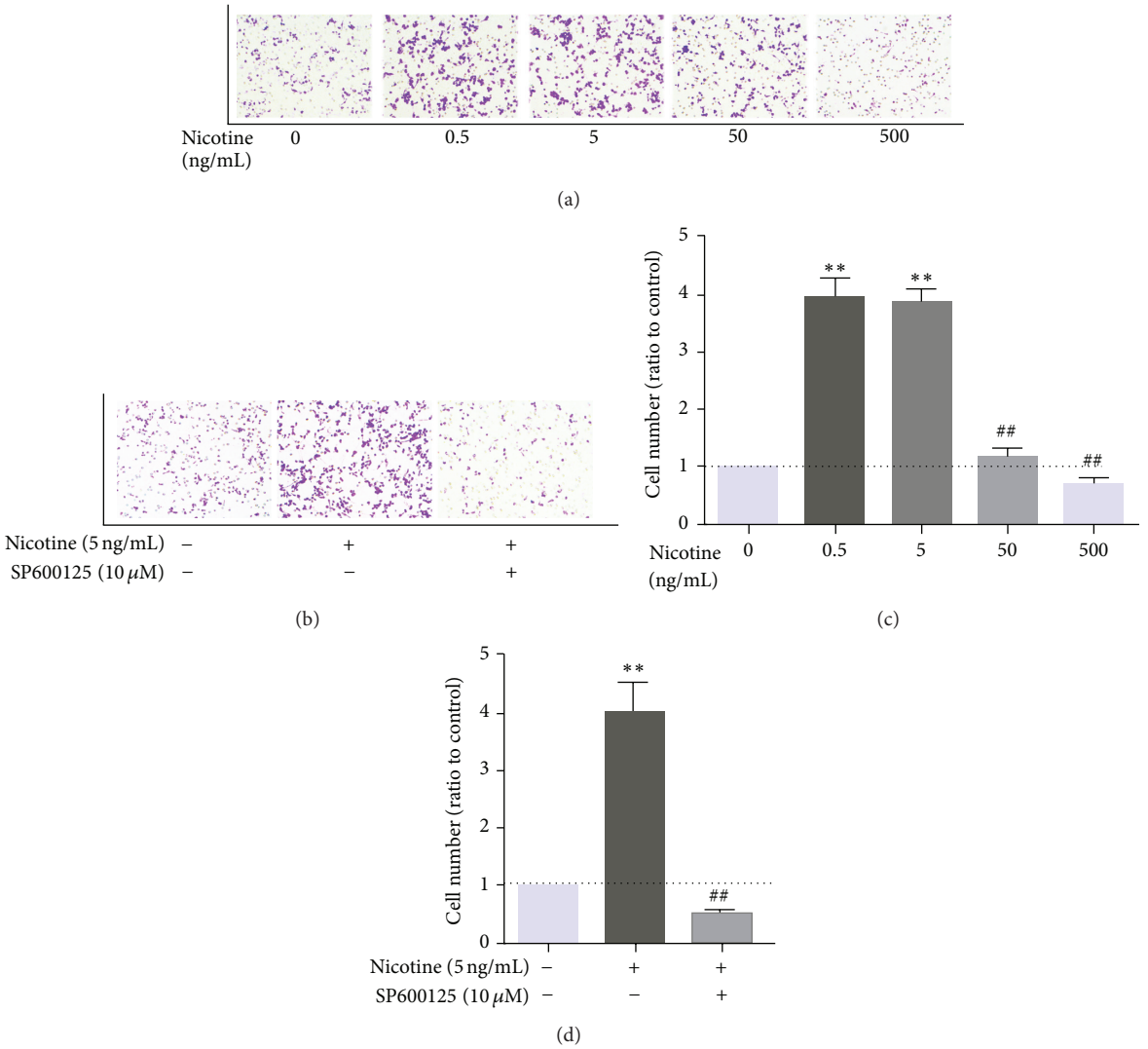

FIGURE 7: Transwell assay of macrophage migration. Representative pictures are shown. (a, b) Macrophages that had migrated to the lower chamber were counted after DAPI staining. (c, d) Bar graphs representing quantification of macrophage migration. Nicotine at 0.5 ng/mL or 5 ng/mL induced the strongest macrophage migration, while lower migration was observed following treatment at 50 ng/mL and a significant inhibitory effect was noted at a 500-ng/mL concentration. SP600125 inhibited the migration of macrophages. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the group treated with 5 ng/mL nicotine.

nonsurgical therapeutic options for AAA, a disease that frequently results in fatal outcome.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Zhen-Zhen Guo and Qun-An Cao contributed equally to this work.

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Review Article

Sphingolipids as Mediators in the Crosstalk between Microbiota and Intestinal Cells: Implications for Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) describes different illnesses characterized by chronic inflammation of the gastrointestinal tract. Although the pathogenic mechanisms leading to IBD are poorly understood, immune system disturbances likely underlie its development. Sphingolipids (SLs) have been identified as important players and promising therapeutic targets to control inflammation in IBD. Interestingly, it seems that microorganisms of the normal gut microbiota and probiotics are involved in sphingolipid function. However, there is a great need to investigate the role of SLs as intermediates in the crosstalk between intestinal immunity and microorganisms. This review focuses on recent investigations that describe some mechanisms involved in the regulation of cytokine profiles by SLs. We also describe the importance of gut microbiota in providing signaling molecules that favor the communication between resident bacteria and intestinal cells. This, in turn, modulates the immune response in the bowel and likely in other peripheral organs. The potential of SLs and gut microbiota as targets or therapeutic agents for IBD is also discussed.

1. Introduction

Inflammatory bowel disease (IBD) is a collection of digestive tract pathologies with chronic inflammation, such as Crohn's disease (CD) and ulcerative colitis (UC). Both conditions usually cause diarrhea, pain, fatigue, and weight loss, among other symptoms. CD causes inflammation in many parts of the gastrointestinal (GI) tract, whereas UC only affects the colon. Currently, the specific etiology of IBD is not well established. However, research in this field points to GI immune system dysfunction as a central pathogenic component [1]. The GI tract is continuously exposed to a great diversity of antigens from foods, bacteria, and parasites. Consequently, immunity in this organ is suppressed to avoid inflammation and maintain homeostasis. Any disruption in the regulatory mechanisms of the GI immune system leads to an excessive response that causes chronic inflammation [2, 3]. Recently, sphingolipids (SLs), particular membrane lipids, have been shown to play an important role in modulating the GI immune response and are promising therapeutic targets for IBD [4]. It has been also observed that the bacteria that naturally colonize the gut, named microbiota, may be involved in the metabolism of SLs, including their biosynthesis. Thus, microbiota and their interaction with probiotics likely are pivotal players in regulating GI immunity. The crosstalk between these components needs to be investigated more deeply. In this review, we will focus on the relationships between SLs, intestinal microbiota, and probiotics with a particular emphasis on their influence upon IBD.

2. Sphingolipids

Sphingolipids (SLs) are plasma membrane components involved in controlling cellular processes such as proliferation, migration, and apoptosis [5]. Moreover, some bacterial species (*Bacteroides*, *Sphingomonas*, etc.) are capable of synthesizing these lipids [6, 7]. SLs are composed of a sphingoid backbone attached to a fatty acid via an amide bond. The main SLs include ceramide (Cer), ceramide-1-phosphate (C1P), glucosylceramide (GC), sphingomyelin (SM), sphingosine (Sph), and sphingosine-1-phosphate (S1P) (Figure 1(a)). S1P is antagonistic to Cer and Sph, since it promotes cell growth and inhibits apoptosis. The enzymes that interconvert Cer, Sph, and S1P regulate their functions. The metabolism of SLs and the participating enzymes is reviewed elsewhere [8, 9]. However, it is important to mention that Cer has a central role in their metabolism, since it can be synthesized by *de novo* pathway or derived from complex SLs such as SM [9, 10] and glycosphingolipids (Figure 1(b)).

3. Cer Plays a Harmful Role in IBD

Cer and related products contribute to varied biological processes as signaling molecules; in addition they are also involved in the development and progression of several human diseases including IBD. The hydrolysis of SM, catalyzed by sphingomyelinases (SMases), is an important source of Cer. However, it can be synthesized by other pathways, which may include the participation of certain microorganisms and cytokines. For instance, the major constituent of the outer membrane of Gram-negative bacteria (lipopolysaccharide or LPS) activates acid SMase in macrophages, which increases Cer content [11, 12]. Once produced, Cer and related lipids participate in inflammatory processes of several tissues, where they stimulate immune cells [13–15] by triggering their mitogen-activated protein kinase (MAPK) pathway [16]. The data shows that IL-1 and Cer augment inflammation via increased eicosanoid production [17, 18]. This possibly contributes to tumor development associated to IBD [19, 20]. Table 1 summarizes both the harmful and beneficial effects of Cer.

It is known that interleukin- (IL-) 1 dose- and time-dependently increases Cer accumulation in intestinal epithelial cells (IEC) *in vitro,* increasing the inflammatory response [21]. The way this IL-1-induced Cer rise modulates the immune response is reported. IL-1 or Cer treatment has no effect on IEC, but a cyclooxygenase- (COX-) 2 inhibitor increases their apoptosis. In addition, IL-1 or Cer increases activation of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in a time and dose dependent manner, by reducing the levels of inhibitor of κ-light-chain-enhancer of activated B cells IκBα and IκBβ. The effect requires the degradation of IκBα and IκBβ by the proteasome [19]. IL-1 or Cer augments the production of the antiapoptotic protein B-cell lymphoma- (BCL-) 2, while reducing the expression of several proapoptotic molecules: BCL-2 associated protein X (BAX), BCL-2 homologous antagonist/killer (BAK), and BCL-2 associated death promoter (BAD) [19, 20]. IL-1 or Cer decreases cyclin-dependent kinase inhibitor p21 levels and the number of cells in the G_0/G_1 phase of the cell cycle, while augmenting cells in the G_2/M phase. All these data suggest that IL-1 and Cer enhance survival of IEC by activating COX-2 and NF-κB, which results in reduced proapoptotic protein expression and increased levels of antiapoptotic molecules. Thus, augmented inflammation and reduced apoptosis of IEC may contribute to tumorigenesis in IBD patients.

On the other hand, tumor necrosis factor- (TNF-) α and interferon-γ induce apoptosis of IEC and also impair their barrier function [21]. These proinflammatory proteins result in Cer production, which may be at least partially responsible for their effects on barrier action. In agreement, exogenous SMase dose-dependently increases IEC permeability *in vitro* [34]. Consistently, SMase treatment diminishes transepithelial resistance and a Cer antibody blocks the augmented permeability caused by platelet activating factor (PAF). Lipid rafts (detergent-insensitive glycosphingolipid-enriched domains) in epithelial cells show high levels of SM, Cer, and cholesterol, plus the tight-junction proteins occludin and claudin-4. In fact, Cer colocalizes with a tight-junction protein. Incubating IEC with exogenous SMase results in a fast elevation of Cer plus a reduction of SM and cholesterol [34]. Thus, the data suggest that proinflammatory stimuli activate SMases, which hydrolyze SM into Cer. Cer accumulates in junctional complexes, reducing their cholesterol levels and provoking their destabilization, which eventually produces a dysfunctional epithelial barrier in the intestine.

In this sense, incubation of a colon cancer cell line with exogenous SMase results in rapid elevation of the mRNA for matrix metalloproteinase- (MMP-) 1 and MMP-10. In fact SMase dose-dependently increases the expression of MMP-1 protein. IL-1β and TNF-α augment MMP-1 production in colon cancer cells and fibroblasts from healthy subjects and patients with UC. MMP-1 degrades the extracellular matrix and is thought to damage the colonic mucosa. Inhibition of acidic SMase with imipramine blocks the effect of IL-1β and TNF-α on MMP-1 [22]. Thus, the results suggest that inhibiting acid SMase activity may be a viable therapeutic option for IBD patients (Table 1).

Accordingly, a SMase inhibitor reduces TNF-α, IL-1β, and IL-6 LPS-induced release from macrophages and diminishes TNF-α secretion from human peripheral blood mononuclear cells (PBMC) in response to LPS [23]. The inhibitor also decreases TNF-α, IL-1β, and IL-6 levels in a colitis animal model produced by administering dextran sulphate sodium (DSS) and reduces the increase in macrophage Cer levels and NF-κB stimulation caused by LPS. This inhibitor also prevents the increase in macrophage acid SMase activity caused by LPS or TNF-α. Finally, SM inhibition also increases the viability of cells incubated in media from macrophages exposed to LPS and decreases colon inflammation [23].

The injury caused by Cer is actually caused by its metabolic products, particularly its phosphorylated forms. The harmful Cer derivatives are produced by key enzymes involved in SL metabolism. In this line, the activity of neutral ceramidase (nCDase), an enzyme that catalyzes Cer breakdown, increases in the epithelial layer of the colon after treatment with DSS [30]. The role of nCDase is clearly

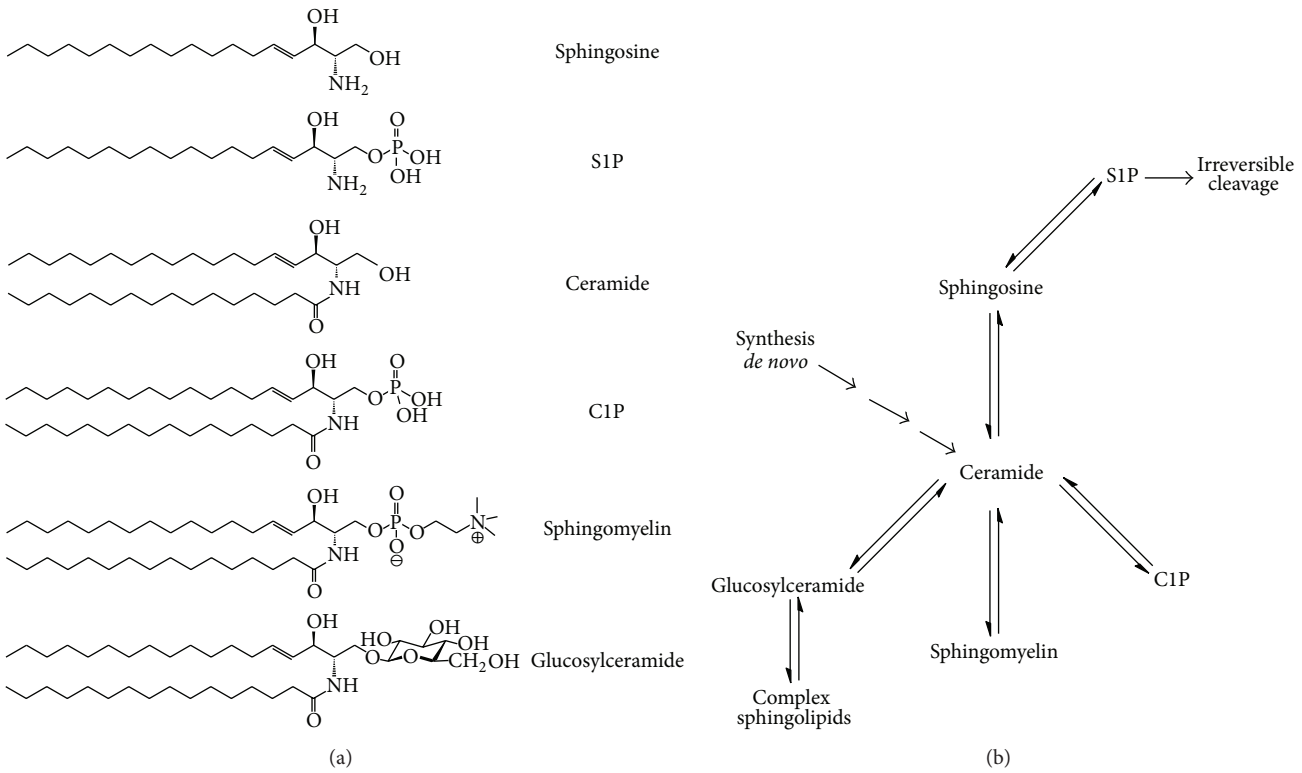

Figure 1: (a) Structure of the main bioactive sphingolipids: sphingosine (Sph), sphingosine-1-phosphate (S1P), ceramide (Cer), ceramide-1-phosphate (C1P), sphingomyelin (SM), and glucosylceramide (GluCer). (b) Schematic representation of the central role of ceramide in sphingolipid metabolism.

demonstrated in a null mutant mouse. After DSS treatment, Cer increases in the epithelial layer of the colon in both wild-type and nCDase$^{-/-}$ mice, while S1P concentration decreases in wild-type mice but increases in nCDase$^{-/-}$ animals. Cer only increases in the blood of mutant mice due to DSS, while systemic S1P levels augment in both wild-type and nCDase$^{-/-}$ mice after DSS administration. TNF-α and COX-2 expression increase after DSS treatment in the epithelial layer of wild-type and mutant mice, respectively. DSS causes systemic inflammation in both genotypes, as indicated by decreased red blood cells and increased white blood cells; though neutrophils and lymphocytes are higher in nCDase$^{-/-}$ mice. Finally, endotoxin levels are increased in the serum of mutant mice after DSS administration. Thus, nCDase may protect against inflammation, since when it is lacking worse UC symptoms develop [30].

Oral SM ingestion increases SM in feces and IEC of DSS-treated mice [24]. SM feeding may be harmful since it increases weight loss, intestinal mucosal inflammation, and epithelial damage in mice exposed to DSS. Inflammation of the intestinal mucosa is also augmented by dietary SM in IL-10$^{-/-}$ mice. Importantly, SM supplementation results in higher cathepsin D activity and IEC apoptosis [24]. SM feeding increases Cer in control and DSS-treated mice [25]. Similarly, a human colon cell line (HT-29) also converts SM into Cer. Cathepsin D and a proapoptotic protein (BCL-2 homology 3 interacting-domain death agonist or BID) augment in HT-29 cells upon SM treatment. Indeed, SM and DSS activate BID and reduce BCL-2 levels. SM causes apoptosis of HT-29 cells and IEC but phosphatidylcholine protects them. Similarly, SM affects tight-junction proteins making tight junctions weaker, whereas phosphatidylcholine has the opposite effect [25].

4. Cer Plays a Beneficial Role in IBD

There are also studies suggesting that Cer and SMases, especially if exogenously applied, may be beneficial for IBD treatment. In this line, it is important to note that their action may depend on how and where Cer is produced. For instance, exogenous acidic and neutral SMases dose-dependently increase Cer levels and trigger NF-κB, mimicking the effect of TNF-α [35]. However, the effect of these SMases on NF-κB activation differs in kinetics and the stimulated κB complexes. Acidic SMase turns on p50/p50 homodimers later (20 hours) than neutral SMase, which activates RelA/p52 or RelA/p50 heterodimers at 30 minutes. In fact, IL-8 expression is more than double with neutral SMase compared to acidic SMase. Lastly, the latter SMase induces apoptosis of colon cancer cells *in vitro* but neutral SMase has no effect unless NF-κB is inactive. Therefore, Cer-induced apoptosis may depend on the enzyme that produces it or the site where it is produced [35].

Alkaline SMase is a nucleotide pyrophosphatase/phosphodiesterase family member that breaks down dietary SM [36]. Interestingly, it hydrolyzes PAF *in vitro*, while neutral

Table 1: Potential harmful and beneficial mechanisms of Cer and S1P in IBD.

SLs	Effect	Target	Tissue/cells	References
		Harmful		
Cer	Increases	NF-κB & COX-2	Intestinal tract	[12, 13]
		BCL-2 expression	Intestinal tract	[12, 13]
		Inflammation	Intestinal tract	[12, 13]
	Decreases	Apoptosis	Intestinal tract	[12, 13]
		Expression of BAX, BAK & BAD	Intestinal tract	[12, 13]
Cer	Activates	Immune cells	Intestinal tract	[14–16]
		MAPK cascade	Intestinal tract	[17]
	Decreases	Cholesterol levels	IEC tight junctions	[20, 21]
		Barrier function	IECs	[20, 21]
SMase	Increases	TNF-α, IL-1β & IL-6 secretion	Intestinal macrophages	[22]
		LPS-induced TNF-α release	PBMCs	[22]
		TNF-α, IL-1β & IL-6 levels in DSS-induced colitis	Colon	[22]
		LPS-induced increase of Cer & NF-κB	Intestinal macrophages	[22]
		Inflammation	Colon	[22]
		LPS-induced cell death	Intestinal tract	[22]
nCDase	Decreases	DSS-induced S1P and COX-2 levels	Colon	[23]
		Endotoxin levels	Serum	[23]
Dietary SM	Increases	DSS-induced inflammation	Colon	[24, 25]
		Cathepsin D activity, BID activation, HT-29 cell & IEC apoptosis	Colon	[24, 25]
	Decreases	BCL-2 levels	Colon	[24, 25]
		Beneficial		
Cer	Decreases	IL-6 synthesis Mast cell degranulation	Colon	[26]
Alkaline SMase	Decreases	PAF	Intestinal tract	[16]
		DSS-induced inflammation	Rectum	[27]
		Tumor incidence	Colon	[28]
		DSS + azoxymethane cancer aggressiveness	Colon	[28]
	Protects		Colonic epithelium	[29]
Acidic SMase	Increases	Apoptosis	Colon cancer cells	[30]
Dietary SM	Decreases	DSS-induced inflammation	Colon	[29]
		Lymphocyte entry	Colon, PPAR-$\gamma^{-/-}$ mice	[31]
		Carcinoma burden	Colon, PPAR-$\gamma^{-/-}$ mice	[31]
		F4/80^{+} macrophages	Mesenteric lymph node	[31]
	Delays Hastens	Inflammation	Intestinal tract	[31]
		Recovery	Intestinal tract	[31]
	Increases	Survival of PPAR-$\gamma^{-/-}$ mice		[31]
		Chemokines and their receptors	Intestinal tract, PPAR-$\gamma^{-/-}$ mice	[31]
		CD4^{+} T cell maturation genes	Intestinal tract, PPAR-$\gamma^{-/-}$ mice	[31]
S1P	Increases	B & T cell survival	Intestinal tract	[26]
	Decreases	T cell proliferation Cytokine synthesis	Intestinal tract	[26]
S1P	Increases	COX-2 & PGE_2, inflammation, metalloproteinase production	Intestinal tract	[32, 33]

SMase does not affect PAF [15]. In fact, alkaline SMase activity against SM or PAF is inhibited by high amounts of PAF or SM, respectively. Moreover, its effect on PAF hydrolysis is dose- and time-dependent and is enhanced by bile salts. Low concentrations of zinc (0.1–0.25 mM) stimulate its activity against PAF, while higher levels dose-dependently inhibit PAF hydrolysis. Importantly, PAF incubation with alkaline SMase eliminates its functional effects: p42 and p44 MAPK phosphorylation, IL-8 release, and leukocyte chemotaxis are inhibited [15]. Since PAF displays proinflammatory effects [29, 37], these results suggest that alkaline SMase plays a protective role against the development of IBD and colon cancer. In fact, two reports show that this is indeed the case. Rectal administration of alkaline SMase diminishes DSS-induced inflammation and preserves the colonic epithelium [27]. Similarly, mice lacking alkaline SMase show higher colon tumor incidence and more aggressive cancers due to azoxymethane plus DSS treatment [28].

Oral SM administration may reduce inflammation caused by DSS [31]. Similarly, dietary SLs block tumor development and repress colon cancer [38, 39]. In line, inflammation onset is delayed by SM feeding in mice lacking peroxisome proliferator-activated receptor- (PPAR-) γ in epithelial and hematopoietic cells and their recovery is accelerated [40]. Dietary SM increases survival, reduces harmful colonic changes, and diminishes tumor area in PPAR-$\gamma^{-/-}$ mice. SM supplementation also lowers lymphocyte infiltration into the colon, reduces carcinoma load, decreases F4/80$^+$ macrophages in the mesenteric lymph node, and tends to reduce cluster of differentiation (CD)4$^+$ T cells in both mutant and wild-type mice. SM feeding augments several chemokines plus their receptors and genes that participate in the differentiation of CD4$^+$ T cells towards both proinflammatory and anti-inflammatory phenotypes. Dietary SM is anti-inflammatory by reducing regulatory gene expression and modifying genes involved in tissue protection or regeneration, suggesting that SM feeding may alter tumor development by reducing inflammation [40]. SM may affect inflammatory processes in a PPAR-γ dependent manner but its effect on cancer seems independent of this receptor. Thus, SM ingestion may be beneficial or harmful (see above), perhaps depending on its source.

The extracellular action of Cer has shown beneficial effects in the damaged colon, maybe by binding to receptors not related to SLs, such as the leukocyte monoimmunoglobulin-like receptor 3 (LMIR3) [41]. LMR3$^{-/-}$ mice are very vulnerable to colitis induced by DSS, shown by increased weight loss and disease activity as well as reduced colon length and survival [26]. These mutants show greater infiltration of neutrophils, eosinophils, and mononuclear cells into the colon. These cells together with mast cells express LMIR3 on their surface and DSS treatment increases its expression in mast cells. DSS augments the number of mast cells in both genotypes, but this is greater in LMIR3$^{-/-}$ mice. Similarly, degranulated mast cells are also higher in mutant mice. IL-6, IL-17A, and TNF-α as well as chemokine transcripts and proteins are increased by treatment with DSS in LMIR3$^{-/-}$ mice. Bone marrow and mast cell transplantation show that the latter cells participate in colitis aggravation in mutant mice. Injury to the colon results in the presence of extracellular ATP which activates P2X7 purinoceptors in mast cells, which release inflammatory molecules [42]. Consistent with this, DSS increases the levels of ATP in the colon. In the absence of Cer, ATP treatment increases mast cell degranulation and secretion of neutrophil chemoattractants such as leukotriene B4, while Cer represses these effects in wild-type but not LMIR3$^{-/-}$ mice. Similarly, Cer inhibits IL-6 synthesis in wild-type mast cells but not in mast cells from mutant mice. Anti-Cer antibodies worsen colitis symptoms in wild-type but not LMIR3$^{-/-}$mice, while Cer liposomes suppress mast cell degranulation in the colon of wild-type mice but not in mutant mice [26]. The data suggest that Cer liposomes may actually be useful as an IBD therapeutic strategy.

5. Sphingosine-1-Phosphate and IBD

S1P is synthesized from Sph, a product of Cer breakdown, by two enzymes: sphingosine kinase 1 and sphingosine kinase 2 (SK1 and SK2). Once produced, S1P exerts its action by two mechanisms: directly via intracellular targets or by binding to one of its five different membrane receptors named S1PRs [43]. S1P degradation is regulated reversibly by S1P phosphatases or irreversibly by the S1P lyase enzyme (S1PL). S1P has a significant role in regulating immune cell trafficking, inflammation, angiogenesis, and enhancing cell survival. S1P treatment enhances the survival of B and T cells and inhibits both homoeostatic proliferation and T cell receptor-induced proliferation of T cells, as well as inhibiting cytokine production [44].

It was proposed that S1P favors cell proliferation and survival, as well as inflammation mediated by prostaglandins since it acts as a chemoattractant agent for basophils, neutrophils, and NK cells by upregulating COX-2 and PGE_2 expression [32, 33, 45, 46]. It is important to mention that the inflammatory response is not limited to the effects of S1P and C1P expression in the intestinal tissue. The resulting PGE_2 expression can induce the production of interleukins 4, 5, and 10 (Th2 profile) and negatively regulate the expression of interferon γ, TNF-α, and interleukins 1β, 2, and 12 [47]. Th2 profile cytokines can then induce further expression of PGE_2 and COX-2 in the intestinal tissue, which sustains and increases inflammation. The rise in prostaglandin and COX-2 induces metalloproteinase expression, which, due to the inflammatory conditions that predominate in the intestinal tissue, favor its destruction (Table 1). The principal effect of inflammation on the intestine is the loss of function and structure of the intestinal mucosa and as a consequence failure in the absorption of nutrients, translocation of microbiota bacteria, and changes in the intestinal microenvironment that favor the development of pathogenic bacteria. Thus, S1P has a harmful role in inflammatory illnesses including IBD [48].

Modulating S1P signaling has been proposed as a therapeutic target in IBD treatment [49]. In this line, several studies have been published which use S1PR antagonists in animal models of colitis. Two independent reports show that treatment with FTY20 significantly attenuates the development

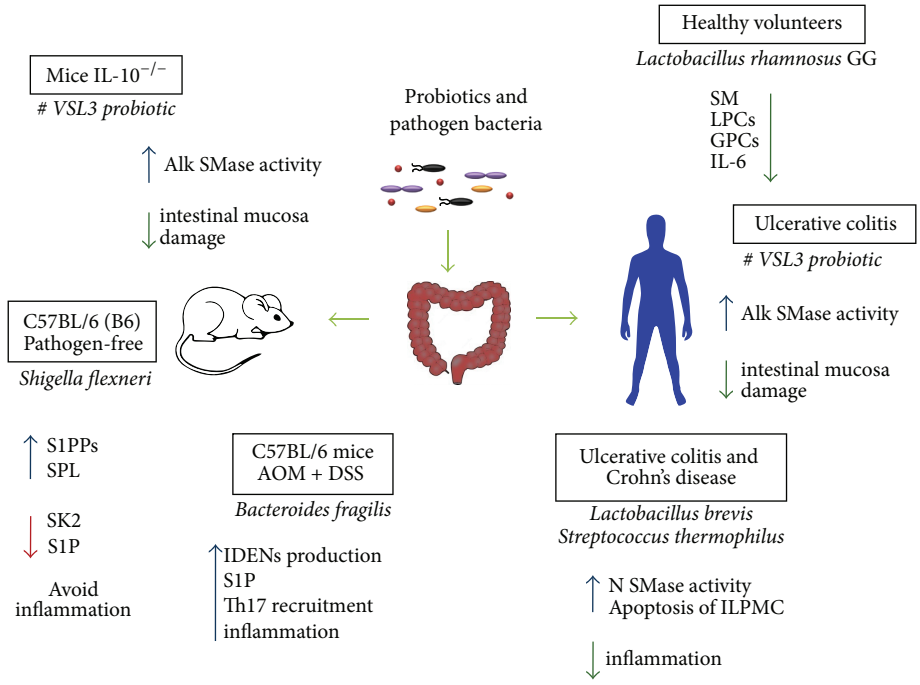

Figure 2: Known effects of pathogenic bacteria, microbiota members, and probiotics on SL signaling in IBD. Probiotics increase SMase activity and diminish intestinal inflammation reducing mucosal damage in both humans and a mouse model. *Bacteroides fragilis,* a known microbiota member, induces inflammation by stimulating epithelial production of IDENs containing high levels of S1P and mediating Th17 recruitment. Conversely, the pathogen *Shigella flexneri* can avoid the inflammatory response by decreasing S1P levels, downregulating SK2 expression and increasing SPL and S1PPs expression. SM: sphingomyelin, LPCs: lysophosphatidylcholines, GPCs: glycerophosphatidylcholines, IL-6: interleukin-6, ILPMC: intestinal *lamina propria* mononuclear cells, IDENs: intestinal derived exosome like nanoparticles, S1P: sphingosine 1 phosphate, SK2: sphingosine 1 phosphate kinase 2, SPL: sphingosine 1 phosphate lyase, and S1PPs: sphingosine 1 phosphate phosphatases.

of colitis induced by DSS or due to genetic deficiency of IL-10. In addition, similar results are obtained with the use of two different antagonists: W-061 and KRP-203 [50–53]. On the other hand, SK1 knockout mice are less susceptible of developing colitis after treatment with DSS [54]. Similarly, administration of SK inhibitors (ABC747080 and ABC294640) reduces the development of colitis induced by DSS in a mice model [55]. Additionally, treatment with a selective S1P1 receptor agonist (SEW2871) improves colitis symptoms in IL-10 deficient mice [56].

6. Probiotics Exert Beneficial Actions by Modifying Intestinal Lipids

Our bodies are colonized by trillions of microorganisms from more than 1000 different species. The majority of microbes colonizes the gut, having an important role in nutrition and may be associated with bowel diseases [57]. It has been proposed that dysbiosis (an imbalance in the quantity or type of gut microorganisms) produces or worsens inflammatory diseases. In this sense, diverse therapies using probiotics have been proposed as treatments for IBD [58]. Probiotics are defined as living microorganisms which, when administered in adequate amounts, confer a health benefit on the host [59].

Probiotics show immunomodulatory actions *in vitro*, in animal models and in humans [60, 61], especially in the context of inflammatory diseases [62]. In this line, treatment with *Lactobacillus rhamnosus* GG causes significant lipid alterations in healthy humans [63]. This treatment increases triacylglycerols but decreases lysophosphatidylcholines (LPCs), glycerophosphatidylcholines, and SMs. These changes correlate moderately with IL-6 levels, especially the diminished LPCs, and may participate in the beneficial effects of this probiotic on intestinal epithelial barrier function [63] as illustrated in Figure 2.

Similarly, a probiotic with eight different bacterial strains has beneficial effects on the intestine [64]. IL-10 knockout mice have lower baseline activity of alkaline SMase compared to wild-type animals and treatment with this probiotic augments its function in the ileum and colon [65]. The probiotic increases alkaline SMase action in wild-type mice and reduces damage to the colonic mucosa in IL-10 knockouts. In humans suffering from UC, the probiotic elevates alkaline SMase function and diminishes disease activity [65]. Thus, the results suggest that augmented activity of alkaline SMase is beneficial in patients with IBD.

Sonicates of probiotic *Lactobacillus brevis* or *Streptococcus thermophilus* show high neutral SMase activity compared to sonicates of nonprobiotic *Escherichia coli* and *Escherichia faecalis* [66]. *L. brevis* sonicates cause apoptosis of intestinal *lamina propria* mononuclear cells (ILPMC) from healthy individuals and patients with UC or CD. Interestingly, the effect

is more pronounced in the latter. Similarly, sonicates from *S. thermophilus* result in elevated apoptosis of ILPMC from people suffering IBD. Activation of PBMC and ILPMC with antibodies for CD3 and CD28 results in higher apoptosis of these cells in response to the probiotic organisms. Exogenous Cer or neutral SMase also augment apoptosis of ILPMC from healthy subjects and individuals with IBD (Figure 2). Again, the effect is less evident in the former. Both *L. brevis* sonicates and exogenous neutral SMase increase c-jun N-terminal kinase activation in ILPMC. *L. brevis* sonicates cause the production of higher levels of reactive oxygen species in ILPMC. Sonicates of *L. brevis* and *S. thermophilus* incubated with glutathione lose their ability to induce apoptosis of ILPMC from healthy individuals and those affected with CD. In a similar fashion, glutathione abrogates the apoptotic effect of the sonicates on activated PBMC. A specific inhibitor of neutral SMase diminishes apoptosis of ILPMC induced by *L. brevis* sonicates [66]. These results suggest that probiotics generate elevated levels of Cer via their endogenous SMase, which exerts anti-inflammatory effects by killing resident and blood-derived immune cells.

Although the exact mechanism is not completely identified, some bacteria can modulate the host immune system by modulating S1P levels. For example, a study showed that, in a mouse model and a human cell line, *Shigella flexneri* downregulates SK2 expression but upregulates the expression of sphingosine 1 phosphate lyase (SPL) and sphingosine 1 phosphate phosphatases (S1PPs), thus decreasing S1P levels as a mechanism to avoid the inflammatory response [67]. Conversely, secreted particles from enterotoxigenic *Bacteroides fragilis* stimulate intestinal epithelial cells to produce intestinal derived exosome like nanoparticles (IDENs). These IDENs contain elevated levels of S1P, CCL20, and PGE_2, which mediate Th17 recruitment and induction contributing to intestinal inflammation and cancer [68] (Figure 2).

7. Invariant Natural Killer T Cells and Bacterial α-Galactosylceramide

Probiotic bacteria may produce compounds quite similar to α-galactosylceramide, which potently activates invariant natural killer T cells (iNKT). These cells are specifically reduced by treatment with azoxymethane followed by DSS, which induces colitis and colon cancer [69]. Mice lacking iNKT cells show more and bigger tumors, as well as worse inflammation indicators than wild-type mice. Azoxymethane/DSS administration augments $CD25^+$ $CD4^+$ T cells and $NK1.1^+$ T cells in mice lacking iNKT cells. The number of IL-13^+ $CD3^+$ cells and IL-13 release in the mesenteric lymph node and colon are increased in these animals. Importantly, α-galactosylceramide administration elevates the Th1/Th2 ratio in the mesenteric lymph node, while inflammation and the number of tumors are decreased in the colon. Treatment with α-galactosylceramide diminishes the number of colonic $NK1.1^+$ T cells and IL-13 release from colonic lymphocytes [69]. The data suggest that probiotics stimulate iNKT cells, which reduce inflammation and development of colon cancer by altering T cell populations and cytokine secretion.

An investigation of the SLPs synthesized by a prominent constituent of the gut microbiota was performed [70]. A putative serine palmitoyltransferase, which catalyzes the first committed step of sphingolipid biosynthesis, was deleted from *Bacteroides fragilis* NCTC 9343. No double mutants are obtained, suggesting that the enzyme is critical for survival. Single mutants lack sphingolipid production, allowing identification of the sphingolipids produced by wild-type *B. fragilis*. These bacteria synthesize Cer phosphorylethanolamine, its matching dihydroceramide analog, and α-galactosylceramide. The latter binds to CD1d to activate mouse and human iNKT cells, as shown by IL-2 and IFN-γ synthesis. This lipid also stimulates human PBMC, as shown by proliferation of $CD3^+$ $V\alpha24^+$ cells, and activated iNKT cells *in vivo*, evidenced by higher CD25, CD69, and IFN-γ expression (Figure 3). Moreover the effect is blocked by CD1d antibodies [70]. The results suggest that an important member of the gut microbiota produces α-galactosylceramide, which stimulates PBMC and iNKT cells.

Another study suggested that α-galactosylceramide did not activate iNKT cells, but instead it reduced their stimulation both *in vitro* and *in vivo* by competing for CD1d binding or impeding detection by iNKT cells [71]. Mice monocolonized with *B. fragilis* that lack their presumed serine palmitoyltransferase have a higher number of colonic iNKT cells and worse colitis symptoms, evidenced by a more pronounced weight loss as well as higher IL-4 and IL-13 secretion. The iNKT cell population is reduced by neonatal treatment with a CD1d antibody; this blocks oxazolone-induced colitis in adult mice with altered *B. fragilis*. These animals have similar colonic bacteria number, comparable chemokine levels, analogous iNKT cell stimulation, and apoptosis plus similar polysaccharide A expression. The bacterial SLs diminish iNKT proliferation, but only when mice were exposed to them prenatally. Ceramides, glycosylceramides, and phosphoethanolamine ceramides are present in wild-type but not in mutant *B. fragilis*. iNKT cell activation is decreased by glycosylceramides, including α-galactosylceramide. Importantly, α-galactosylceramide treatment during the neonatal period diminishes the number of colonic iNKT cells and improves oxazolone-induced colitis in mice with altered bacteria [71]. Thus, α-galactosylceramide produced by a prominent constituent of the gut microbiota is beneficial. The issue of whether this lipid stimulates or inhibits iNKT cells remains to be resolved.

Glycosylceramides contained in mammalian milk and serum activate iNKT cells; indeed human iNKT cells are stimulated by cow and human milk [72]. But glycosylceramides from the spleen of Gaucher's disease patient do not affect iNKT cells. This suggests that an unknown ingredient in mammalian milk and serum is responsible for the observed effect because the glycoceramides within cow's milk and Gaucher's spleen are comparable. Upon further analysis, the ingredient is likely α-galactosylceramide which is also present in the thymus of mammals and activated iNKT cells [72]. The most probable explanation is that mammalian microbiota is responsible for producing this α-galactosylceramide, although the intriguing possibility that it can be synthesized by mammals cannot be dismissed. Of note, in this study

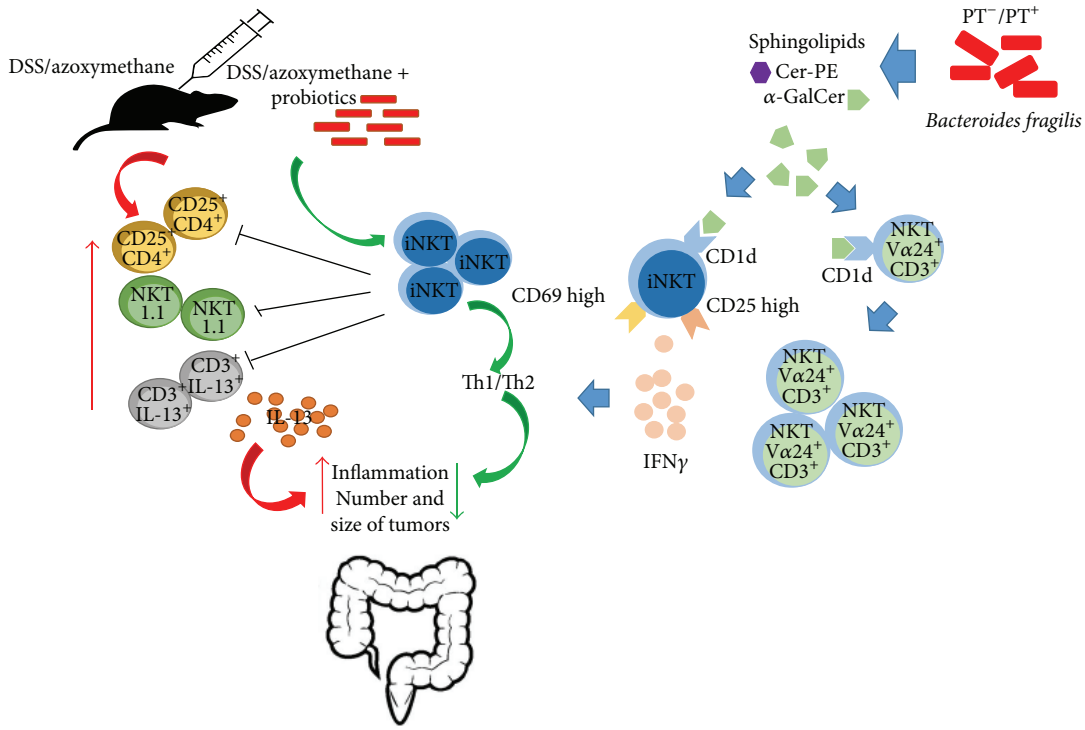

FIGURE 3: Bacterial α-galactosylceramide as iNTK regulator in colon cancer. Treatment with azoxymethane followed by DSS reduces the number of iNTK cells and augments $CD25^+$ $CD4^+$ T cells, $NKT1.1^+$ T cells, and IL-13^+ $CD3^+$ cells, as well as IL-13 release. All this contributes to form a greater number of tumors of a larger size. Probiotic bacteria can produce compounds similar to α-galactosylceramide, which prevents inflammation and reduces the number and size of tumors. *B. fragilis* produces α-galactosylceramide which stimulates iNKT cells binding to CD1d, increasing production of IFN-γ and proliferation of $CD3^+$ $V\alpha24^+$ cells. DSS: dextran sulphate sodium, iNKT: invariant natural killer, NKT: natural killer T cell, and PT: serine palmitoyltransferase.

galactosylceramides stimulate iNKT cells as explained in Figure 3.

8. Conclusion

IBD has become an emergent public health problem. As discussed previously, although its exact etiology remains unclear, the role of SLs in contributing to the inflammatory process is evident. Indeed, besides its role in digestive function, the gut is actually considered as an immune organ. This is in large part due to the signaling molecules that are produced within it, particularly those comprised by SLs, which affect not only the intestinal tract but the whole immune system. As mentioned above, Cer is a central molecule in defining the role of the immune response through its different metabolic byproducts: SM, Sph, and S1P, which mediate specific responses. SMases are of particular interest because of their differential activities. Thus, research on therapeutic agents able to modulate SMases and formulations for tissue-specific delivery is mandatory. The conversion of Cer into different SLs, which enhance or prevent the inflammatory response, depends on cell molecular signals and the cell microenvironment. In this sense, gut microbiota may provide the conditions that define the source and fate of SLs as modulators of the immune response. For instance, microorganisms provide enzymes or their activators to promote Cer metabolism and thereby regulate cytokine production. Thus, gut microbiota is a new and attractive target for the control of IBD and even other inflammatory conditions.

In this sense, prebiotic and probiotic agents emerge as pivotal players in the control of the immune response in this organ. Further therapies may include the preservation of natural microbiota in the bowel in order to avoid IBD and new therapies based on nutritional programs including food that favors beneficial species or even seeding the intestinal tract with strains that can produce specific SLs, depending on the pathological situation. These promising possibilities deserve further scientific and clinical investigation.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Anti-Inflammatory Effects of Hyperbaric Oxygenation during DSS-Induced Colitis in BALB/c Mice Include Changes in Gene Expression of *HIF-1α*, Proinflammatory Cytokines, and Antioxidative Enzymes

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Reactive oxygen species (ROS) and nitrogen species have an indispensable role in regulating cell signalling pathways, including transcriptional control via hypoxia inducible factor-1α (*HIF-1α*). Hyperbaric oxygenation treatment (HBO_2) increases tissue oxygen content and leads to enhanced ROS production. In the present study DSS-induced colitis has been employed in BALB/c mice as an experimental model of gut mucosa inflammation to investigate the effects of HBO_2 on *HIF-1α*, antioxidative enzyme, and proinflammatory cytokine genes during the colonic inflammation. Here we report that HBO_2 significantly reduces severity of DSS-induced colitis, as evidenced by the clinical features, histological assessment, impaired immune cell expansion and mobilization, and reversal of *IL-1β*, *IL-2*, and *IL-6* gene expression. Gene expression and antioxidative enzyme activity were changed by the HBO_2 and the inflammatory microenvironment in the gut mucosa. Strong correlation of *HIF-1α* mRNA level to *GPx1*, *SOD1*, and *IL-6* mRNA expression suggests involvement of *HIF-1α* in transcriptional regulation of these genes during colonic inflammation and HBO_2. This is further confirmed by a strong correlation of *HIF-1α* with known target genes VEGF and *PGK1*. Results demonstrate that HBO_2 has an anti-inflammatory effect in DSS-induced colitis in mice, and this effect is at least partly dependent on expression of *HIF-1α* and antioxidative genes.

1. Introduction

Relapsing chronic inflammation found in the gut of individuals affected by inflammatory bowel diseases (IBD) is a result of several overlapping factors, including dysregulation of the immune response to the enteric microbiota, genetic susceptibility, and environmental factors [1, 2]. Reactive oxygen (ROS) and nitrogen (RNS) species generated by inflammatory cells during an immune response create oxidative stress and are considered as important factors contributing to the pathogenesis of IBD. Lymphocytes, neutrophils, and macrophages activated during the gut inflammation produce high amounts of ROS/RNS destroying surrounding tissue [3]. Although oxidative stress is a major factor in the inflamed tissue leading towards necrosis, DNA damage, and carcinogenesis, certain amounts of ROS and other free radicals have an indispensable role in regulating different cell signalling pathways [4]. In addition, some immune cells, namely, the macrophages and neutrophils, use ROS to combat the microorganisms responsible for the infection.

Current therapies of IBD, including immunosuppressive drugs, antibiotics, and biological drugs, are efficient in controlling the course of the disease. However, for many affected individuals, conventional therapy becomes an inadequate choice for long-term treatment because of significant side effects and risk factors, such as increased cancer risk, development of tuberculosis, and heart failure [5–7]. In recent years, hyperbaric oxygen (HBO_2) therapy has been introduced as a possible additional treatment for IBD patients, especially in the case of refractory disease when the standard therapy is ineffective.

HBO_2 involves exposure to 100% oxygen under pressure greater than 1 atmosphere of absolute pressure (ATM). It is a well-established procedure frequently applied in the medical practice, especially effective in treating wounds of various aetiologies [8–11]. HBO_2 increases blood and tissue oxygen saturation resulting also in enhanced production of ROS and RNS [12]. Previous studies have verified that the clinical efficacy of HBO_2 derives from modulation of intracellular transduction cascades, leading to synthesis of growth factors which promote wound healing, neoangiogenesis, and ameliorates postischemic and postinflammatory injuries [13]. Additional investigations on the mechanism underlying HBO_2-induced wound healing have revealed a central role of hypoxia inducible factor-1 alpha (*HIF-1α*) as transcriptional regulator of genes involved in angiogenesis, energy metabolism, and cell proliferation [9, 14, 15]. A further important function attributed to the *HIF-1α* is modulation of the immune responses, including the helper T-cell differentiation towards regulatory (Treg) versus Th17 phenotype [16, 17] and its strong anti-inflammatory activity in the gastrointestinal mucosa and hypoxic epithelium as a result of the transactivation of specific genes encoding for barrier-protective elements such as mucins [18–20].

Hypoxia and ROS/RNS can induce stabilization of *HIF-1α* leading to the activation of the hypoxia signal transduction pathway [21]. Increased oxidative stress in the gut mucosa has been verified in humans suffering from ulcerative colitis [22, 23], as well as in experimentally induced colitis in animals [24, 25]. A recent study revealed increased activity of antioxidative enzymes and reduced oxidative stress in the inflamed gut mucosa following HBO_2 exposure [24]; however, specific mechanisms inducing activation of antioxidative enzymes in the inflamed colonic tissue upon HBO_2 remain unknown.

Since there is evidence that the intracellular redox status is in a close correlation with the inflammatory microenvironment, and it can also be changed by HBO_2, the aim of this study was to investigate the effects of HBO_2 on the mRNA expression of *HIF-1α*, proinflammatory cytokines, and antioxidative enzymes in the gut and peripheral lymphoid organs of BALB/c mice with DSS-induced colitis. An additional aim was to assess the activity of antioxidative enzymes and whether *HIF-1α* gene expression regulation during the gut inflammation and HBO_2 treatment correlates with the changes in antioxidative and proinflammatory gene expression. Our findings reveal that HBO_2 treatment may effectively modulate the intestinal milieu in inflammatory conditions involving *HIF-1α*-mediated regulation of antioxidative gene expression.

2. Materials and Methods

2.1. Animals. BALB/c mice, obtained from Charles River (Calco, Italy), were bred at the animal facility of the Medical Faculty Osijek (Croatia). Mice were provided with standard rodent chow (Mucedola, Settimo Milanese, Italy) and water *ad libitum*. The experimental facility was maintained at 22 ± 2°C, 55 ± 5% humidity, and 12-hour light/dark cycle. All procedures involving live animals were conducted in accordance with the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609/EEC) and were approved by the local Ethical Committee (Faculty of Medicine, University of Osijek) and Croatian Ministry of Agriculture.

2.2. Experimental Design. For each experiment male mice at the age of 10–12 weeks were randomized into 4 groups (n = 4-5 mice/group/experiment): control mice (CTRL), control mice undergoing HBO_2 (CTRL + HBO_2), mice receiving dextran sodium sulphate (DSS), and DSS treated mice undergoing HBO_2 (DSS + HBO_2). The average body weight of the mice at the time of inclusion into the study was 22.8 ± 0.4 g. Colitis was induced by 5% (w/v) of DSS (Mr 36.000–50.000, MP Biomedicals, Illkrich, France) in drinking water *ad libitum* for 7 consecutive days [26, 27]. The HBO_2 treatment was initiated at day 1 and was administered twice a day, 12 hours apart, until the end of experiment (the last session was applied in the morning of day 8; 15 sessions in total). During one HBO_2 session, mice were exposed to 100% O_2 for 60 minutes at 2.4 bars with addition of 15 minutes for gradual compression and decompression. Mice were sacrificed by cervical dislocation on day 8, after the morning HBO_2 session. Disease activity index (DAI) was assessed by daily measurement and scoring of animal body weight loss, stool consistency, and the presence of occult or gross blood per rectum. Measurements were performed at the same time each day until the end of experiment (day 8). DAI was determined as a sum of body weight loss score (0, none; 1, 1–5% loss; 2, 5–10%; 3, 10–15%; 4, >15%), stool consistency score (0, normal; 2, loose stool; 4, diarrhea), and score of occult/gross bleeding (0, normal; 2, occult bleeding; 4, gross hematochezia). Mice were sacrificed by cervical dislocation on day 8 following the last morning HBO_2 treatment. Colons, mesenteric lymph nodes (MLN), and spleens were collected for further analysis. Colon length was determined for each animal.

2.3. Histological Assessment of Colitis. Colonic tissue was removed immediately after the animals were sacrificed, washed in PBS, and fixed in 4% paraformaldehyde. After 72 hours fixed tissue was embedded in paraffin and cut into a series of 6 μm thick sections. Slides were dried, deparaffinised, rehydrated, and stained with haematoxylin and eosin. Histological disease activity was assessed by an experienced histologist, blinded for clinical information.

Histological evaluation was preformed according to modified Geboes score as follows [28]:

Grade 0 (structural (architectural changes)):

0: no abnormality,

1: mild abnormality,

2: mild or moderate diffuse or multifocal abnormalities,

3: severe diffuse or multifocal abnormalities.

Grade 1 (chronic inflammatory infiltrate):

0: no increase,

1: mild but unequivocal increase,

2: moderate increase,

3: marked increase.

Grade 2 (lamina propria leukocytes):

0: no increase,

1: mild but unequivocal increase,

2: moderate increase,

3: marked increase.

Grade 3 (intraepithelial neutrophils):

0: none,

1: <5% crypts involved,

2: <50% crypts involved,

3: >50% crypts involved.

Grade 4 (crypt destruction):

0: none,

1: probable, local excess of neutrophils in part of crypt,

2: probable, marked attenuation.

Grade 0 (structural (architectural changes)):

3: unequivocal crypt destruction.

Grade 5 (erosion or ulceration):

0: no erosion, ulceration, or granulation tissue,

1: recovering epithelium + adjacent inflammation,

2: probable erosion focally stripped,

3: unequivocal erosion,

4: ulcer or granulation tissue.

Slides were analysed using light microscopy at magnifications 40x, 100x, 200x, and 400x and photographed at 200x (Olympus® BX50 microscope, Olympus C-5050 digital camera, and QuickPHOTO PRO imaging software (Promicra s.r.o., Prague, Czech Republic)).

2.4. Isolation of Lamina Propria Lymphocytes. After isolation, the colon was cleaned of intestinal content and freed of fat tissue, washed in DMEM (Sigma Aldrich, Steinheim, Germany), cut into 5 cm long pieces, and, while shaken at 100 rpm, incubated at 37°C for 20 minutes in DMEM with 25 mM EDTA. The tissue was then thoroughly washed in PBS buffer for at least 5 times, cut into 2 mm long strips, and digested in DMEM containing 5 U/mL DNase (Roche, Mannheim, Germany) and collagenase II (Gibco, Paisley, UK) at 37°C for 20 min. Following this, supernatant was removed, and the previous step was repeated until complete digestion of the tissue was achieved. The supernatant was then filtered through a 100 μm sized filter; DMEM + 2% FBS (Sigma Aldrich, Steinheim, Germany) was added and centrifuged at 800 ×g for 10 min at room temperature. Cells were resuspended in 5 mL of 40% Percoll (GE Healthcare, Uppsala, UK), overlaid on 4 mL of 80% Percoll, and centrifuged at 900 ×g/20 min/4°C. Lymphocytes from the interphase were collected and analysed by flow cytometry.

2.5. Flow Cytometry. Lymphocytes were isolated from the mesenteric lymph nodes (MLN) and spleen by teasing apart the organs between the frosted ends of two microscopic slides. The cells were incubated with a mixture of PE anti-CD4 (clone GK1.5, ExBio antibodies), FITC anti-B220 (clone RA3-6B2, obtained from the American Type Culture Collection and conjugated with FITC using standard procedures), and APC anti-CD3 antibodies (clone 145-2C11, ExBio antibodies) and the other panel with PerCP anti-CD45 (clone 30-F11, BD Biosciences), FITC anti-Gr-1 (clone RB6-8C5, BD Biosciences), and PE anti-F4/80 (clone BM8, StemCell Technologies Inc.) or PE anti-CD4 (clone MEM-241, ExBio antibodies) and PerCP anti-CD8 (clone MEM-31, ExBio antibodies) antibodies. Dead cells were excluded based on 7-aminoactinomycin D (7-AAD) (Applichem, Darmstadt, Germany) staining. At least 20,000 live cells were collected by a BD FACS Canto II cytometer (FACS Canto II, Becton Dickinson, San Jose, CA, USA) and analysed using the FlowLogic software (Inivai Technologies, Mentone, Australia).

2.6. Measurement of Intracellular ROS Level. To assess hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) level, 10^6 lymphocytes isolated from MLN and spleens were incubated for 30 min on +4°C with 10 μM dichlorofluorescein diacetate (DCF-DA) (Biomol, Hamburg, Germany), washed for 5 min at 400 ×g on +4°C, and analysed with the FACS Canto II. Following this, cells were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA, Calbiochem, Darmstadt, Germany), incubated for 30 min, and analysed for the second time. At each measurement minimum of 10,000 target cells were analysed. Data are expressed as the median fluorescence intensity (MFI) ± s.e.m.

2.7. Real-Time PCR. Colon, MLN, and spleen samples were isolated, snap frozen in liquid nitrogen, and stored at −80°C till analysis. Total RNA was extracted using ONE STEP RNA Reagent (BIO BASIC Inc., Markham, Ontario, Canada)

Table 1: Primer sequences, PCR product length, and primers annealing temperature used for qPCR analysis.

Gene		Sequence	PCR product length/bp	Annealing temperature/°C
HIF-1α	For	TGACGGCGACATGGTTTACA	280	63
	Rev	AATATGGCCCGTGCAGTGAA		
SOD1	For	GGAAGCATGGCGATGAAAGC	80	56
	Rev	GCCTTCTGCTCGAAGTGGAT		
GPx1	For	TCCAGTATGTGTGCTGCTCG	249	63
	Rev	GTGTCCGAACTGATTGCACG		
CAT	For	GGTGCCCCCAACTATTACCC	141	61
	Rev	GAATGTCCGCACCTGAGTGA		
IL-1β	For	GCCTTGGGCCTCAAAGGAAAGAATC	282	66
	Rev	GGAAGACACAGATTCCATGGTGAAG		
IL-2	For	CTCTGCGGCATGTTCTGGAT	163	65
	Rev	AGAAAGTCCACCACAGTTGCT		
IL-6	For	GCTGGAGTCACAGAAGGAGTGGC	117	63
	Rev	GGCATAACGCACTAGGTTTGCCG		
HPRT1	For	TCAGTCAACGGGGGACATAAA	142	59
	Rev	GGGGCTGTACTGCTTAACCAG		

according to manufacturer's protocol. RNA purity and concentration was assessed by NanoPhotometer® P-Class P330-30 (Implen, Munich, Germany). In order to purify RNA from all polysaccharides, including DSS, an additional purification step using 8 M LiCl was performed [29], followed by the standard genomic DNA purification step using Deoxyribonuclease I kit (Sigma Aldrich, St Louis, MO, USA). One microgram of RNA was used for cDNA synthesis by High Capacity cDNA kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on CFX96 system (Bio Rad, Singapore) to assess relative expression of catalase (*CAT*), glutathione peroxidase 1 (*GPx1*), superoxide dismutase 1 (*SOD1*), *HIF-1α*, *IL-1β, IL-2,* and *IL-6*. Gene expression was normalized to *HPRT1* gene. Primers list is given in the supplementary data (Table 1). Except for the primers for *IL-6* gene published by Jeong et al. [30], all other primers were custom made using Primer 3 software. Messenger RNA expression was determined using SsoFast EvaGreen Supermix (Bio Rad, Singapore). Data are presented as mean ± s.e.m.

2.8. Antioxidative Enzymes Activity Measurement. Frozen tissue samples were homogenized with liquid nitrogen and weighed. Tissue powder was additionally homogenized in 100 mM phosphate buffer solution (pH 7.0) containing 1 mM EDTA (1 : 10, w/v) using Ultra turrax T10 homogenizer (IKA, Staufen, Germany) while kept on ice. Tissue homogenates were sonicated for 30 seconds on ice in three 10 seconds intervals and then centrifuged at 20,000 ×g for 15 minutes at 4°C. Supernatant was collected, aliquoted, and stored at −80°C till analysis. CAT, GPx, and SOD enzyme activities were determined using a Lambda 25 UV-Vis spectrophotometer equipped with UV WinLab 6.0 software package (Perkin Elmer for the Better, Massachusetts, USA).

Catalase (EC 1.11.1.6) activity was estimated spectrophotometrically using H_2O_2 as a substrate [31]. The reaction mixture consisted of 10 mM H_2O_2 in 50 mM phosphate buffer pH 7.0. Changes in absorbance of the reaction mixture were measured at 240 nm during 2 minutes after the sample addition. One unit of activity corresponds to the loss of 1 μmol of H_2O_2 per minute. CAT activity was calculated using molar extinction coefficient (ε = 0.04 mM cm^{-1}) and expressed as U mg^{-1} protein.

To assess glutathione peroxidase (EC 1.11.1.9) activity a modified method described by Wendel [32] using H_2O_2 as a substrate was employed. GPx activity was determined indirectly by measuring the rate of NADPH oxidation to NADP+, accompanied by a decrease in absorbance at 340 nm. The assay mixture consisted of 50 mM phosphate buffer with 0.4 mM EDTA and 1 mM sodium azide (pH 7.0), 0.12 mM NADPH, 3.2 units of GR, 1 mM glutathione, and 0.0007% (w/w) H_2O_2 in a total volume of 1.55 mL. One unit catalyses the oxidation by H_2O_2 of 1.0 μmole of reduced glutathione to oxidized glutathione per minute at pH 7.0 and 25°C. GPx activity was calculated using molar extinction coefficient for NADPH (ε = 6.220 mM cm^{-1}) and expressed as U mg^{-1} protein.

Superoxide dismutase (EC 1.15.1.1.) activity was determined using cytochrome C (0.05 mM) as an inhibitory molecule in PBS buffer saline with 0.1 mM EDTA in system xanthine (1 mM)/xanthine oxidase (50 U) by Flohe method [33].

Total soluble protein concentration in protein extracts was determined by Bradford reagent (Sigma Aldrich, Steinheim, Germany) following manufacturer's protocol and using bovine serum albumin as a standard.

2.9. Statistical Analysis. Normal distribution was assessed by the Shapiro Wilk test. Within groups differences were tested by one-way ANOVA or Kruskal-Wallis test followed by the Holm-Sidak/Tukey or Dunn's *post hoc* multiple comparison procedure, respectively (Sigma plot 11.0, SigmaStat Inc., San Jose, CA, USA). In some cases, the Student *t*-test and Mann-Whitney *U* Statistic were used to compare the differences

between the two groups in the case of normally distributed variables and variables that violated assumption of normality, respectively. Spearman's correlations were calculated where appropriate (Sigma plot 11.0, SigmaStat Inc.). DAI results were analysed by two-way ANOVA and Bonferroni *post hoc* test (GraphPad Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA). A P value of < 0.05 was considered statistically significant for all procedures. All data are presented as mean values ± standard error of mean (s.e.m.).

3. Results

3.1. HBO_2 Ameliorates the Course of DSS-Induced Colitis. In order to determine the effects of HBO_2 on the course of acute colitis, BALB/c mice were exposed to 5% DSS in the drinking water *ad libitum* and daily monitored for body weight, stool consistency, and occult/gross rectal bleeding to calculate DAI. In this study the DSS treatment induced substantial weight loss, rectal bleeding, loose stool, and colon shortening resulting in significantly higher DAI compared to the control (CTRL) group, starting from day 3 until the end of the experiment ($P < 0.01$; Figure 1(a)). Mice that received DSS and underwent HBO_2 treatment (DSS + HBO_2 group) also presented with significantly higher DAI compared to the CTRL group; however, in this group of mice HBO_2 treatment significantly reduced DAI compared to the DSS mice, starting from day 5 throughout day 8 (days 5 and 8 $P < 0.01$; days 6 and 7 $P < 0.05$; Figure 1(a)). In addition, average colon length in the DSS group of mice was significantly shorter (8.14 ± 0.23 cm) compared to the CTRL group (13.88 ± 0.45 cm; $P < 0.0001$), while this effect was significantly ameliorated by HBO_2 treatment in the DSS + HBO_2 group (11.34 ± 0.38 cm) compared to the DSS group ($P = 0.0001$, Figure 1(b)). DSS induced colitis resulted in significant body mass loss compared to CTRL group, irrespective of HBO_2 treatment (11.89 ± 0.03% and 8.24 ± 0.01% in the DSS and DSS + HBO_2 group, resp.; $P < 0.001$). Mice undergoing HBO_2 presented with reduced body mass loss. CTRL and CTRL + HBO_2 gained body mass during the experiment, 8.25 ± 0.2% and 0.33 ± 0.01%, respectively.

Histological assessment of the colon revealed severe inflammation and ulceration extending into the deep portions of the mucosa with loss of crypts and with increased number of lamina propria leukocytes in DSS group of mice. We also found structural changes of mucosa in the colonic tissue of DSS + HBO_2 mice but with reduced infiltration of inflammatory cells and decreased crypt distortion. When compared to the DSS + HBO_2 group, the DSS group had significantly higher total histological score as well as individual scores (see modified Geboes score in Section 2.3, Figure 1(d), $P < 0.001$), except for the intraepithelial neutrophil infiltration score ($P = 0.104$).

Distribution of inflammatory cells among the peripheral lymphoid organs, including Gr-1^+ leukocytes (monocytes and neutrophils), F4/80^+ leukocytes (monocytes), $CD3^+$ T lymphocytes, and $B220^+$ B lymphocytes (Figure 2), was assessed at the end of the experiment. In the MLN, frequencies of Gr-1^+ cells did not differ among the experimental groups, while DSS induced a significant increase in F4/80^+ and decrease in $CD3^+$ cell frequencies ($P < 0.05$ and $P = 0.032$, resp.; Figure 2(b)). These findings in the DSS group were accompanied by a B-cell increase which was not statistically significant. HBO_2 alone had no effect on the cell frequencies in MLN of control mice, whereas it substantially ameliorated these changes in mice with DSS-induced colitis (DSS + HBO_2 group) but without reaching statistical significance.

In the spleen, Gr-1^+ cell frequencies were significantly decreased in the DSS group compared to CTRL ($P = 0.006$) and CTRL + HBO_2 groups ($P < 0.001$; Figure 2(a)). HBO_2 treatment reversed Gr-1^+ cell frequencies to control values in the DSS + HBO_2 group ($P = 0.056$; Figure 2(a)). In addition, the DSS mice showed reduced frequencies of $B220^+$ lymphocytes compared to the CTRL group ($P = 0.016$), and HBO_2 treatment abolished these effects in the DSS + HBO_2 group ($P = 0.034$; Figure 2(c)). In addition, our study revealed that DSS-induced immune responses in the MLN and the spleen were significantly dampened by HBO_2 treatment.

Frequency of $CD4^+$ cells among the colon lamina propria lymphocytes of DSS and DSS + HBO_2 groups was significantly increased compared to the CTRL group ($P = 0.015$ and $P = 0.047$, resp.), while the frequency of $CD8^+$ cells was significantly increased only in the DSS group when compared to the CTRL group ($P = 0.011$; Figure 3).

3.2. Inflammation of Colonic Mucosa and HBO_2 Treatment Induce Changes in Antioxidative Enzymes Gene Expression and HIF-1α Gene Regulation. Inflammatory conditions have been known to include the enhanced production of ROS and other oxidative mediators that may affect transcriptional regulation via *HIF-1α*. To investigate the role of *HIF-1α* in the regulation of the antioxidative response/capacity during DSS-induced colitis and HBO_2 treatment, *HIF-1α*, *CAT*, *GPx1*, and *SOD1* mRNA expressions were determined using quantitative PCR method. *HIF-1α* mRNA expression was significantly changed by the HBO_2 treatment and the inflammatory microenvironment in the gut mucosa. DSS-induced colitis resulted in significant upregulation of *HIF-1α* gene in colonic mucosa ($P = 0.008$ for DSS group compared to CTRL), and the HBO_2 treatment further increased *HIF-1α* mRNA expression in the DSS + HBO_2 group ($P = 0.028$ compared to CTRL; Figure 3(a)). In addition, the activity of *HIF-1α* protein was indirectly confirmed by measuring mRNA expression of well-established *HIF-1α* target genes, *VEGF* and *PGK1*. Both genes showed strong positive correlation to the *HIF-1α* mRNA (Supplementary Figure 1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/7141430). There was also a tendency for upregulation of *HIF-1α* gene in MLN and spleens of the DSS group and its downregulation via HBO_2 in the DSS + HBO_2 group (Figure 4(a)); however, these changes did not reach statistical significance.

Inflammation during DSS-induced colitis and the HBO_2 treatment also induced significant changes in mRNA expression of target antioxidative genes. DSS-treated mice presented with significant downregulation of the *CAT* gene in the colon compared to the CTRL group ($P = 0.031$), while there was a significant upregulation of *CAT* gene in the spleen of

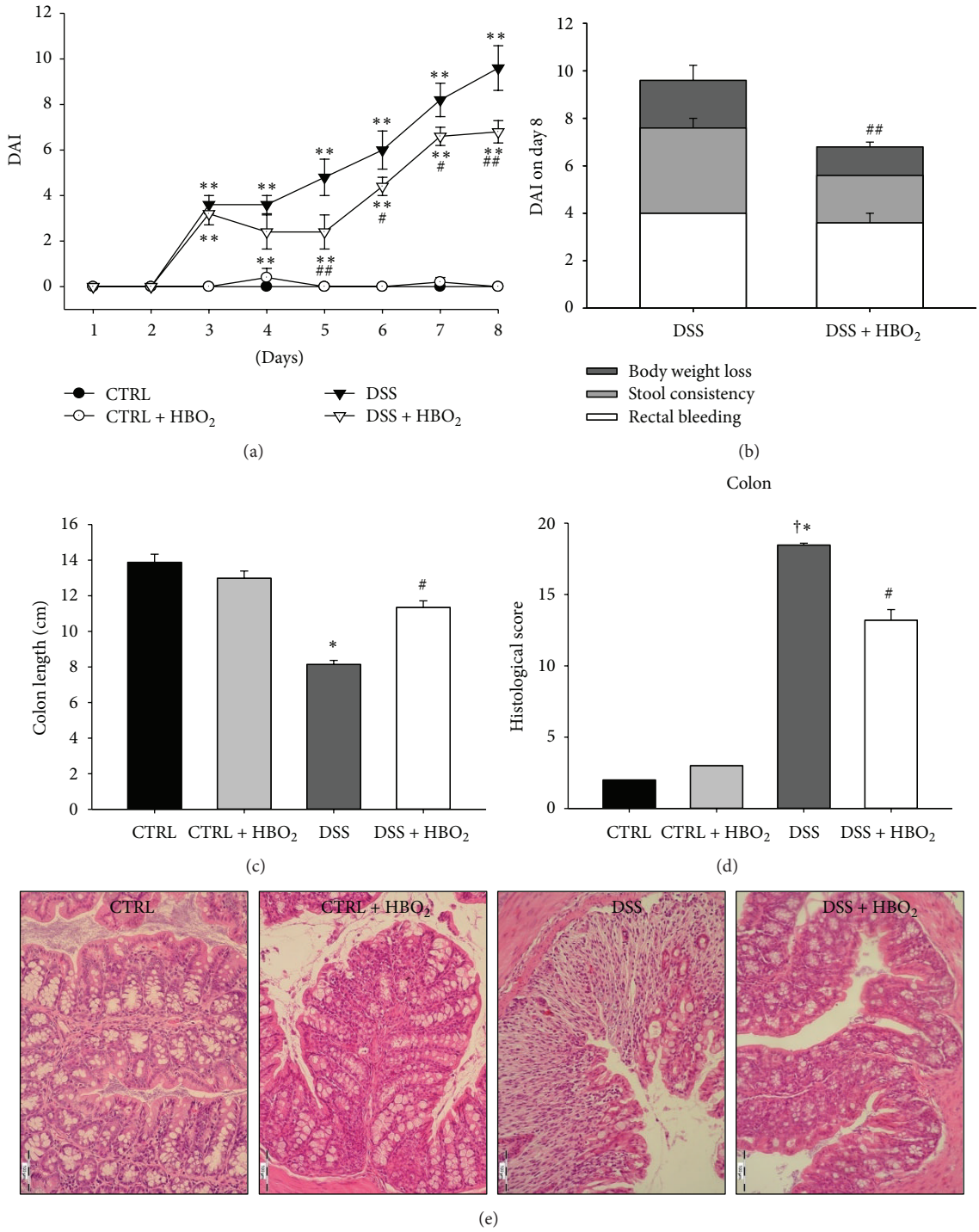
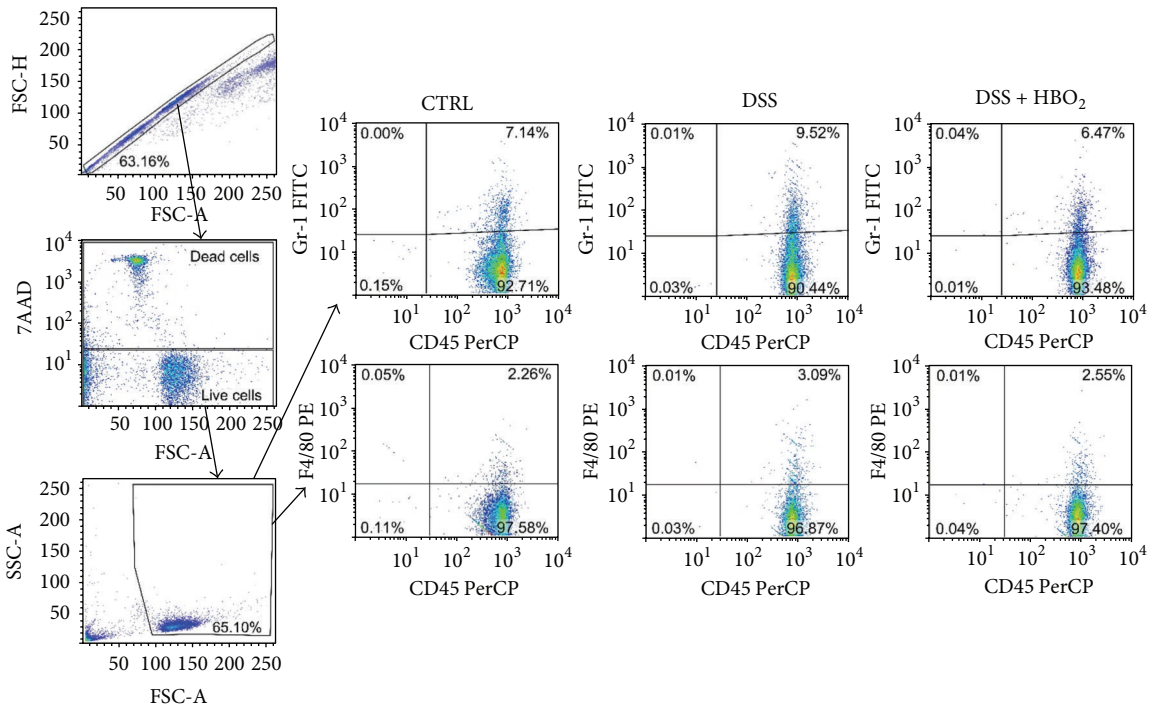
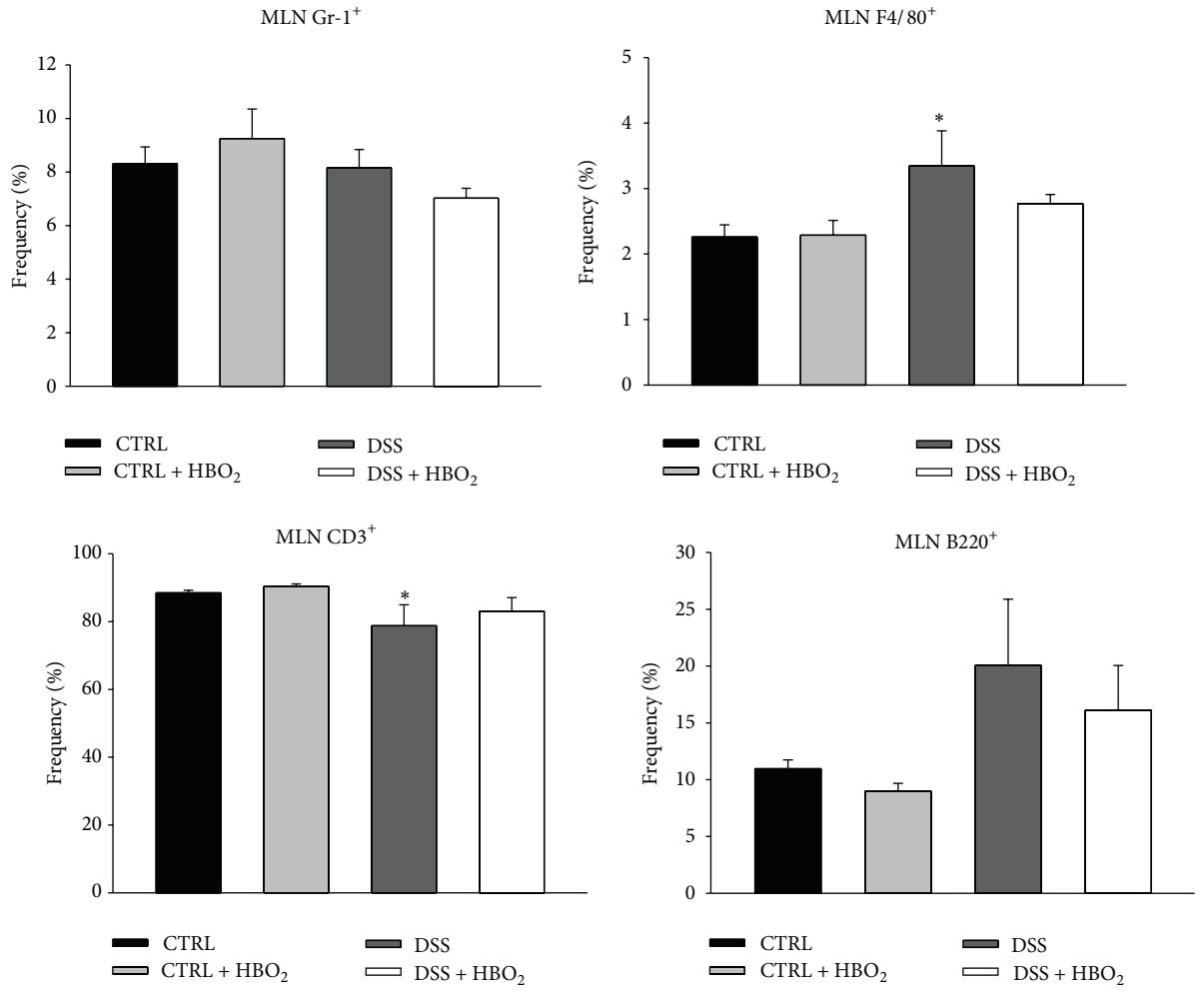


Figure 1: HBO_2 ameliorates the course of DSS-induced colitis in BALB/c mice. Male BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = 5 mice/group/experiment): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. (a) Disease activity index (DAI) assessed by daily scoring of the body weight change, stool consistency, and occult/gross rectal bleeding; (b) the stacked bars on the bar chart represent average score of each symptom in the total DAI score for particular experimental group, including weight changes, stool consistency, and rectal bleeding score; (c) colon length measured in cm; (d) histological samples of gut tissue were stained with haematoxylin and eosin and severity of colitis assessed using modified Geboes score; (e) representative histological samples of distal colon, CTRL and CTRL + HBO_2: normal intact colonic mucosa, DSS: the mucosa shows severe inflammation and ulceration extending into the deep portions of the mucosa with loss of crypts, and DSS + HBO_2: structural restoration with mild inflammatory infiltration and crypt distortion; magnification 200x. Presented data (mean ± s.e.m.) are representative results from one experiment with n = min. 5 mice/group. *Statistically different from CTRL $P < 0.05$ and $^{**}P < 0.01$; $^{\#}$statistically different from DSS $P < 0.05$ and $^{\#\#}P < 0.01$; †statistically different from CTRL + HBO_2.

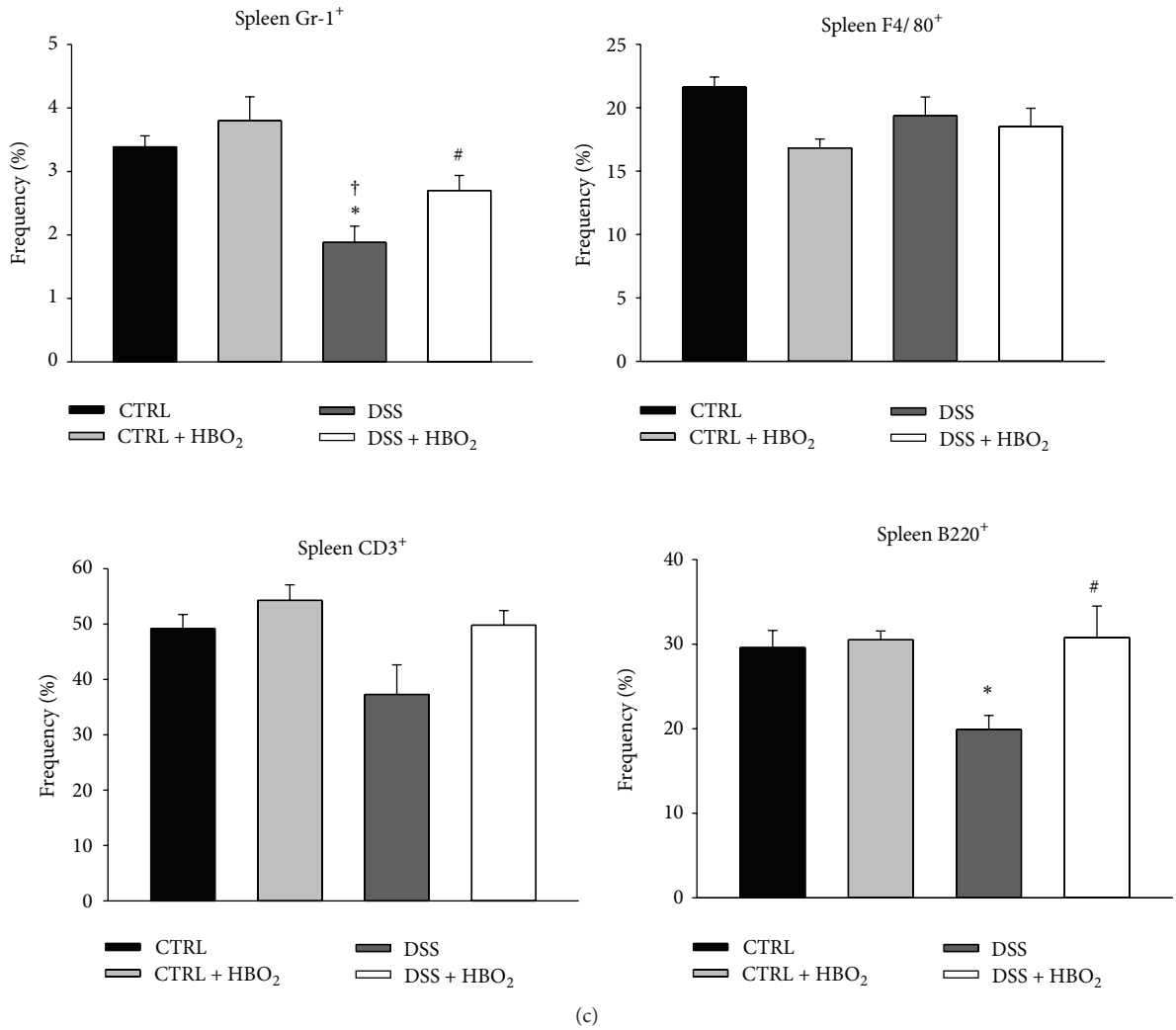


(a)



(b)

Figure 2: Continued.



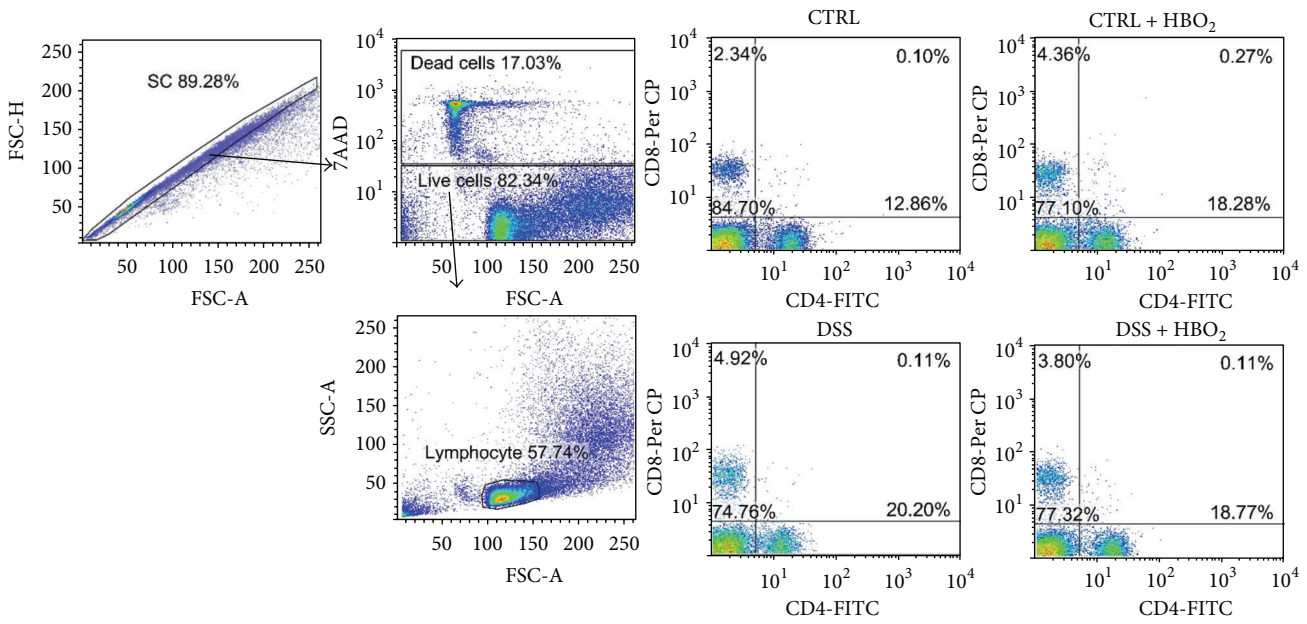
(c)

Figure 2: HBO_2 changes immune cell frequencies in the mesenteric lymph nodes (MLN) and the spleen of BALB/c mice with DSS induced colitis. BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = min. 5 mice/group/experiment): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (DSS, 5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. Representative dot plots of MLN obtained by flow cytometry, illustrating the gating strategy for $CD45^+$ $Gr\text{-}1^+$ and $CD45^+$ $F4/80^+$ cells in MLN of CTRL, DSS, and DSS + HBO_2 groups. Doublets were excluded by forward scatter area (FSC-A) versus forward scatter height (FSC-H) and the dead cells using 7-AAD. (a) Frequency of $CD45^+$ $Gr\text{-}1^+$cells, $CD45^+$ $F4/80^+$ cells, $CD3^+$ T cells, and $B220^+$ B cells in MLN (b) and spleen (c). Data are presented as mean ± s.e.m.% of single, live leukocytes (for $Gr\text{-}1^+$ and $F4/80^+$ cells) or single, live lymphocytes (for $CD3^+$ and $B220^+$ cells). *Statistically different from CTRL, $P < 0.05$; †statistically different from CTRL + HBO_2, $P < 0.05$; #statistically different from DSS, $P < 0.05$. 7-AAD, 7-aminoactinomycin D.

the DSS + HBO_2 mice compared to the CTRL group (P = 0.026; Figure 4(b)). In the colon, *GPx1* mRNA expression was increased in the DSS (P = 0.034) and the DSS + HBO_2 (P = 0.003) group compared to CTRL group. The upregulation was even greater in mice with DSS-induced colitis that underwent the HBO_2 treatment (DSS + HBO_2 group; Figure 4(b)). *SOD1* mRNA expression was significantly reduced in the colon of the DSS + HBO_2 group compared to CTRL (P = 0.008) and CTRL + HBO_2 (P = 0.007) groups. Similar changes in *SOD1* gene expression were also found in the MLN of the DSS + HBO_2 group (P = 0.025 compared to CTRL; Figure 4(b)). To summarize, colitis resulted in *GPx1* gene upregulation and *CAT* gene downregulation, while HBO_2 downregulated *SOD1* and further upregulated *GPx1* in a tissue-specific manner.

To examine the possible role of *HIF-1α* in transcriptional control of antioxidative genes in the colon, Spearman correlations were calculated. The results revealed a strong negative correlation between *HIF-1α* and *SOD1* (r = −0.651, P = 0.001) and a positive correlation of *HIF-1α* to the *GPx1* gene (r = 0.750, P < 0.001), while there was no significant correlation between the *HIF-1α* and the *CAT* gene in the colonic tissue (Figure 4(c)).

3.3. HBO_2 Treatment Reduces Expression of Proinflammatory Genes Upregulated during DSS-Induced Colitis. The early



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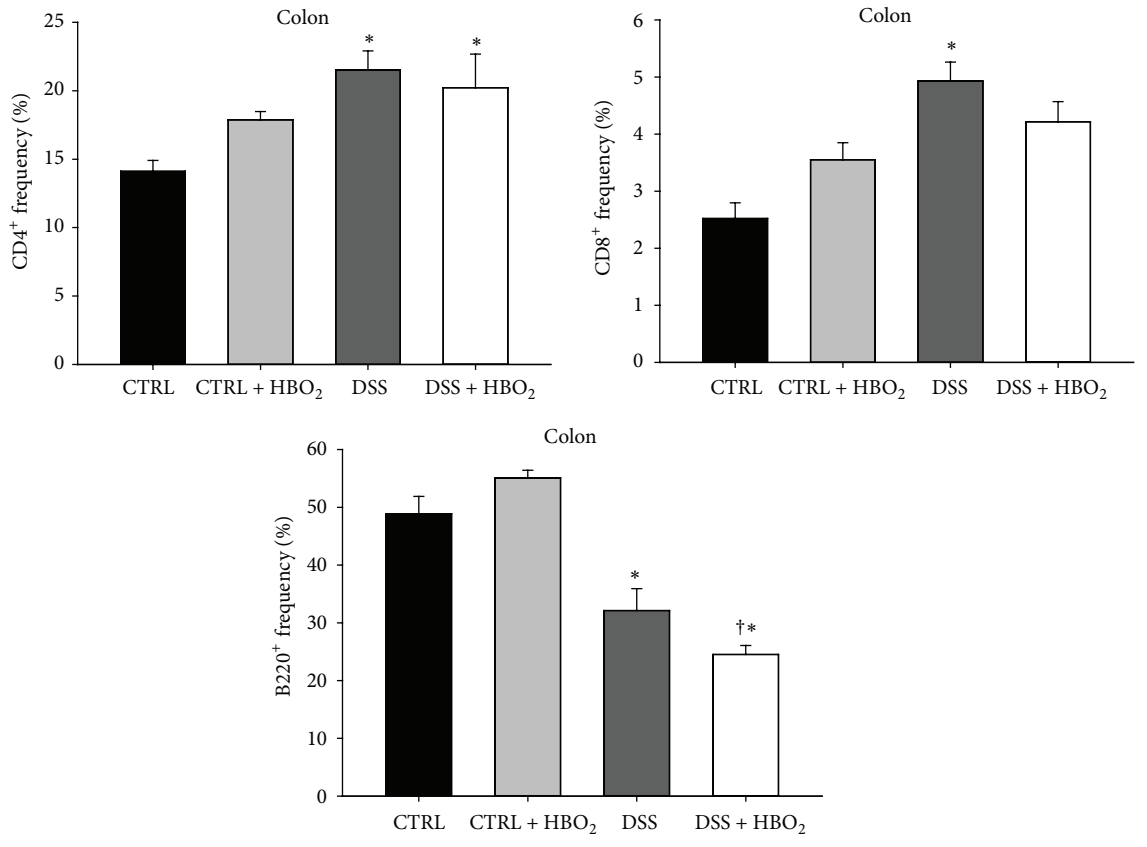
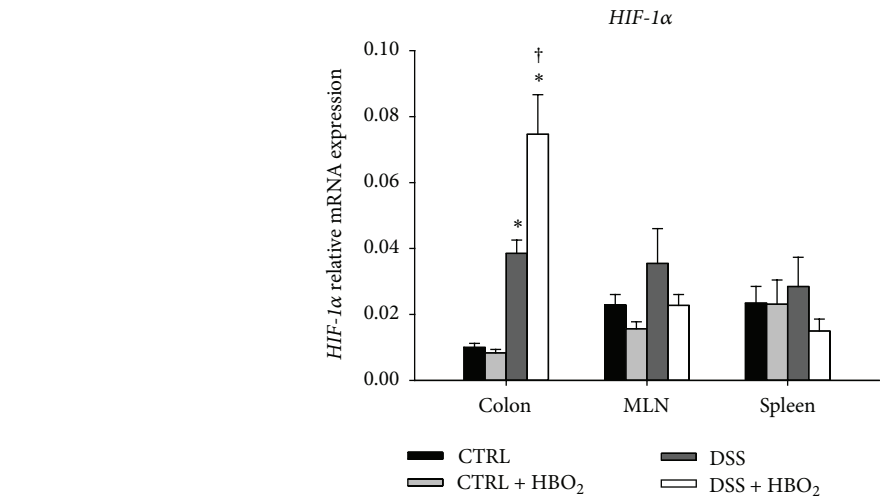


(b)

Figure 3: HBO_2 induced changes in $CD4^+$ and $CD8^+$ T cell frequencies in the colon of BALB/c mice with DSS induced colitis. BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = min. 4 mice/group/experiment): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (DSS, 5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. (a) Representative dot plots of colon lamina propria lymphocyte obtained by flow cytometry, illustrating the gating strategy for $CD4^+$ and $CD8^+$ lymphocytes, and (b) measured frequencies of colon $CD4^+$ and $CD8^+$ lymphocytes. Doublets were excluded by forward scatter area (FSC-A) versus forward scatter height (FSC-H) and the dead cells using 7-AAD. Data are presented as mean ± s.e.m.% of single, live lymphocytes. *Statistically different from CTRL, $P < 0.05$; †statistically different from CTRL + HBO_2.



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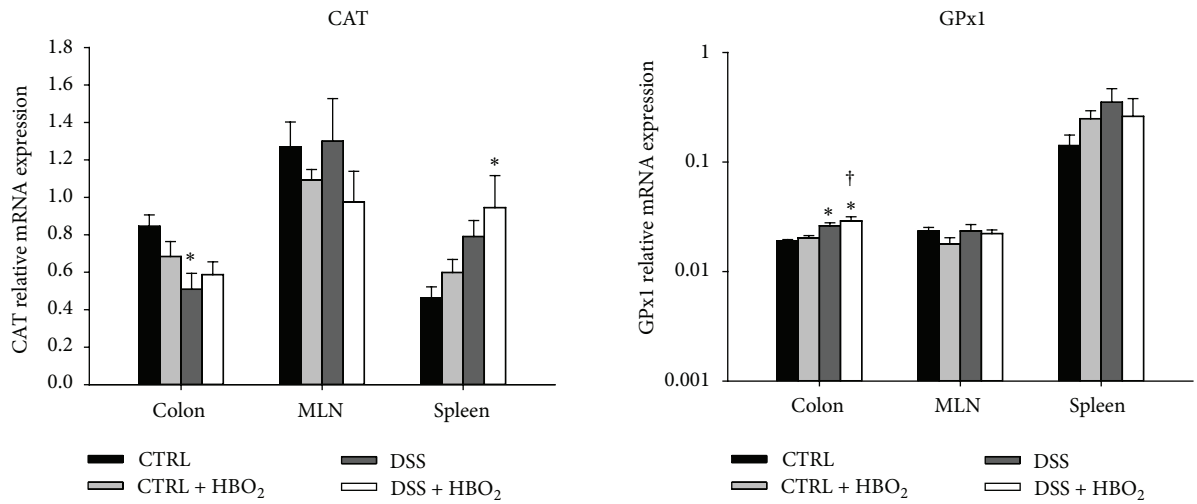

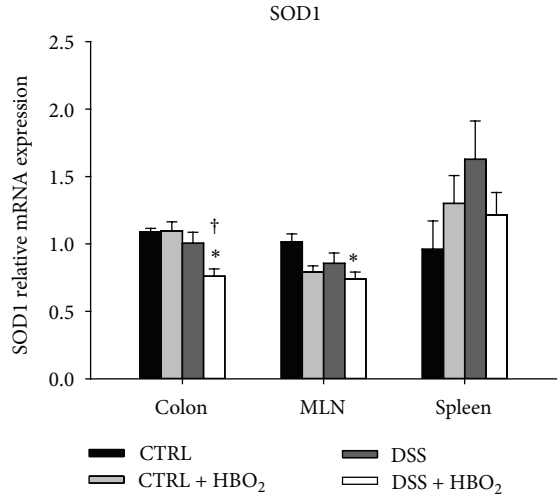

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Figure 4: Continued.

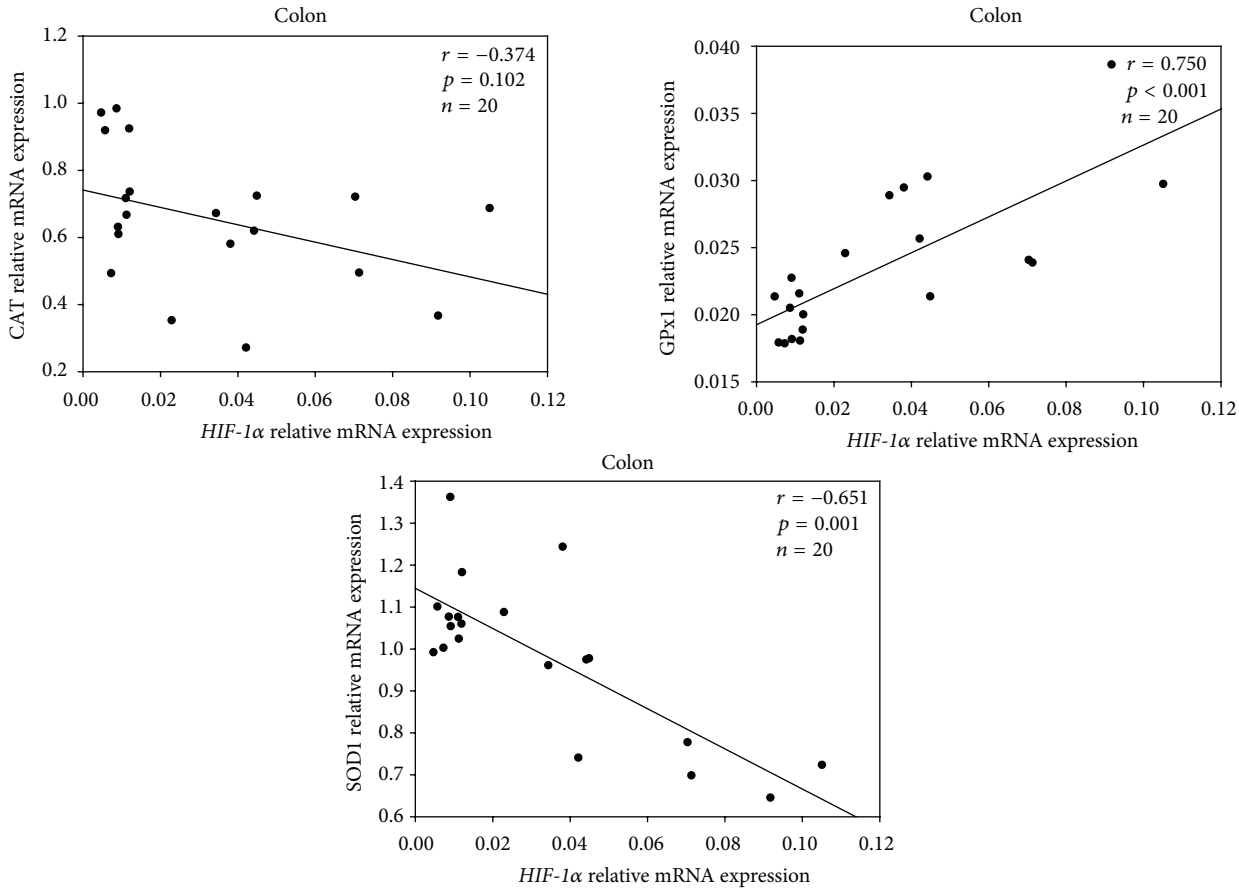

Figure 4: Relative mRNA expression of *HIF-1α*, *CAT*, *GPx1,* and *SOD1* in colon, MLN, and spleen (a, b) and their correlation to the *HIF-1α* gene expression in colonic tissue (c). Relative mRNA expression was measured by real-time PCR; all measured genes were normalized to the *HPRT1* gene expression. BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = min. 5/group/experiments): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (DSS, 5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. Data are presented as mean ± s.e.m. of two independent experiments, each with min. 5 mice/group. *Statistically different from CTRL, $P < 0.05$; †statistically different from CTRL + HBO_2, $P < 0.05$.

phase of inflammation is mediated by several proinflammatory mediators, which prompted us to assess how HBO_2 treatment affects their production. We found that gut mucosa inflammation was accompanied with a significant increase in *IL-1β* and *IL-6* gene expression. In the case of *IL-6* gene, this was significant for DSS and DSS + HBO_2 groups compared to the CTRL + HBO_2 group ($P = 0.024$ and $P = 0.021$, resp.; Figure 5(a)) in the colon. Furthermore, *IL-6* gene was significantly upregulated in the MLN of the DSS group ($P = 0.001$ compared to CTRL), and HBO_2 reduced its expression almost to control values in the DSS + HBO_2 group ($P = 0.016$ compared to DSS; Figure 5(a)). *IL-1β* mRNA expression in the colonic mucosa was significantly increased during inflammation in DSS group compared to CTRL ($P = 0.014$) and CTRL + HBO_2 groups ($P = 0.041$). *IL-1β* mRNA levels in the colon of the DSS + HBO_2 group did not significantly differ from the control groups, suggesting that HBO_2 treatment blocked the increase of *IL-1β* gene expression in the inflamed mucosa.

IL-2 gene was significantly upregulated in the MLN of the DSS group compared to the CTRL group ($P = 0.003$), and HBO_2 treatment resulted in its significant downregulation in the DSS + HBO_2 group ($P = 0.032$). Similarly, *IL-2* gene was significantly downregulated in the spleen of mice from the DSS + HBO_2 group compared to the CTRL group ($P = 0.025$). In addition, there was a strong positive correlation between *HIF-1α* and *IL-6* gene ($r = 0.749$, $P < 0.001$; Figure 5(b)), while there was no correlation between *HIF-1α* and *IL-1β* or *IL-2* genes in the colonic tissue (Figure 5(b)).

3.4. Colonic Inflammation and HBO_2 Treatment Induce Changes in the Activity of Antioxidative Enzymes. In addition to their mRNA expression, we also tested the enzymatic activity of antioxidative enzymes. We found that both the inflammation and the HBO_2 treatment *per se* were able to change the activity of antioxidative enzymes in the colonic mucosa and the peripheral lymphoid organs (MLN and spleen). In spleen HBO_2 treatment *per se* induced significant increase of SOD activity ($P = 0.040$ compared to CTRL). Mice with DSS-induced colitis presented with significantly increased activity of SOD ($P = 0.012$ compared to CTRL) in the colon and reduced CAT activity in MLN and spleen ($P = 0.023$ and

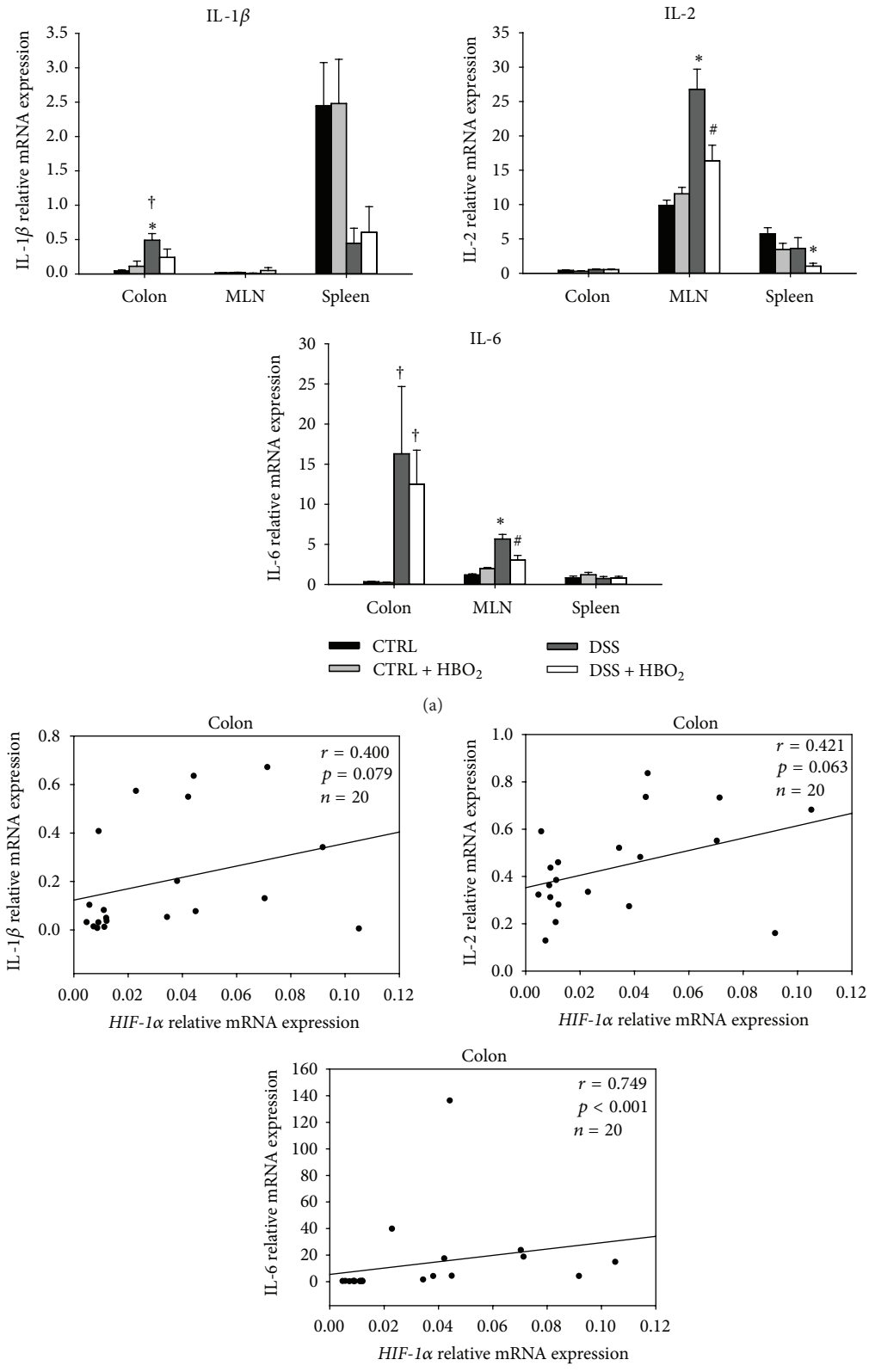

Figure 5: Relative mRNA expression of *IL-1β*, *IL-2,* and *IL-6* in the colon, MLN, and spleen (a) and their correlation to *HIF-1α* gene expression in colonic tissue (b). Relative mRNA expression was measured by real-time PCR; all measured genes were normalized to expression of *HPRT1* gene. BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = min. 5/group/experiment): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (DSS, 5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. Data are presented as mean ± s.e.m.; *statistically different from CTRL, $P < 0.05$; $^{\#}$statistically different from DSS, $P < 0.05$; †statistically different from CTRL + HBO_2, $P < 0.05$.

$P = 0.032$, resp.) compared to CTRL group. HBO_2 treatment did not change SOD activity in the inflamed colonic mucosa, which was comparable to the levels found in DSS and CTRL + HBO_2 groups (significantly increased compared to CTRL, $P = 0.010$). On the other hand, HBO_2 treatment significantly increased CAT ($P = 0.020$) and GPx ($P = 0.001$) activities in the spleens of the DSS + HBO_2.

3.5. Lymphocyte H_2O_2 and $ONOO^-$ Production Remains Unchanged during DSS-Induced Colitis and HBO_2 Treatment. Immune cells at the site of inflammation and in the peripheral lymphoid organs are an important source of ROS [3]. Therefore we assessed the basal levels of intracellular H_2O_2 and $ONOO^-$ and their production upon PMA-induced activation in the lymphocytes isolated from MLN and spleens of the mice from all experimental groups (Figure 6). Basal H_2O_2 and $ONOO^-$ production in the MLN was not significantly different among the groups, except for the lymphocytes from the DSS + HBO_2 group which presented with a significant increase of H_2O_2 and $ONOO^-$ levels compared to the CTRL group ($P = 0.033$). PMA stimulation resulted in increased intracellular H_2O_2 and $ONOO^-$ production, although statistically significant only for CTRL ($P = 0.031$) and DSS + HBO_2 ($P = 0.012$) groups.

In the spleen, HBO_2 increased lymphocyte H_2O_2 and $ONOO^-$ production in CTRL + HBO_2 and DSS + HBO_2 groups ($P = 0.004$ and $P = 0.007$ compared to the CTRL; and $P = 0.005$ and $P = 0.009$ compared to the DSS group). Their production after PMA-induced activation was decreased in all experimental groups except the CTRL group; however, this effect reached statistical significance only in the CTRL + HBO_2 group ($P = 0.018$ compared to unstimulated lymphocytes).

4. Discussion

In the present study, the experimental model of DSS-induced colitis in BALB/c mice was employed to explore the effects of HBO_2 on the antioxidative enzymes, transcription factor *HIF-1α,* and proinflammatory cytokine genes during colonic inflammation and their role in modulating the course of the disease via HBO_2 treatment. The most important findings are that (a) HBO_2 significantly reduces symptoms and severity of DSS-induced colitis, as evidenced by clinical appearance, contraction of the immune cell expansion and mobilization, and reversal of *IL-1β, IL-2,* and *IL-6* gene expression; (b) HBO_2 modulates the expression of antioxidative enzyme genes and enzyme activities during colitis; and (c) HBO_2 enhances *HIF-1α* mRNA expression in the inflamed colonic tissue which is in a strong correlation with *GPx1, SOD1,* and *IL-6* mRNA expression.

Several previous studies in animals and humans demonstrated the positive effects of HBO_2 treatment in influencing the severity of colitis and reducing gut mucosa inflammation [27, 34]; however, data on the precise underlying mechanisms are scarce. Considerably more data on the beneficial anti-inflammatory effects of HBO_2 are available for other conditions such as septic shock, ischemia/reperfusion injuries, and atherogenesis, where the previous studies reported reduced proinflammatory cytokine expression, suppressed development of Th cells, shrinking of spleen and lymph nodes, decreased responses to antigens, and reduced frequencies of circulating leukocytes [35–42]. Although this is the first animal study investigating the effects of HBO_2 performed on DSS-induced colitis in BALB/c mice and correlating it with the immune cell frequencies, our results are in line with previous findings on the changes associated with DSS-induced acute immune response, as well as on the effects of HBO_2 on the antioxidative enzyme activities determined in other animal models, such as TNBS and acetic acid induced colitis in rats [27, 43, 44]. During colitis mice presented with decreased T and B cell frequencies in the spleen and reduced T cell frequencies in the MLN, suggesting that lymphocytes are recruited from the peripheral lymphoid organs and probably migrate to the inflamed colonic mucosa. One element of the beneficial effect of HBO_2 may be linked to normalized T and B cells frequency in the MLN and spleen of mice with DSS-induced colitis after hyperbaric treatment (Figures 1 and 2). By measuring CD4 and CD8 lymphocyte in colon we confirm our hypothesis of T-cell recruitment from the peripheral lymphoid organs and their migration to the inflamed colonic mucosa, as well as immunomodulatory effect of HBO_2. In our model HBO_2 did not affect cell frequencies in the peripheral lymphoid organs of control mice, in contrast to previous findings where HBO_2 treatment *per se* was able to change lymphocyte subset populations in the spleen [45]. The observed differences may be due to different oxygen tension applied in our study.

For a long time macrophages and neutrophils have been considered as immune cells exclusively producing proinflammatory cytokines, chemokines, and large amounts of ROS/RNS contributing to aggravated inflammation. We have found decreased spleen $Gr\text{-}1^+$ cell frequencies during colitis and their normalization upon HBO_2 treatment (Figure 2). In addition, we showed increased MLN frequencies of $F4/80^+$ cells in DSS group, while HBO_2 treatment reversed their frequencies almost to control values. These findings indicate that HBO_2 can modulate distribution of phagocytes by retaining neutrophils in the spleen and instigating macrophage migration towards the site of inflammation, in agreement with previous findings describing inhibited neutrophil infiltration into the gut of mice with DSS-induced colitis [46]. Furthermore, HBO_2 treatment alone did not change the expression of proinflammatory cytokines in the colon, MLN, or spleen of the control mice; however, DSS-induced colitis resulted in a significant *IL-1β* and *IL-6* gene upregulation in the colonic tissue and *IL-2* gene upregulation in the MLN (Figure 5). Consistent with previous studies, HBO_2 treatment abolished these effects, further confirming its anti-inflammatory potential [47–49].

Several animal studies on the effects of HBO_2 on the experimental colitis reported an increased antioxidative capacity and changes in antioxidative enzyme activity [24, 25]. It has been proposed that an optimal HBO_2 treatment could generate ROS which would function primarily as intermediates in the antioxidative signalling pathways leading to increased expression of antioxidative enzymes, reduced inflammation, and ameliorated colitis symptoms but would

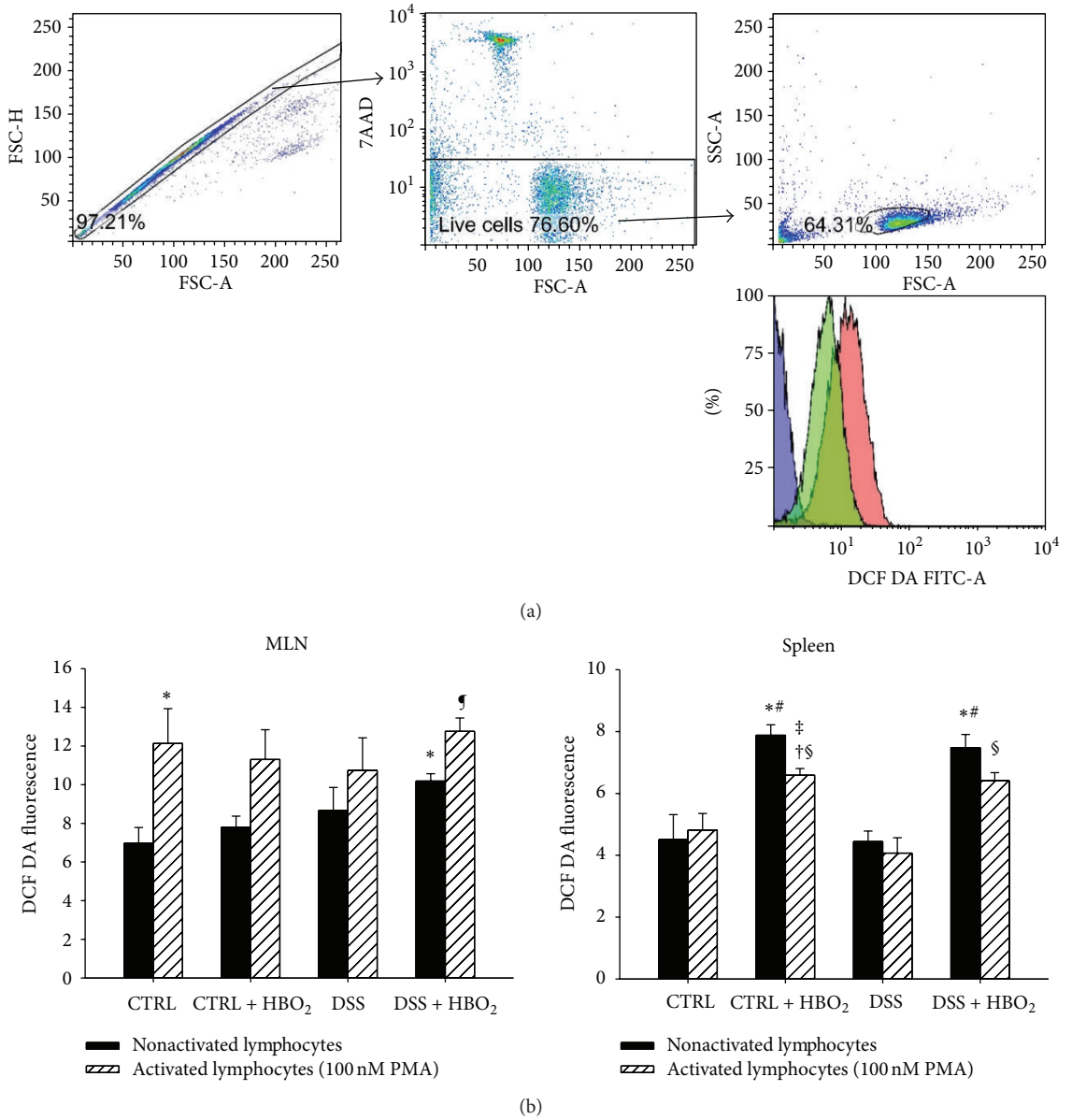

FIGURE 6: Lymphocyte H_2O_2 and $ONOO^-$ production during colitis and HBO_2 treatment. BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups (n = min. 4 mice/group/experiment): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving dextran sodium sulphate (DSS, 5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. To assess the basal intracellular H_2O_2 and $ONOO^-$ levels, lymphocytes isolated from MLN and spleens were stained with 10 μM DCF DA and analysed by flow cytometry (black bars, (b)). Next, lymphocytes were activated with 100 nM PMA for 30 min and the ROS production measurement repeated (lined white bars, (b)). (a) shows a representative lymphocyte gating strategy panel and a histogram of DCF-DA signal from the negative control (blue), resting lymphocytes (green), and the PMA activated lymphocytes (red). Doublets were excluded by forward scatter area (FSC-A) versus forward scatter height (FSC-H) and the dead cells by 7-AAD staining. Data are presented as mean ± s.e.m.; *statistically different compared to CTRL, $P < 0.05$; $^\#$statistically different compared to DSS group, $P < 0.05$; †statistically different from CTRL + HBO_2, $P < 0.05$; ‡statistically different compared to activated lymphocytes of CTRL group, $P < 0.05$; §statistically different from the activated lymphocytes of DSS group, $P < 0.05$. DCF DA: dichlorofluorescein diacetate; PMA: phorbol myristate acetate; 7-AAD: 7-aminoactinomycin D; ¶statistically different from nonactivated lymphocytes DSS + HBO_2.

not further damage the colonic tissue [50, 51]. Drenjancevic et al. showed that 24 hours after a two-hour HBO_2 treatment at 2 bars in rats oxidative stress is not elevated, as evidenced by assessing ferric reducing antioxidant power ability of plasma (FRAP) and thiobarbituric acid reactive substances (TBARS) level [12]. In addition, a recent study also suggests that ROS produced by NADPH oxidase complex are important mediators inducing anti-inflammatory response in autoimmune diseases [52]. Data on the *CAT* mRNA level during DSS-induced colitis and upon HBO_2 treatment were not available prior to this study. We found that *CAT* mRNA expression is tissue and treatment specific (Figure 4). Colitis resulted

Table 2: Catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activity in the colon, MLN, and spleen during colitis and HBO_2 treatment.

Enzymatic activity (U mg^{-1} P)	Tissue	CTRL	CTRL + HBO_2	DSS	DSS + HBO_2
CAT	Colon	6.77 ± 3.04	5.39 ± 1.07	4.11 ± 0.33	4.23 ± 0.20
	MLN	13.90 ± 0.39	11.78 ± 1.71	**9.06 ± 0.22***	12.62 ± 0.42
	Spleen	10.44 ± 1.94	14.00 ± 0.97	**6.75 ± 0.53***	**14.28 ± 1.11**$^{\#}$
GPx	Colon	0.084 ± 0.028	0.097 ± 0.012	0.080 ± 0.008	0.072 ± 0.003
	MLN	0.164 ± 0.006	0.158 ± 0.021	0.175 ± 0.004	0.172 ± 0.007
	Spleen	0.098 ± 0.005	0.109 ± 0.003	0.114 ± 0.007	**0.134 ± 0.004***†
SOD	Colon	15.40 ± 3.29	**27.86 ± 3.55***	**30.56 ± 2.70***	**30.97 ± 1.58***
	MLN	36.53 ± 2.80	33.96 ± 3.33	34.48 ± 1.62	35.30 ± 1.75
	Spleen	19.87 ± 1.15	17.91 ± 0.72	20.93 ± 1.03	**25.34 ± 1.61***†

BALB/c mice at the age of 10–12 weeks were randomly assigned into 4 groups ($n = 5$/group): CTRL: control mice, CTRL + HBO_2: control mice undergoing HBO_2 (60 min/2.4 ATM, 2x/day, days 1–8), DSS: mice receiving DSS (5% w/v, days 1–7), and DSS + HBO_2: DSS treated mice undergoing HBO_2. Presented data (mean ± s.e.m.) are representative results from one experiment with min. five mice/group; $P < 0.05$ was considered significant; *statistically different from CTRL, $P < 0.05$; $^{\#}$statistically different from DSS, $P < 0.05$; †statistically different from CTRL + HBO_2, $P < 0.05$.

in a significant downregulation of *CAT* mRNA expression in the colonic mucosa, and the HBO_2 treatment induced its upregulation in the spleen of DSS + HBO_2 group of mice. These results were largely in accordance with our finding on enzymatic catalase activity that was decreased in all measured tissues in the DSS group and reversed to control values in the spleen of DSS + HBO_2 group (Table 2), as well as with a previous study demonstrating decreased catalase activity in colonic tissue upon DSS treatment [53]. This is also in line with a study on skin transplanted BALB/c mice where HBO_2 treatment increased catalase, GPx, and SOD activity in the spleen [54]. Furthermore, upregulation of protein and mRNA catalase levels 14 days after HBO_2 treatment, but not after 7 days, was also observed in the ulcer tissue of patients with diabetic foot, indicating a time-course for the effect of HBO_2 to prevail [55].

We found that *GPx1* mRNA level was upregulated in the colon of DSS treated mice, irrespective of the HBO_2 treatment, and there were no significant differences in the *GPx1* mRNA expression in MLN and spleen. In contrast to our findings on mRNA expression, colon GPx enzyme activity was slightly reduced in DSS + HBO_2 group compared to other groups, which is consistent with previous results obtained in acetic acid induced colitis in rats receiving combined HBO_2 and ozone treatment [56]. However, other reports indicate decreased GPx and SOD activity in the inflamed distal colon mucosa and the plasma of rats with acetic acid induced colitis, and HBO_2 normalized GPx but not SOD activity in the colon [24]. The observed discrepancies in the results may be related to the differences in experimental models used among the studies. In addition, in our study HBO_2 treatment induced enhanced GPx and SOD activity in the spleen of DSS mice which is in contrast to reduced *SOD1* mRNA expression and might be explained by additional SOD2 and SOD3 function in regulation of antioxidative capacity. Although intracellular H_2O_2 and $ONOO^{-}$ levels were slightly increased during inflammation and HBO_2 treatment in the MLN and HBO_2 *per se* increased its level in spleen, impaired lymphocyte function was not observed.

Intensive research on the beneficial wound healing effects of HBO_2 revealed its capacity to induce neovascularization, reduce oedema, decrease leukocyte adhesion, stimulate fibroblast expansion, and inhibit bacterial growth [14, 57]. Some of these processes are transcriptionally regulated by *HIF-1α*, namely, the vascular endothelial growth factor (VEGF) expression [58], regulatory T lymphocyte differentiation [59], and preservation of epithelial thigh junction integrity [60]. In addition, previous studies employing conditional deletion of epithelial *HIF-1α* or pharmacologic activation of *HIF-1α* in a murine model of colitis demonstrated a protective role for *HIF-1α* in colitis [61]. It has also been shown that *HIF-1* increases expression of barrier-protective genes (multidrug resistance gene-1, intestinal trefoil factor, *CD73*) [62], decreases *TNFα* mRNA expression [61], and enhances antimicrobial activity by transcribing beta-defensin 1 [63]. In the present study we found increased expression of *HIF-1α* gene in inflamed colonic tissue, and HBO_2 further increased its level. In the peripheral lymphoid organs *HIF-1α* gene expression was changed (upregulated) by the inflammation while HBO_2 treatment showed a tendency to reverse this increase. These data suggest involvement of different mechanisms controlling *HIF-1α* gene expression at the site of inflammation (colon) and the peripheral lymphoid organs (MLN and spleen), responsible for the initiation of the immune response and the T/B-cell expansion and differentiation, respectively. We also demonstrated a strong positive correlation between *HIF-1α* and *GPx1* mRNA levels in the colon (Figure 4(b)). This is in line with *in vitro* studies where overexpressed *HIF-1α* in colorectal cancer cells resulted in enhanced *GPx1* expression through TGF-*β*RI/Smad2/ERK1/2/*HIF-1α* signalling cascade, suggesting transcriptional regulation of *GPx1* by *HIF-1α* [64].

In the present study we found strong negative correlation between *HIF-1α* and *SOD1* mRNA expression. This is in accordance with a previous study showing that docosahexaenoic acid downregulates *SOD1* gene transcription through an HRE-mediated mechanism (HRE, hypoxia-response element), involving HIF signalling in human cancer cells [65];

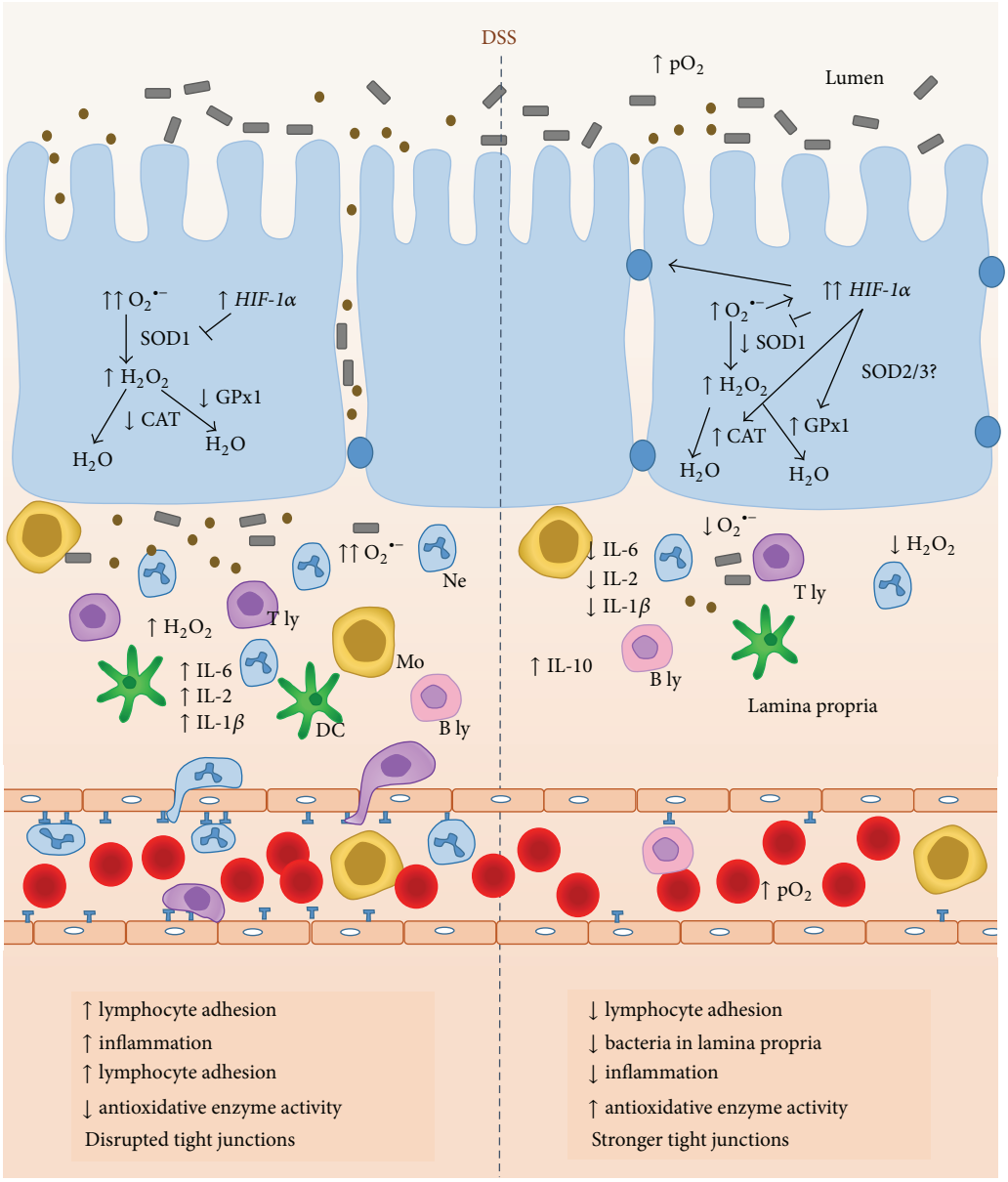

Figure 7: Proposed mechanisms mediating effects of HBO_2 on the inflammation during DSS-induced colitis. Compared to untreated DSS-induced colitis (left of the vertical dotted line), high partial pressure of oxygen during HBO_2 (right) activates *HIF-1α* signaling which leads to higher *CAT* and *GPx1* mRNA expression resulting in reduced oxidative stress in the inflamed colonic mucosa. *HIF-1α* mediated (negative) transcriptional regulation of proinflammatory cytokine IL-6 and reduced oxidative tissue injury leads to decreased neutrophil, monocyte, and lymphocyte adhesion and infiltration to the gut mucosa. In addition, HBO_2 activates genes encoding for barrier-protection and genes responsible for improving tight junction integrity, all together resulting in reduced number of bacteria entering the lamina propria of gut mucosa. T ly, T lymphocyte; B ly, B lymphocyte; DC, dendritic cell; Ne, neutrophil; Mo, monocyte/macrophage; *HIF-1α*, hypoxia inducible factor 1 alpha; CAT, catalase; GPx1, glutathione peroxidase 1; SOD1, superoxide dismutase 1; pO_2, partial O_2 pressure; ↑, increased; ↓, decreased.

thus our results indicate similar mechanism involved in SOD1 control in the murine colon mucosa *in vivo* during colitis and HBO_2.

Previous studies revealed that *HIF-1α* mediated transcriptional regulation of different proinflammatory cytokines and growth factor genes are tissue and cell specific and include regulation trough alternative splicing, mRNA stability, and interactions with other transcription factors like NF-κB [66–69]. In our study we found a strong correlation between *HIF-1α* and *IL-6* mRNA levels suggesting involvement of *HIF-1α* in transcriptional regulation of *IL-6* gene during colonic inflammation and HBO_2.

In conclusion, our results confirmed that HBO_2 exerts an anti-inflammatory effect on DSS-induced colitis in mice, and this effect at least involves *HIF-1α* and antioxidative genes expression regulation (as outlined in Figure 7). However,

further studies are necessary to identify the cells that may contribute to or are influenced by the effects upon HBO_2 treatment.

Abbreviations

CAT: Catalase
CTRL: Control
DAI: Disease activity index
DCF DA: Dichlorofluorescein acetate
DSS: Dextran sodium sulfate
GPx1: Glutathione peroxidase 1
HBO_2: Hyperbaric oxygen
HRE: Hypoxia-response element
HIF-1α: Hypoxia inducible factor-1 alpha
IBD: Inflammatory bowel disease
MFI: Median fluorescence intensity
MLN: Mesenteric lymph node
$O_2^{\bullet-}$: Superoxide radical
$ONOO^-$: Peroxynitrite
PMA: Phorbol 12-myristate 13-acetate
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
s.e.m.: Standard error mean
SOD1: Superoxide dismutase 1
TCR: T cell receptor
Treg: T regulatory lymphocyte
VEGF: Vascular endothelial growth factor.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Martina Mihalj and Sanja Novak conceived and designed the study. Sanja Novak, Martina Mihalj, Rosemary Vukovic, Zoltán Kellermayer, and Anita Cosic performed the experiments. Maja Tolusic Levak performed pathohistological evaluation and analysis and wrote the paper. Rosemary Vukovic, Sanja Novak, Martina Mihalj, Péter Balogh, and Ines Drenjancevic contributed to data analysis and interpretation. Sanja Novak wrote the first draft of the paper. Martina Mihalj, Péter Balogh, and Zoltán Kellermayer critically revised the paper, and all authors read and approved its final version.

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Review Article

Inflammatory Mechanisms Associated with Skeletal Muscle Sequelae after Stroke: Role of Physical Exercise

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Inflammatory markers are increased systematically and locally (e.g., skeletal muscle) in stroke patients. Besides being associated with cardiovascular risk factors, proinflammatory cytokines seem to play a key role in muscle atrophy by regulating the pathways involved in this condition. As such, they may cause severe decrease in muscle strength and power, as well as impairment in cardiorespiratory fitness. On the other hand, physical exercise (PE) has been widely suggested as a powerful tool for treating stroke patients, since PE is able to regenerate, even if partially, physical and cognitive functions. However, the mechanisms underlying the beneficial effects of physical exercise in poststroke patients remain poorly understood. Thus, in this study we analyze the candidate mechanisms associated with muscle atrophy in stroke patients, as well as the modulatory effect of inflammation in this condition. Later, we suggest the two strongest anti-inflammatory candidate mechanisms, myokines and the cholinergic anti-inflammatory pathway, which may be activated by physical exercise and may contribute to a decrease in proinflammatory markers of poststroke patients.

1. Introduction

Chronic stroke is the second leading cause of death and the third cause of disability worldwide. Moreover, the overall incidence of stroke is increasing exponentially. From 1990 to 2010, the number of deaths and disabilities related with stroke rose by 26% and 19%, respectively, regardless of the age group [1, 2]. Indeed, every year approximately 795,000 people experience a new or recurrent chronic stroke, and every four minutes someone dies from stroke in the United States [3].

Furthermore, it has been shown that stroke prevalence is greater in low-income countries when compared to developed countries. A systematic review comprising 56 epidemiological studies carried out between 1970 and 2008 showed that in ten countries of low and middle income the incidence and premature mortality due to chronic stroke more than doubled, reaching 5.6% increase annually [4].

Muscle atrophy in the paretic and nonparetic limbs is a phenotypic change caused by different factors (e.g., hemiparesis and immobilization) observed in poststroke patients [5]. Since this atrophy is associated with decrease in muscle strength and power, cardiovascular fitness, and mobility, some researchers have suggested that poststroke patients show stroke-related sarcopenia, similar to the muscle weakness found in elderly people [6, 7]. Regardless of the molecular pathway responsible for eliciting this phenomenon, several studies have indicated that increased inflammatory markers may be a trigger factor for this condition [8, 9]. In fact, some findings from research have pointed that proinflammatory cytokines (e.g., TNF-α and IL-6) may activate

the molecular pathways involved muscle atrophy (e.g., ubiquitin proteasome system).

On the other hand, physical exercise has been widely suggested as a beneficial tool for rehabilitation of stroke patients, since it may be able to counterregulate most of the stroke sequelae on the organic system [10]. Indeed, several reviews have suggested that physical exercise may elicit improvement on cognition, upper and lower limb motor function, cardiovascular performance, cardiovascular risk factors (e.g., triglycerides and insulin resistance), fatigue resistance, balance, gait, and mobility [10–12]. Also, physical exercise has been effectively used as an anti-inflammatory therapy in chronic diseases [13–16].

Unfortunately, data about the mechanisms underlying the beneficial effects of physical exercise on poststroke patients are limited. However, several experiments have already demonstrated that physical exercise may decrease the inflammatory state through myokines and the cholinergic anti-inflammatory pathway; as such, we may hypothesize that both mechanisms may also be present in stroke patients. In this sense, the present study attempted to demonstrate the potential role of inflammation on muscle atrophy, regardless of specific molecular pathways in poststroke patients. We also assessed the compensatory role of myokines and the cholinergic anti-inflammatory pathway in counterbalancing this phenomenon and consequently improving prognosis.

2. Functional and Skeletal Muscle Complications Associated with Stroke

Stroke complications are numerous and variable depending on the site of impairment and the degree of obstruction of the blood vessels involved. Generally, stroke complications may be characterized as morphological (e.g., decrease in muscle mass and increase in muscle mass fat infiltration), physical (e.g., hemiparesis, spasticity, rigidity, balance and coordination changes, tremors, deficit in gross and fine motor skills, and sensory changes), psychoaffective (e.g., depressive disorders, anxiety, and aggressiveness), and cognitive (memory, attention, and concentration issues, language disorders and executive functions, difficulty in action planning, and perceptual deficit) [6, 7, 17].

Regarding physical alterations, hemiparesis or muscle weakness contralateral to brain lesion, which is characterized by weakness/palsy in one of the sagittal body sides and central nervous system injury, is the most frequent disability found in poststroke patients, affecting around 50% of the patients [6, 18–21].

Hemiparesis is closely associated with muscular abnormalities, which, in turn, cause impairment in muscle functionality [6, 18]. Indeed, studies have found poststroke atrophy in the contralateral limb, whereas results on the ipsilateral limb have demonstrating maintaining or even trend to increase in the muscle mass [20, 22, 23]. Moreover, evidences demonstrate nondifferences between the muscle volume of the quadriceps from the ipsilateral limb of poststroke patients in comparison with the limb of control group matched by age and sex [23]. In the following 3 months after a stroke, patients show small differences in muscle mass content between the contralateral and ipsilateral limb with women and men displaying 1.88% and 3.74% lower muscle mass in the paretic limb and nonparetic limb, respectively [22]. However, with the progression of the condition, differences between sagittal sides may amount to 24% in 6 months [20, 23].

In line with these findings, a systematic review, which evaluated 14 studies, undertaken in three different continents (i.e., America, Europe, and Asia) and involved 450 poststroke patients (i.e., 53.5–75 years), has shown that the contralateral limb displayed 4.5% atrophy when compared to the ipsilateral limb, with a high magnitude of atrophy observed in the midthigh (14.5%) [5].

Decrease in muscle mass due hospitalization is associated with poor prognosis, including length of stay in intensive care unit in different population [24, 25]. In poststroke patients, decrease in muscle mass few days after the phenomenon is associated with impairment in the capacity to walk again [26]. Furthermore, evidence suggests that this association is dependent on the affected limb, as demonstrated by Prado-Medeiros et al. [23], who found a moderate but significantly negative association between paretic limb atrophy (but not in ipsilateral limb) and the capacity to generate strength and power of the muscle knee flexors and extensors [23].

The paretic limb shows lower isometric and isotonic strength than ipsilateral limb [20, 27]; in turn, both show lower strength than nonaffected healthy control limbs [27]. These physical characteristics involved in the capacity to generate strength and power in poststroke patients deserve attention, since low strength is positively associated with low scores in tests that mimic the activities of daily life (ADL), such as six-minute walk test (6MWT), stair climbing (i.e., normal and fast pace), gait speed (i.e., normal and fast pace), moving from a sitting to a standing position, flexing the affected knee, and scales, which evaluate muscle function (i.e., Rivermead motor assessment), in poststroke patients [23, 28, 29]. Moreover, patients who suffered stroke seem to spend more energy to perform these daily tasks [29].

Due these stroke-induced alterations on muscle mass, physical function, and functionality, some studies have suggested that the term sarcopenia should be employed to describe this phenotype found in poststroke patients [6, 7]. According to the these investigations, this aspect is clinically important since sarcopenia has been found to be a trigger factor to development of syndromes associated with low resilience in elderly people, such as frailty syndrome [30]. However, muscle and functional alterations in poststroke patients seems to occur in different times and magnitudes, unlike the changes observed in elderly people [6, 7, 18, 31].

Muscle fiber shifting helps explain these differences. In older people, predominantly, histological analyses show type II fast-twitch shift toward to type I low-twitch muscle fibers [32, 33]. On the other hand, poststroke patients undergo an inverse phenomenon, whereby type I low-twitch muscle fibers shift forward to type II fast-twitch muscle fibers [6, 18]. These histological changes are inversely associated with gait speed and are likely to be caused by altered neural activation patterns [34]. However, consensus about sarcopenia diagnosis in poststroke patients has not yet been reached, as well as

the role it plays in the physical function in this population [6, 7, 19].

3. Possible Mechanisms Associated with Muscle Atrophy in Poststroke Patients

Some mechanisms have been suggested to explain muscle atrophy in poststroke patients [6, 7]; however, little is known about them [6, 7]. Muscle atrophy is caused by an imbalance between protein synthesis and breakdown and can be elicited by two phenomena: (a) when the rate of protein breakdown (i.e., proteolysis) exceeds the rate of protein synthesis and (b) when the rate of protein synthesis decreases [35, 36].

The insulin-like growth factor 1 (IGF-1)/Akt/mammalian target of rapamycin (mTOR) pathway (i.e., IGF-1/Akt/mTOR) has been known to be the most important pathway to muscle protein synthesis [36].

IGF-1 is a circulating growth factor synthesized in different tissues, including the skeletal muscle [37, 38]. IGF-1 may act either as a hormone, due to its systemic characteristic, or in an autocrine fashion, as a local growth factor [38]. Activity of IGF-1 on skeletal muscle is a strong stimulus to muscle hypertrophy [38, 39]. Indeed, a transgenic mice model with overexpressing of IGF-1 gene has shown higher muscle mass in fore and hind limbs, due to increase in the muscle cross-sectional area of type II fibers, during adult life and older age, in comparison with the wild type [38].

The activity of IGF-1 is mediated by its binding to tyrosine kinase IGF-1 receptor in the lipid bilayer [36, 40, 41]. IGF-1 recruits insulin receptor substrate (IRS-1), which is also activated by insulin and, consequently, leads to the phosphorylation and activation of phosphatidylinositol 3-kinase (PI3K) [36, 40]. PI3K phosphorylates phosphatidylinositol (4, 5) bisphosphate, synthetizing phosphatidylinositol (3, 4, 5) [36, 39–41]. This process allows the creation of a lipid-binding site to Akt [36, 39–41]. Downstream targets of Akt are the mammalian target of rapamycin (mTOR), which, in turn, phosphorylates and activates p70s6k, also called S6K1, through the essential regulatory subunit eIF3f, ribosomal proteins, which eventually leads to increased ribosomal biogenesis and protein translation [41–44]. Still regarding eIF3f, this regulatory subunit acts as a scaffold, allowing the activation of mTOR downstream targets (i.e., $p70^{S6K}$) [42]. This phenomenon occurs from the binding of mTOR on C-terminal of the eIF3f subunit, phosphorylating and activating $p70^{S6K}$, which is found anchored in the mov34 domain [42]. Evidences indicate that increase of MAFbx in response to food deprivation causes eIF3f loss, leading to marked decrease on phosphorylation of $p70^{S6K}$, without alterations in mTOR activity, indicating the association of mTOR downstream targets [42]. On the other hand, overexpression of eIF3 subunit elicits significantly an increase in $P70^{S6K}$ [42]. It is important to mention that 4E-BP1, a dual activity molecule, can be a key factor in muscle mass regulation, since its molecule can activate eIF3f, after be phosphorylate by mTOR, which leads to its dissociation of eIF3f [45]. However, eIF3f inactivation can occur if 4E-BP1 is dephosphorylated [45].

Moreover, results from experiments *in vitro* and *in vivo* have demonstrated that IGF-1/PI3K/Akt/mTOR pathway may revert muscle proteolysis and muscle myofibrillar breakdown (i.e., actinin), by inhibiting E3 ligases (i.e., MAFbx and MuRF-1), FoxO1—which also activates MAFbx and MuRF-1—, and decrease in the activity of the ubiquitin proteasome system [36, 37, 39, 46].

In an animal model of stroke, atrophied paretic and nonparetic muscles show a downregulation of the IGF-1/PI3K/Akt/mTOR pathway [47]. In poststroke patients, few studies have focused on the association between IGF-1 and muscle atrophy [48]. In the studied performed by Silva-Couto et al. [48], chronic stroke patients with hemiparesis showed lower serum concentrations of IGF-1 and IGFB-3, an IGF-1 transporter, than healthy controls. Concomitantly, muscle atrophy was found in poststroke patients [48]. Other studies have shown a positive correlation between muscle function and IGF-1 serum concentrations, which may indicate a modulation by muscle mass [49]. However, this variable was not measured. Therefore, inferences about the IGF-1 pathway on muscle atrophy in poststroke patients are still sensitive and we must be proceeding with caution.

There is more considerable evidence in the literature for muscle atrophy elicited by the increased rate of protein breakdown than by protein synthesis.

As aforementioned, muscle atrophy is one of the components of the poststroke phenotype [6, 7, 18]. Besides the contribution of hemiparesis to this phenomenon, bed rest time, which occurs from one to three days after the event, immobilization, and decrease in physical activity levels negatively affect muscular homeostasis in poststroke patients, leading to muscle atrophy due to denervation, unloading, and disuse [7, 26, 50].

Although disuse atrophy is probably not regulated by a single mechanism, but a complex one [36], the ATP-dependent ubiquitin proteasome system (UPS) has been widely suggested as the main mechanism responsible for muscle atrophy, since UPS genes were found to be increased in muscular atrophy due to different factors (i.e., denervation, unloading, and disuse) [36, 37, 40, 43]. Ubiquitin is a small peptide that targets the protein, leading to ubiquitin-dependent protein catabolism and forming the core of a much larger protease, the 26S proteasome [40, 43]. The process of ubiquitination, protein labeling and targeting, is dependent on three ubiquitin-enzymes: E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligating enzyme [36, 40]. These enzymes act in a chain reaction fashion creating a polyubiquitination chain, where E1 activates, through an ATP-dependent pathway, and transfers the ubiquitin to E2, which is later replaced by E3 [36, 37, 40, 51], which, in turn, catalyzes the conjugation of the ubiquitin with the target protein [40, 51].

In the skeletal muscle, two muscle-specific E3 enzymes have been studied and were found to be associated with the muscle atrophy phenotype [36, 37, 40]. Muscle ringer finger 1 (MuRF1) and muscle atrophy F-box (MAFbx), also called Atrogin-1, are two E3 ligases widely expressed on muscle mass during muscular atrophy and activated by FoxO family of transcription factors, which remained inactivated by Akt

phosphorylation [9, 36, 37, 40]. Besides the activity of MuRF1 and MAFbx on muscle catabolism as an E3 ligase, these genes are associated with the inhibition of the components of muscular anabolism, such as a myogenic regulatory factor and eIF3-f [42, 51].

Both genes seem to be increased in response to different models of muscle atrophy (i.e., denervation and disuse) [43]. In fact, in healthy subjects, for example, MAFbx and MuRF1 expression (i.e., mRNA) has been found to remain increased after the first 10 days of disuse atrophy in lower limb biopsies [52, 53]. Data from experiments in animals deficient in MAFbx and MuRF-1 ($MAFbx^{-/-}$ and $MuRF\text{-}1^{-/-}$) genes corroborate evidence about the role of both factors in muscle atrophy, since $MAFbx^{-/-}$ and $MuRF\text{-}1^{-/-}$ animals show lower magnitude of loss in muscle mass and fibers during atrophy when compared to the wild type [43].

Although the results in healthy subjects demonstrate a strong association between UPS, particularly MAFbx and MuRF-1 activity, and muscle atrophy, evidence about post-stroke patients are limited, and only data from animal studies are available [47, 54].

In the experiment developed by Desgeorges et al. [47], mice undergoing transient focal cerebral ischemia have shown quadriceps, soleus, and tibialis anterior atrophy in the paretic side. Concomitantly, the expression of MuRF-1 and MAFbx did increase in the paretic muscle. Data from Springer et al. [54] corroborate these findings and show increased proteasome activity in the muscle under atrophy after transient focal cerebral ischemia [54].

Together with UPS, autophagy represents the two major proteolytic systems in mammalian cells [55, 56]. Autophagy may be characterized as a homeostatic process which controls the degradation of damaged organelles, toxic proteins, and intracellular pathogens [55, 56]. The extreme activity of the autophagic complex is harmful to muscle mass, since up- and downregulation of autophagic genes leads to muscle wasting [55]. Regarding downregulation, $atg7^{-/-}$ mice, which present autophagy inhibition, show degenerative changes in muscle mass, lower myofiber size (~40%), and muscle strength when compared to the controls, together with increased activity of atrogenes (i.e., MuRF-1 and MAFbx), which can indicate a relation between both factors [55, 56]. Furthermore, during catabolic condition (i.e., fasting and denervation), inhibition of autophagy increases the magnitude of muscle loss [55].

In poststroke patients, autophagy has been associated with muscle atrophy [47, 57]. However, these findings remain controversial. In an animal model of stroke, the expression of genes associated with autophagy complex (i.e., Ulk1, LC3, and cathepsin L) was increased in paretic and nonparetic limbs [47]. However, protein content did not follow the expression of mRNA and remained unaltered. Yet according to some researchers, the lack of changes in autophagy genes does not rule out the association between the autophagic complex and poststroke muscle atrophy, since a delayed increase in proteins cannot be discarded [47]. In humans, data from a number of studies seem to corroborate the findings of Masiero et al. [55]. In fact, patients with chronic spastic hemiplegia and visible muscle atrophy show decreased expression of autophagy genes when compared to healthy older adults [57].

Myostatin has also been suggested as one of the main pathways regulating muscle atrophy in poststroke patients [47, 58]. Myostatin, also known as growth differentiation factor 8 (GDF8), is key muscle protein regulator factor of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors [59, 60]. GDF8 seems to act as a negative factor against muscle mass development from embryogenesis to adult life, impairing muscle synthesis and increasing muscle catabolism [59–61].

Data from observations in animal models knockout to myostatin gene show that the mutant animal displayed from two- to threefold more muscle mass when compared to the control animals throughout life [61–63]. Although this phenomenon is marked by an increased number of muscle fibers (i.e., hyperplasia) during the first years of life, increase in muscle cross-sectional area (i.e., hypertrophy) is predominant in animals undergoing treatment with a myostatin inhibitor (JA16) during adult life [61, 63]. In humans and in cattle breeds, a mutation in the myostatin gene leads to its downregulation, causing abnormal development of muscle mass [64, 65].

Myostatin consists of two terminals: an N-terminal propeptide, which inhibits the activity of myostatin, and a C-terminal, which is the active form of the protein [60]. Before activation, myostatin is secreted and remains in a latent form [60]. Once activated, the activity of myostatin is regulated by binding with the serine/threonine transmembrane receptor [59, 60, 66]. After being activated by myostatin, the activin type II receptor recruits, phosphorylates, and activates the activin type I receptor, which is associated with SMAD proteins [59, 60, 66]. These proteins are one of the mechanisms responsible for the effect of myostatin on muscle cells, since phosphorylated SMAD 2 and SMAD 3 build a complex with SMAD 4, which is then translocated to the nucleus and changes the transcription of target catabolic genes [59, 67].

Some researchers have also suggested that myostatin may activate genes associated with UPS and maximize a proteolytic process [68]. However, experiments did not show the modulation of MuRF-1 and MAFbx by myostatin [68].

On the other hand, myostatin seems to inhibit the activity of important factors associated with muscle protein synthesis and regeneration, such as Akt, satellite cells and myogenic factors (e.g., MyoD) [58, 67, 69, 70]. In fact, McCroskery et al. [70] showed that myostatin knockout mice displayed higher number of satellite cells activated in the cell cycle and in steady state per unit of muscle fibers, together with faster proliferation of myoblast when compared to the controls [70].

Myostatin was increased in some animal and human models of muscle atrophy, for example, food deprivation, muscle disuse, and muscle denervation [68, 69, 71, 72]. Moreover, gene electrotransfer of a myostatin expression vector was found to induce 20% of muscle atrophy in tibialis anterior muscle of rats [58].

It has been suggested that in poststroke patients, atrophy disuse and denervation would account for increased myostatin. Moreover, myostatin is sensitive to glucocorticoids, which are highly consumed by poststroke patients [4].

However, data remain controversial. In animals undergoing transient focal cerebral ischemia, myostatin increases exponentially in both paretic and nonparetic limb 3 days after cerebral occlusion [47, 73]. However, SMAD 2 and SMAD 3 present downregulation [47]. The only study in humans, to our knowledge, was performed by von Walden et al. [57]. They investigated atrophied lower limb muscle in patients who suffered a stroke in the previous 9 years and found that their biopsy presented lower myostatin expression when compared to older healthy adults [57].

Thus, it is possible that myostatin modulates muscle atrophy at the beginning of the phenomenon, but not during its progression. These findings are corroborated by experiments involving nonstroke patients, which demonstrated that myostatin levels decrease few days after the beginning of muscle atrophy, during the peak loss of muscle mass [58, 69].

In view of such a range of candidate mechanisms, it is difficult to pinpoint the precise process responsible for muscle atrophy in poststroke patients, despite the indications provided by the fact that some pathways (i.e., UPS, autophagy, and myostatin) are increased during this process. Furthermore, further studies involving both pathways in animal models of stroke and in humans are needed, mainly to understand the molecular signaling.

Figure 1 shows the anabolic and catabolic pathways indicated to regulate muscle mass in poststroke patients.

4. Low-Grade Inflammation as a Trigger Factor to Activation of Muscle Atrophy Pathways

Inflammatory process is the mechanism of the immune system in charge of protecting the organic system against harmful agents and restoring homeostasis. Once activated, the components of the immune system return to prestress levels in few days or, at most, weeks. However, this is due to the decreased capacity of the physiological organic system to cope with stressful factors (e.g., reactive oxygen species (ROS)), as may occur due to aging—leading to inflammatory phenotype—or even in response to a pathological state, leading to a chronic low-grade inflammation condition [74, 75].

A number of physiological disorders, such as chronic pulmonary obstructive disease (CPOD), rheumatoid arthritis (RA), cancer, and inflammatory myopathies, also called myositis (i.e., polymyositis, inclusion body myositis, and dermatomyositis), have been found to be associated with elevated proinflammatory markers [8, 9, 76]. Furthermore, inflammation has been suggested to be a common factor leading to increased activity of atrophy-related catabolic pathways, such as UPS and autophagy, during aging (i.e., senescence and senility) and in chronic degenerative states (i.e., cachexia) [8, 9].

Animal studies have demonstrated the regulation of proinflammatory cytokines in myoplasticity. Evidence in the literature has shown that infusion of recombinant tumor necrosis factor-alpha (TNF-α) and TNF-α plus interleukin type-1 (IL-1), both proinflammatory cytokines, increases muscle catabolism in rats [77]. In mutant mice with overexpression of proinflammatory cytokines (i.e., interleukin type-6 (IL-6)), muscle mass presents exacerbated atrophy when compared to the wild type [78, 79]. However, inhibition of IL-6 receptor prevents muscle wasting and increase in UPS activity [79].

Inflammatory process may be also associated with the muscle atrophy phenotype caused by cachexia syndrome in some pathological conditions, since proinflammatory cytokines expression (i.e., mRNA) and production are increased in animal models of heart failure [80, 81] and cancer cachexia [82].

Regarding human beings, cross-sectional studies have found an association between increased concentrations of proinflammatory cytokines (e.g., IL-6 and TNF-α), their soluble receptors, and acute phase proteins (i.e., C-reactive protein (CRP)) and syndromes associated with low muscle mass in elderly [83, 84], cancer cachexia [85], and chronic heart failure [86]. Moreover, although not conclusive, a number of longitudinal studies have suggested that high concentrations of inflammatory markers may indicate a higher degree of muscle atrophy after 3 years [87].

Between the myriad of cytokines which may be associated with muscle atrophy, TNF-α (also called cachectin) seems to be the most likely candidate, due the high number of catabolic pathways that its molecule are involved in [88]. In fact, increased TNF-α is found in muscle wasting related conditions, such as cancer, heart failure, COPD, and sarcopenia [83–86, 89].

However, in poststroke patients, this scenario remains unclear. Indeed, the expression of mRNA TNF-α was increased in the vastus lateralis muscle, contralateral to the lesion, in chronic stroke survivors when compared to ipsilateral limb [90]. Nevertheless, this result was not confirmed in a more recent study [57]. Regarding systemic measurements, an increased level of TNF-α in the serum and cerebrospinal fluid was found in patients 24 hours, one week, and two weeks after the stroke. These increases were associated with infarct volume and severity of neurological impairment [91]. Corroborating these clinical findings, TNF-α blocking was found to reduce the volume of infarction after occlusion of the cerebral artery in mice [92].

Taken together, these data indicate a possible modulation of TNF-α in the skeletal muscle and in the central nervous system (e.g., motor cortex), which in turn affects functionality and, consequently, muscle mass (e.g., hemiparesis, spasticity, rigidity, balance, and coordination changes).

TNF-α effects on muscle wasting my be said to be due to inhibition of protein synthesis (because of alterations in the levels of anabolic hormones, such as IGF-1), a result of phosphorylation of IRS-1 and IRS-2 receptors, inhibition of satellite cells activity, and reduction of MyoD expression [88, 93–95]. Moreover, TNF-α seems to downregulate the synthesis of myosin heavy chain of slow-twitch fibers and increase their degradation [96, 97], which may account for the changes in fiber phenotype in the muscles of chronic stroke survivors.

Regarding proteolysis, we may hypothesize that the process is mediated by TNF-α through direct (e.g., apoptosis)

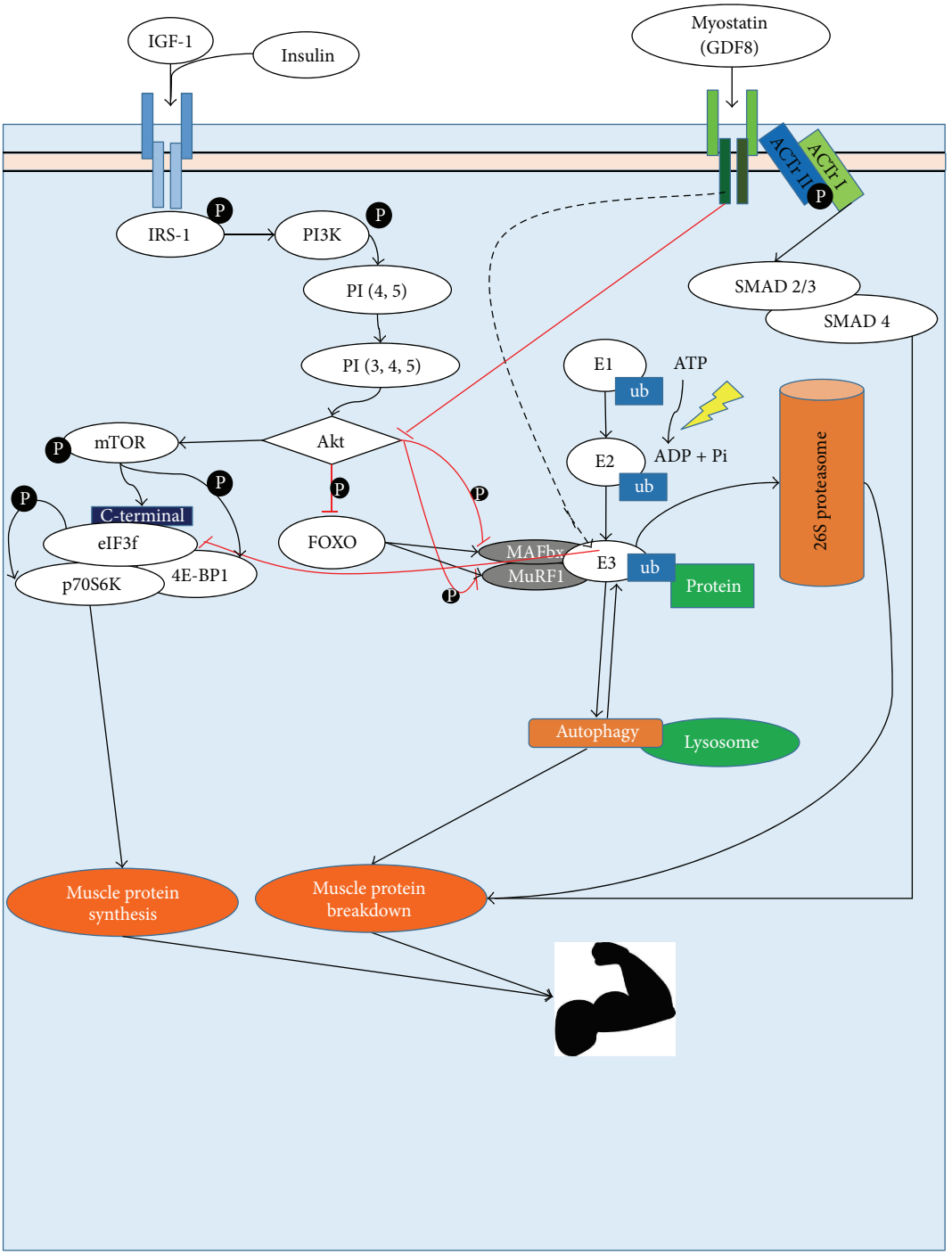

Figure 1: Anabolic and catabolic pathways regulating muscle mass. P = phosphorylation; Ub = ubiquitin.

and indirect mechanisms [88]. The indirect mechanisms are based on the capacity to recruit other proinflammatory cytokines and immune system cells, particularly by the stimulus to cause nuclear factor kappa-light-chain-enhancer of NF-κB activation [88, 96, 98]. In this sense, some researchers have suggested that muscle atrophy mediated by TNF-α/NF-κB activity may be the most powerful stimulus to muscle atrophy [18, 19].

NF-κB is a molecular signaling pathway originating from an evolutionary process, which plays a critical role in the activity of the immune system, regulating some physiological and pathological process, increasing the levels of inducible

nitric oxide (iNOS), with subsequent formation of reactive oxygen species, which results in oxidative damage [99]. Moreover, given its capacity to increase proinflammatory cytokines synthesis, NF-κB is known as the master regulator of inflammatory state [99, 100].

NF-κB may be activated by some stimulus, such as proinflammatory cytokines and ROS [96, 99–102]. However, when not activated, NF-κB remains in the cytoplasm, inhibited by IκBα activity, a molecule from IκB kinase (IKK) family [100, 102]. However, due to stimulation, another IKK molecule, IκBβ (IKK), phosphorylates and activates IκBα, leading to its ubiquitination and eventually to the degradation on 26S proteasome [100, 102]. Since IκBα acts by inhibiting the activity of NF-κB, degradation makes NF-κB free to translocate to the nucleus and change the gene transcription of proinflammatory cytokines [100, 102]. Regarding TNF-α, NF-κB activation by this proinflammatory cytokine is mediated through 1 TNF-α receptor (TNFR1) and/or 2 TNF-α receptor (TNFR2) [88].

Data from literature points to the capacity of NF-κB to contribute to muscle atrophy, since this molecule is increased in different models of muscle atrophy, that is, disuse and cancer cachexia [100]. Besides, in a seminal study, Cai et al. [100] have developed transgenic mice with NF-κB overexpression and found severe muscle atrophy in these animals, due to a sharp decrease in the cross-sectional area of muscle fibers, when compared to the wild type. Interestingly, this phenotype was followed by increased MuRF-1 and proteasome activity, indicating a possible interaction between NF-κB and UPS. Moreover, when the authors blocked NF-κB, muscle mass was restored [100].

In view of these findings, it is possible to infer that NF-κB activation by NF-κB in poststroke patients may contribute not only to muscle atrophy, but also to endothelial dysfunction, causing a positive feedback, ROS production, and inflammation, while maintaining NF-κB activation. This phenotype has been already suggested for other pathological states, such as diabetes mellitus type II [99]. However, data concerning poststroke patients remain unclear in the literature.

Myosteatosis is another condition found in the paretic limb of poststroke patients. This condition is characterized by fat deposition in the skeletal muscle, as well as by the amount of fat mass around the muscle. Evidence in literature suggests that myosteatosis is increased in the atrophied paretic limb in poststroke patients, when compared to the ipsilateral limb [103, 104]. The differences in intramuscular fat content between contralateral and ipsilateral limb can reach 48% in favor of the impaired limb [20]. This may account for the larger increase in fat mass relative to muscle area in the paretic limb relative to ipsilateral limb [20, 73, 103].

The white adipose tissue (WAT) is not just a deposit of triacylglycerol and, consequently, energy but also an active endocrine organ capable of synthesizing and secreting proinflammatory cytokines [85, 105–109]. Increased expression and protein content of proinflammatory cytokines are found in syndromes related with muscle wasting, as cancer cachexia [85, 105–110].

Indeed, visceral (i.e., mesenteric, epididymal, and retroperitoneal) and subcutaneous WAT in cancer cachexia present increased expression of proinflammatory cytokines (i.e., TNF-α, IL-6, and IL-1β), acute phase proteins (i.e., C-reactive protein (CRP)), and chemotaxis factors (i.e., monocyte chemoattractant protein-1 (MCP-1)), concomitant to increased NF-κBp65, IKK-α, and toll-like receptor 2 (TLR2) expression [85, 105, 106, 109, 110]. Moreover, cancer patients with cachexia have a lower number of macrophages (Mϕ) with an anti-inflammatory phenotype (M2) when compared to weight-stable cancer patients, and this may be associated with fat deposition [110].

However, data showing an association between muscle atrophy and WAT in patients with cachexia should be carefully extrapolated to poststroke patients, since data so far have indicated that proinflammatory phenotype may be tumor-dependent [106, 110]. On the other hand, muscle skeletal remodeling is also observed during aging, and older adults show up 2.5-fold more myosteatosis than young adults [111, 112]. Interestingly, intramuscular fat infiltration is negatively associated with muscle volumes of 15 muscles of the lower limbs in the elderly [112], which points to a strong association between myosteatosis and muscle atrophy. Moreover, intramuscular fat infiltration is associated with increased expression of proinflammatory cytokines (i.e., IL-6) [113].

Thus, we may hypothesize that the increased myosteatosis observed in poststroke patients is associated with muscle atrophy due a low-grade inflammatory state, possibly modulated by NF-κB activity. Moreover, some researchers have found that peroxisome proliferator activated receptor (PPAR) may contribute to myosteatosis, leading to muscle atrophy [114].

PPAR is a member of a nuclear receptor family of ligand-dependent transcriptions factors and comprises 3 PPAR subtypes: PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3), which have wide range of effects in the physiological system [114–116].

The main activity of PPAR is located in the adipose tissue, where this molecule regulates positively (i.e., upregulation) the network of adiposity-specific genes, controlling lipid metabolism (i.e., adipogenesis), adipocyte differentiation (i.e., white and brown), and glucose homeostasis (i.e., insulin sensitivity) [114, 115, 117].

Moreover, recent evidence has suggested that PPAR may have an anti-inflammatory propriety, inhibiting inflammatory mediators, such as cytokines (i.e., IL-6 and TNF-α), adhesion molecules (vascular adhesion protein 1 (VCAM1)), acute phase proteins (CRP), IKK, and NF-κB [114–117]. Moreover, PPAR may counterregulate inflammatory conditions induced by pathological states (i.e., obesity and liver fibrosis) [115]. Besides, PPAR may contribute to increasing insulin sensitivity, through adiponectin synthesis [117].

However, inflammatory cytokines, such as TNF-α, may inhibit PPAR, leading to a wide range of alterations, contributing to impaired glucose metabolism, hyperinsulinemia, ROS production, and possibly atherosclerosis, along with increased inflammatory state [114]. Thus, decrease in PPAR activity may lead to a catabolic environment associated with muscle atrophy. Nonetheless, the role of NF-κB activity and

PPARγ in the muscle atrophy of poststroke patients should be further studied and tested.

Interestingly, the increase in intramuscular fat content in poststroke patients has also been widely suggested as a trigger factor to impaired glucose metabolism, a condition that may reach about 80% of the chronic stroke patients [73, 103, 118]. In fact, increased intramuscular fat infiltration, regardless of visceral fat, contributes to the genesis of dyslipidemia, and impaired insulin sensitivity and glucose uptake, causing hyperinsulinemia and hyperglycemia, thus providing a favorable environment for chronic conditions, such as hypertension and diabetes mellitus type II [103, 104, 118, 119]. Therefore, PPAR maybe the pathway underlying impaired glucose metabolism due to increased intramuscular fat content in poststroke patients.

Figure 2 shows the inflammatory factors indicated to regulate muscle atrophy in poststroke patients.

5. Physical Exercise and Stroke

As aforementioned, poststroke patients are generally affected by morphofunctional and cognitive complications, which impairs their capacity to perform the daily life activity and basic and advanced self-care, leading to sedentary behavior and increased hospitalization.

On the other hand, physical exercise (PE) has been postulated by international organizations (i.e., American Heart Association (AHA) and American Stroke Association (ASA)) as a useful tool for the rehabilitation of poststroke patients, since PE may counterregulate the most of the deleterious effects of stroke in the organic systems [10]. Indeed, several reviews have pointed out that PE can elicit improvement in cognition, upper and lower limb motor function, cardiovascular performance, cardiovascular risk factors (e.g., triglycerides), fatigue resistance, balance, gait, and mobility [10, 12, 120].

Most studies on the role of PE in poststroke patients have focused on aerobic exercise. A recent systematic review showed that most clinical trials studying the effect of aerobic physical exercise on stroke patients have used short-term interventions (6–8 weeks), with a mean frequency of 3 sessions per week, and a 30–40-minute exercise duration [120]. Regarding exercise intensity, moderate and progressive (moderate to moderate-intense) intensities prevailed [120].

Increase in cardiorespiratory fitness (15–18%) is the most cited alteration after moderate aerobic exercise, and it occurs even after short-term exercise protocols (i.e., 8 weeks) [10, 120–123]. However, the beneficial effects of moderate aerobic exercise in poststroke patients are not restricted to cardiorespiratory fitness, and studies have demonstrated increase in mobility (e.g., get up and go test (GUG)) and motor function, [123], as well as decrease in cardiovascular risk factors, such as hyperinsulinemia [122].

Changes in the cognitive domain after moderate aerobic exercise have also been the focus of some studies. In the experiment of Quaney et al. [123], the authors did not observe significant changes in selective attention, resistance to interference, working memory, and learning after 8 weeks of moderate aerobic exercise (70% of HRmax), which comprised 45 m sessions. A recent meta-analytic review [124] has challenged those findings, arguing that the practice of physical exercise is able to improve cognition in poststroke patients, even when patients presented depressive symptoms and high anxiety levels [124]. However, these findings should be carefully evaluated since not many studies have focused on cognitive improvements, and these were generally assessed as a secondary outcome. Also, cognitive assessment would require more specific methodological tools.

Some studies have dealt with outcomes that are not generally investigated as clinical outcomes but are nevertheless useful to understanding the phenomenon associated with stroke and the impact of physical exercise on this condition. Ivey et al. [121], for example, have studied the effects of PE on blood flow in poststroke patients. Impaired blood flow is usually associated with endothelium dysfunction, ROS, and inflammation, posing a risk factor to myocardial infarction and recurrent stroke. After 6 months of moderate intensity aerobic exercise (60–70% HR reserve), volunteers showed significant increase in rest blood flow, 25% and 23% in paretic and nonparetic limb and in reactive hyperemia blood flow, 25% and 22% in paretic and nonparetic limb [121].

Some researchers have argued that although aerobic exercises may have beneficial effects on poststroke patients, they would be poorly tolerated in this population [125]. In this context, resistance training exercises have been suggested as an interesting alternative, since they are easier to modulate than aerobic exercise and as such more manageable for poststroke patients [12, 125]. Also, resistance training is able to elicit increase in physical function (i.e., muscle strength and power) and mobility [126, 127]. Nevertheless, resistance training has been poorly studied and the outcomes assessed are generally restricted to physical function. Further studies are required to evaluate other important outcomes, such as improved cognition [125].

Interestingly, few studies aimed to record the effects of PE on muscle mass. Regarding aerobic exercise, Ivey et al. [122] have undertaken one of the few studies investigating this issue, but they did not observe significant alterations. In relation to resistance exercise, Ryan et al. [73] developed the only study that reported the effects of this kind of intervention on muscle mass in poststroke patients. Other researchers have concentrated on the effects of resistance training on myostatin expression. Their findings demonstrated that 12 weeks of resistance exercise until muscle failure was able to elicit an increase in muscle mass of the paretic limb (13%) and nonparetic limb (9%) [73]. A decrease of 49% of myostatin mRNA on paretic limb and 27% on the nonparetic limb was also reported [73].

The rather limited number of studies in the literature dealing with the effects of physical exercise on muscle mass makes it difficult to offer a well-informed assessment of the effects of physical exercise on muscle mass. More studies on this association are required, since muscle atrophy and sarcopenia are linked to hemodynamic [128–130], metabolic [131], and functional alterations [132, 133], which contribute to poor outcomes in poststroke patients. Additionally, increase

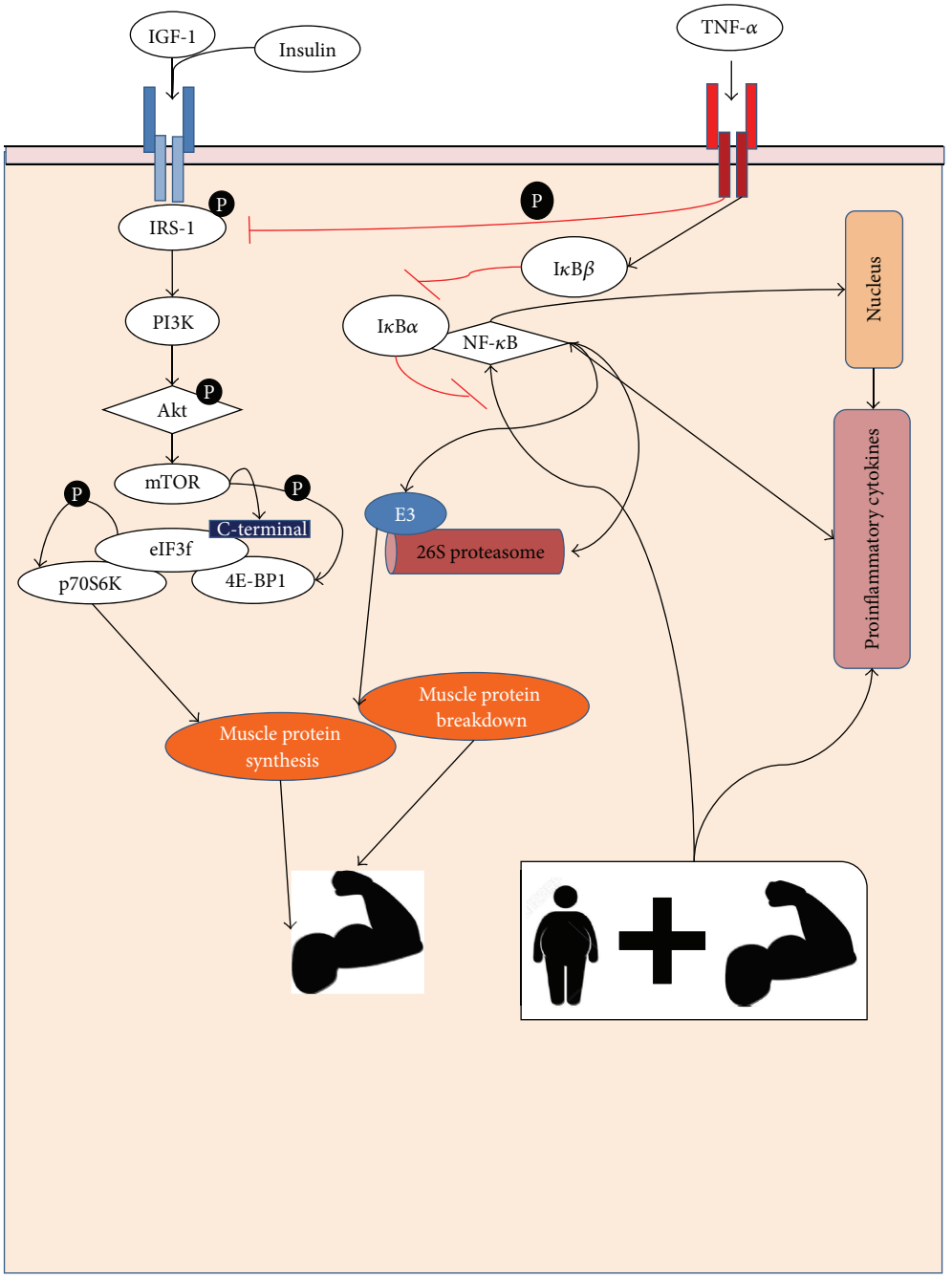

Figure 2: Influence of the inflammatory factors in the regulation of muscle mass.

in muscle mass is associated with increase in physical function and mobility [134–136].

6. The Anti-Inflammatory Effects of Physical Exercise and the Role of Myokines

Physical exercise has been indicated as a powerful non-pharmacological therapy to decrease inflammatory markers, ameliorate the anti-inflammatory environment, and, consequently, lower chronic inflammation in several diseases (e.g., chronic pulmonary obstructive disease, atherosclerosis, heart failure, and myocardial infarction) [13–16, 137].

In fact, it has been found that chronic moderate and moderate-to-high intensity physical exercise may elicit a decrease in inflammatory factors (e.g., TNF-α, IL-1β, IL-6, intercellular adhesion molecule (CAM-1), acute phase proteins (CRP), vascular cell adhesion molecule (VCAM-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF)), in different animal models of diseases, such as heart failure [80], myocardial infarction [138], and cancer

cachexia-anorexia [139] in healthy [140] and senescent animals [141], as well as in human patients with moderate to severe chronic heart failure (~24% ejection fraction) [142], overweight and obesity [143, 144], diabetes mellitus [143–150], myopathies (i.e., dermatomyositis and polymyositis), rheumatoid arthritis [148], spinal cord injury [149], and systemic lupus erythematosus [151], and in elderly people: those with chronical conditions [150] and the healthy [152].

These alterations can occur locally, affecting the expression of these factors in the cardiac muscle [138], skeletal muscle [80, 140, 146, 147, 150], and adipose tissue [153], in the central nervous system [139, 141] or systemically [142–145, 151, 152]. Furthermore, PE also seems to be effective in increasing anti-inflammatory markers (e.g., IL-10) [80, 107]. Besides, data from literature indicate that alterations in inflammatory factors may also be associated with improved physiological function (e.g., ventricular function; aerobic capacity; insulin resistance) [138, 142, 143, 154].

There are no evidences about the effects of chronic physical exercise on the inflammatory markers in stroke patients. Besides being associated with muscle atrophy and poor prognosis, high inflammatory markers have been linked to elevated risk of recurrent ischemic stroke and cardiovascular events even after adjustment for age, sex, race, comorbidities, and statin use [155].

Several mechanisms may be associated with the anti-inflammatory effects of physical exercise: decrease in the expression of toll-like receptors on monocytes and macrophages, inhibition of the infiltration of immune cells on adipose tissue, changes in the phenotype of macrophages on adipose tissue, and decrease in adipose tissue [13].

For many years, the skeletal muscle was predominantly known by its capacity to generate strength, power, and, consequently, physical movement. Later, researchers hypothesized that some or a single humoral factor would be secreted by the active skeletal muscle and would act by altering the signalization of different molecular pathways [14, 137]. As knowledge about the activity and the properties of these molecules was scarce, they were initially called "exercise factor," "work stimulus," and "work factor" [14, 137].

However, accumulated evidence now views the skeletal muscle acting as an active endocrine organ, since the contraction of skeletal muscle in response to a determined load, as observed during physical exercise, may elicit synthesis and release of peptides, hormone-like factors and cytokines—pro- and anti-inflammatory—which, in turn, alter the functioning of tissues and organs [13–15, 137, 156]. Once aggregated, these molecules are termed myokines, and they have been found to be responsible for the interaction between the skeletal muscle and the organic system, due to their action in a paracrine and endocrine and, possibly, autocrine fashion [14, 156].

Regarding the anti-inflammatory effects of the myokines, interleukin-6 (IL-6) is one of the most well-known and studied, being the first to be denominated as a myokine [13, 15, 137, 157]. Besides, IL-6 is considered one of the key myokines which provide the anti-inflammatory effects of physical exercise [13, 15, 137].

Several studies in human beings have demonstrated an increase by over 100-fold in IL-6 levels, as well as mRNA expression, during and after PE, independently of exercise-induced muscle damage and inflammation [158–160]. Moreover, IL-6 is not just an anti-inflammatory cytokine, but evidences indicate its action on glucose metabolism and bioavailability, contributing to beta-oxidation and glucose uptake [160, 161].

In relation to physical exercise, IL-6 seems to be sensitive to alterations on the variables of PE, since running intensity and volume are positively associated with IL-6 levels on plasma [159, 160, 162]. In humans, moderate aerobic physical exercise (75%VO_{2max}) and rhIL-6 infusion are able to increase IL-6 levels in blood plasma and, concomitantly, attenuate the increase in TNF-α levels after endotoxin infusion [163]. Also, IL-6 plasma levels decreased after cessation of PE or rhIL-6 infusion [158, 159, 162]. Interestingly, cessation of low-levels of rhIL-6 infusion was accompanied by a decrease in anti-inflammatory cytokines: IL-1 receptor antagonist (IL-1ra) and IL-10 [159]. On the other hand, decreased IL-6 levels were found 1,5 h after the end of a strenuous physical exercise, showing a sharp increase (>100x) after the end of exercise session. This was accompanied by IL-1ra values 45-fold higher than preexercise levels, which, in turn, were positively correlated with IL-6 levels [162].

Therefore, these data indicate that the anti-inflammatory effect of physical exercise can be mediated by myokines, mainly IL-6. However, there is not a consensus on whether IL-6 acts directly or through other anti-inflammatory cytokines (IL-1ra, IL-10). Both theories are plausible, and the fact remains that myokines do contribute to an anti-inflammatory environment. Additionally, other myokines, IL-8, IL-15, and the brain derived neurotrophic factor (BDNF), have been linked to angiogenesis, metabolism, neurogenesis, and memory formation [14, 137, 161]. But their effect on inflammatory state have yet to be elucidated.

Therefore, these data indicate that the anti-inflammatory effect of physical exercise may be mediated by myokines, mainly IL-6. However, these myokines have not been studied in a stroke context and inferences are therefore limited. Nevertheless, increase in myokines and decrease in the inflammatory milieu of poststroke patients after physical exercise may improve the prognosis of this population, due to a better physiological environment, one which increases muscle mass, strength, power, and mobility while decreasing cardiovascular risk factors.

Figure 3 shows a schematic representation of the myokines activation in response to physical exercise and its inhibitory anti-inflammatory activity on the inflammatory environment.

7. Cholinergic Anti-Inflammatory Pathway

Findings from studies conducted by Tracey group [164–166], as well as from other groups [138, 167, 168], have suggested that the central nervous system (CNS) may act by regulating the central and peripheral inflammatory process [164–166]. This phenomenon occurs due to the activity of the two

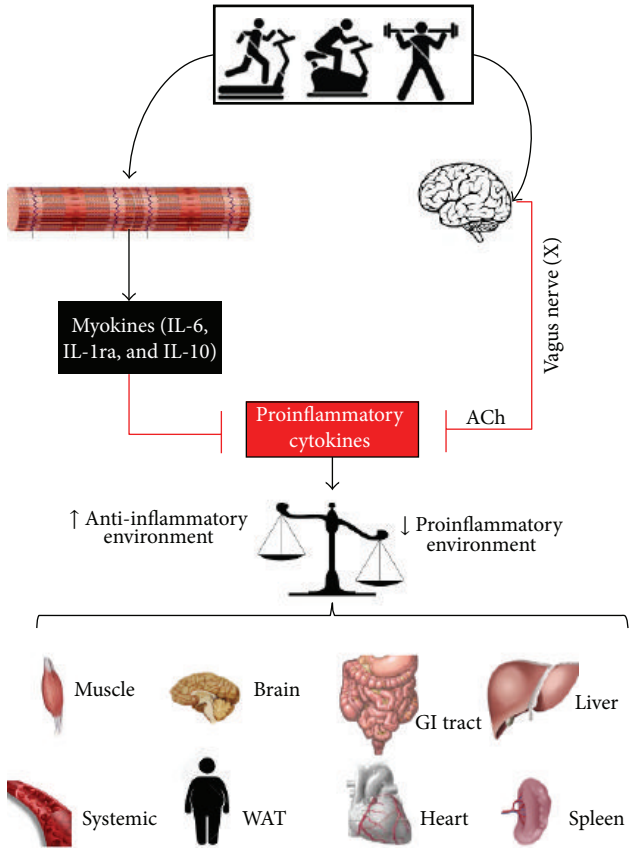

FIGURE 3: Possible anti-inflammatory pathways modulated by physical exercise. Ach = acetylcholine; $\alpha 7$nAChR = receptor $\alpha 7$ subunit; WAT = white adipose tissue.

functional divisions of the autonomous (also called visceral) nervous system—sympathetic (SNS) and parasympathetic nervous system (PNS)—so that each would regulate in a different fashion the immune system and, consequently, the inflammatory process [165, 166, 169].

SNC is known to be activated during "flight or fight" conditions, since it offers the organic system a large blood supply (i.e., increased heart rate and blood pressure), energy substrate (i.e., increased lipolysis), oxygen supply (i.e., bronchial dilation), visual acuity, adrenalin and noradrenalin concentrations, and muscle strength (which could help with survival during Paleolithic and Neolithic period [169].) SNC is increased during situations of allostasis (e.g., hypotension and electrolyte imbalance in order to reestablish the normal functioning). Moreover, this system also acts during dynamic homeostasis, helping to control several physiological systems (e.g., gastrointestinal, cardiovascular, and endocrinal) [165, 166].

Catecholamines (adrenalin and noradrenalin) are synthesized and secreted by the adrenal medulla in the adrenal gland in response to sympathetic activity. Their role in immune cells lies in the fact that they alter their functioning by the modulation of cytokine release, since these cells have α and β receptors to catecholamines [165].

In turn, PNS acts in an anti-inflammatory fashion, a phenomenon denominated cholinergic anti-inflammatory pathway (CAIP) [164–166, 169–173]. The vagus nerve is an inherent component of this pathway. This nerve is the decimal (X) nerve of the SNC and has afferent, motor, and efferent projections [174]. Regarding afferent projections, these conduct information from sensitive periphery receptors, as chemoreceptors, baroreceptors, and visceral receptors on the thorax and abdomen to the CNS [174].

Concerning CAIP, the afferent vagus nerve fibers act as a peripheral sensory component of the PNS and identify the increase in proinflammatory cytokines from the inflammation, linking the CNS to the immune system [164–166, 169–173].

In the brain, the vagus nerve is found in the nodose ganglion and inside the dorsal vagal complex of the medulla oblongata, which is formed by the nucleus of the solitary tract (NST), dorsal motor nucleus of the vagus, and the area postrema [174]. From there, a neurohumoral and a cholinergic pathway can occur and counterbalance inflammation [174]. The neurohumoral pathway is activated due to the synapse of the NTS with the paraventricular nucleus, a hypothalamic nucleus, which stimulates the synthesis and release of the corticotropin releasing hormone (CRH) [174]. However, the functioning of this pathway remains poorly understood.

On the other hand, projections from the NTS form the efferent arc of the inflammatory reflex, which would act

through efferent vagus nerve fibers and neurotransmitters [169]. In summary, CAIP occurs after the inflammatory signalization on the afferent vagus nerve fibers to the NTS; a reflex response mediated by efferent vagus nerve fibers will propagate and culminate in acetylcholine (i.e., ACh), the mainly parasympathetic neurotransmitter, release [165, 166, 169].

Several studies have demonstrated the protective effect of CAIP stimulation, as well as the need of the efferent vagus nerve, on animal models of systemic inflammation [164, 168, 175–177]. In fact, results from experimental studies have found that intravenous (IV) and intracerebroventricular (ICV) pretreatment with CNI-1493 (a pharmacological stimulator of the vagus nerve), carbachol (a cholinergic agonist), pyridostigmine (a peripheral cholinesterase inhibitor agent), and electrical stimulation protected rats against inflammatory factors associated with acute hypovolemic hemorrhagic shock, endotoxin-induced shock, myocardial infarction, and carrageenan-induced acute inflammation, such as increase in serum TNF-α levels, edema, neutrophil aggregation, macrophage infiltration, NF-κB protein levels, and loss of IkBa [164, 168, 175–177]. Also, these alterations on inflammatory markers may be associated with increased M2 macrophages, which are characterized by producing mainly anti-inflammatory cytokines and regulatory T cells (Tregs), known by their immunosuppressive capacity [168].

On the other hand, surgical and chemical (i.e., atrophin, an antagonist of cholinergic pathway) vagotomy eliminated the protective effects of chemical and electrical vagus nerve stimulation on inflammatory markers [164]. Furthermore, after surgical vagotomy, the researchers performed new electrical stimulation, but this time on the distal end of the transected right vagus nerve and found an attenuation of the acute inflammation [176].

The activity of CAIP seems to be mediated by ACh, which is the most important neurotransmitter, being the main neurotransmitter release in postganglionic efferent vagal neurons [165, 166, 172]. After the activation of central muscarinic receptor M1 and/or inhibition of the negative regulator of the ACh release (the M2 receptor), ACh is released and acts by using two G protein-coupled receptors, muscarinic and nicotinic [169, 170].

Classically, the most well-known activity of ACh in the organic system is mediating muscarinic receptors, which are found in the hearth and neurons and in the smooth muscle, for example. However, regarding the immunological activity of the ACh, the muscarinic receptor did not seem to have a role in this response. A specific unit of nicotinic receptors, nicotinic acetylcholine receptor α7-subunit (α7nAChR), is found in immune cells and, once activated, it inhibits cytokine secretion [178, 179]. In fact, Wang et al. [179] have demonstrated that α7nAChR is essential to the effectiveness of the anti-inflammatory effect of the cholinergic efferent arc on TNF-α, IL-1β, and IL-6 concentration in endotoxemic rats, since α7nAChR-deficient mice present higher TNF-α, IL-β, and IL-6 concentrations when compared to the wild type [179].

Furthermore, increased ACh levels, due to activation and inhibition of M1 and M2 muscarinic receptors, respectively, and consequently increase in vagus nerve activity (as demonstrated by Pavlov et al. [170]) will cause inhibitory effects on inflammatory markers (i.e., TNF-α, IL-1B, IL-6, and IL-18) through a posttranscriptional mechanism, since ACh did not alter proinflammatory cytokines mRNA in LPS-stimulated macrophage [170, 176].

In relation to stroke patients, evidences in the literature have been indicating that poststroke patients present a phenotype of dysautonomia, mainly characterized by decreased on vagal tonus [11, 180, 181]. Indeed, cross-sectional studies showed impairment of the autonomic control, diagnosed by decreased parasympathetic activity in time domain measures (i.e., root mean square of successive differences (RMSSD) and standard deviation of the normal-to-normal R-R intervals (SDNN)) and frequency domains (high frequency (HF)) of HRV—in right-sided and left-sided ischemic stroke patients when compared to aged-matched healthy control [11, 180, 181]. Also, these results are more evident in patients with right insular involvement, probably due to the association with cardiac control [180].

Besides its negative impact on cardiovascular complications (e.g., increase in ventricular and supraventricular arrhythmias) [11, 180, 181], dysautonomia may be one of the mechanisms responsible for the aforementioned increased inflammatory markers observed locally and systematically in poststroke patients, due to impairment in the CAIP functioning.

Interestingly, CAIP was thought at first to act only as an arc reflex, controlling acutely the inflammatory state and preventing cellular damage [169, 173]. However, some researchers have suggested that CAIP may also act in a chronic fashion, in the pharmacological treatment, and it may be responsible for the anti-inflammatory effects of physical exercise, for example [154, 182]. However, only few experiments have tested this hypothesis [138, 183].

In the research undertaken by Conti et al. [183], the authors tested the hypothesis that physical exercise would positively modulate the deleterious effects of menopause, mimicked through ovariectomy, on autonomic nervous system and inflammatory profile in metabolic syndrome (i.e., SHR more fructose diet) female rats. Animal underwent 8 weeks of low-to moderate intensity combined physical exercise, aerobic (60% maximal running speed) plus resistance training (60% of the maximum load), which was performed 5 days per week. Results showed that combined physical exercise was able to restore dysautonomia and TNF-α levels on cardiac tissue. Furthermore, oxidative stress, one of the mechanisms associated with NF-κB activation, was decreased in both cardiac and renal tissue of trained metabolic syndrome female rats when compared to sedentary metabolic syndrome rats [183].

Rodrigues et al. [138] have studied the effect of PE on autonomic control and the potential impact on inflammatory markers in infarcted rats. In this study, infarcted rats underwent a moderate intensity (50–70 of the VO_{2max}) exercise program, 1 h per day, 5 days per week, during 3 months. Researchers observed increased HF of the pulse interval, which characterizes parasympathetic activity, in trained group when compared to the sedentary infarcted

control group. Moreover, TNF-α concentration and TNF-α/IL-10 ratio were lower in trained infarcted rats than in sedentary control. Interestingly, one group of rats trained for only two months, followed by a 1-month detraining period. Results of this group were similar to the trained group, presenting increased parasympathetic activity and lower inflammatory profile when compared to the infarcted group. Also, in the same study, the researchers found that CAIP was a plausible candidate mechanism associated with decreased inflammatory profile, since HF was negatively correlated with IL-6 and TNF-α concentration on the left ventricle [138].

Unfortunately, the effects of physical exercise on cardiovascular autonomic control and their impact on tissue and systemic inflammation profile remain poorly understood and further studies focusing on this issue are needed. However, recent studies have been demonstrated that direct cortical vagus nerve stimulation (Cvns) and indirect (noninvasive transcutaneous vagus nerve stimulation (nVNS)) vagal nerve stimulation lead to decreased infarct volume (up to 33%), neurological damage, and increased grip strength in rats undergoing acute cerebral ischemia [184, 185]. Furthermore, experiments have shown that the middle cerebral artery occlusion (MCAO) animal model undergoing vagal nerve stimulation presented lower Iba1 and CD68 (microglial markers indicating immune response of brain tissue) and TNF-α, IL-6, and IL-1β concentrations than sham-control [184, 185].

Taken together, data on the anti-inflammatory effect of physical exercise on the inflammatory markers in animal models of myocardial infarction and menopause and the effectiveness of electrical stimulation of vagus nerves in decreasing inflammation in MCAO animals, make it possible to infer that physical exercise may activate CAIP in poststroke patients and, consequently, contribute to a decrease in inflammatory markers, allowing recovery of the organic system.

Figure 3 shows a schematic representation of the CAIP activation in response to physical exercise, and its inhibitory anti-inflammatory activity on the inflammatory environment.

8. Conclusion

We presented several findings from a range of studies which may indicate that proinflammatory markers (e.g., TNF-α and IL-6) may be responsible for the activation of pathways associated with muscle atrophy in poststroke patients (e.g., UPS system). On the other hand, physical exercise seems to be a powerful tool to counterbalancing these phenomena, due its capacity to elicit a decrease in inflammatory markers in different animal models of disease (e.g., cancer, myocardial infarction, and heart failure), as well as in human beings. These beneficial outcomes may take place because physical exercises seem to act in an anti-inflammatory fashion, through myokines and the cholinergic anti-inflammatory pathway. Even if these data are mostly inference-based, they may point to the possible mechanism to be further studied and encourage research on inflammation and muscle atrophy and on the effect of physical exercise in poststroke patients.

Abbreviations

TNFR1: 1 TNF-α receptor
TNFR2: 2 TNF-α receptor
ACh: Acetylcholine
ADL: Activities of daily live
AHA: American Heart Association
ASA: American stroke association
CAIP: Cholinergic anti-inflammatory pathway
CPOD: Chronic pulmonary obstructive disease
Cvns: Cortical vagus nerve stimulation
CRP: C-reactive protein
GUG: Get up and go test
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GDF8: Growth differentiation factor 8
HF: High frequency
IL-1ra: IL-1 receptor antagonist
iNOS: Inducible nitric oxide
IRS-1: Insulin receptor substrate
IGF-1: Insulin-like growth factor 1
IL-6: Interleukin type-6
ICV: Intracerebroventricular
IV: Intravenous
KO: Knockout
Mϕ: Macrophages
mTOR: Mammalian target of rapamycin
MCAO: Middle cerebral artery occlusion
MCP-1: Monocyte chemoattractant protein-1
MAFbx: Muscle atrophy F-box
MuRF1: Muscle ringer finger 1
nVNS: Noninvasive transcutaneous vagus nerve stimulation
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B
PNS: Parasympathetic nervous system
PI3K: Phosphatidylinositol 3-kinase
ROS: Reactive oxygen species
Tregs: Regulatory T cells
RA: Rheumatoid arthritis
RMSSD: Root mean square of successive differences
6MWT: Six-minute walk test
SDNN: Standard deviation of the normal-to-normal R-R intervals
SNS: Sympathetic nervous system
TLR2: Toll-like receptor 2
TGF-β: Transforming growth factor-β
TNF-α: Tumor necrosis factor-alpha
UPS: Ubiquitin proteasome system
VCAM1: Vascular adhesion protein 1
WAT: White adipose tissue.

Competing Interests

The authors have no conflict of interests to declare.

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Research Article

Interferon Tau Affects Mouse Intestinal Microbiota and Expression of IL-17

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This study was conducted to explore the effects of interferon tau (IFNT) on the intestinal microbiota and expression of interleukin 17 (IL-17) in the intestine of mice. IFNT supplementation increased microbial diversity in the jejunum and ileum but decreased microbial diversity in the feces. IFNT supplementation influenced the composition of the intestinal microbiota as follows: (1) decreasing the percentage of *Firmicutes* and increasing *Bacteroidetes* in the jejunum and ileum; (2) enhancing the percentage of *Firmicutes* but decreasing *Bacteroidetes* in the colon and feces; (3) decreasing *Lactobacillus* in the jejunum and ileum; (4) increasing the percentage of *Blautia*, *Bacteroides*, *Alloprevotella,* and *Lactobacillus* in the colon; and (5) increasing the percentage of *Lactobacillus*, *Bacteroides*, and *Allobaculum*, while decreasing *Blautia* in the feces. Also, IFNT supplementation decreased the expression of IL-17 in the intestines of normal mice and of an intestinal pathogen infected mice. In conclusion, IFNT supplementation modulates the intestinal microbiota and intestinal IL-17 expression, indicating the applicability of IFNT to treat the intestinal diseases involving IL-17 expression and microbiota.

1. Introduction

Interferon tau (IFNT) is produced by trophectoderm cells of conceptuses of ruminant species and is the maternal recognition of the pregnancy signal. Besides its critical roles in implantation and establishment of pregnancy in ruminants [1, 2], it has a plethora of physiological functions in various cell types such as macrophages, lymphocytes, and epithelial cells in humans and mice [3–5]. It is a type I interferon (IFN), which includes IFN alpha (IFNA), IFN beta (IFNB), IFN delta (IFND), and IFN omega (IFNW). After binding to a common receptor, IFNA receptor 1 (IFNAR1), and IFNAR2, type I IFNs affect the production of inflammatory cytokines such as interleukin- (IL-) 1β and tumor necrosis factor α (TNF-α) [6, 7]. Thus, type I IFNs have widely recognized roles in inflammatory diseases, such as experimental allergic encephalomyelitis, multiple sclerosis, and spontaneous autoimmune diabetes [5, 8–10]. Notably, unlike other members of type I IFN family, IFNT has few adverse effects and low cytotoxicity even at high dosages [11, 12], suggesting its therapeutic potential as an alternative to other type I IFNs due to its anti-inflammatory effects. Recent compelling findings about the anti-inflammatory effects of IFNT include lower NLRP3 (nucleotide-binding oligomerization domain-like receptor, pyrin domain-containing 3) inflammasome-driven IL-1β secretion by human macrophages [4], mitigation of obesity-associated systemic tissue inflammation in mice [5], and promotion of Th2 biased immune response in mice [9].

The influence of IFNT on intestinal microbiota is unknown. The intestinal microbiota provides important benefits for the development of immune responses; however, the disturbances in the intestinal microbiota are associated with numerous chronic inflammatory diseases [13, 14]. Also, the effect of IFNT on expression of IL-17 in the intestine is not known. The potential effect of IFNT on expression of IL-17 is important as IL-17 promotes local chemokine production to recruit monocytes and neutrophils to sites of inflammation that leads to development and pathogenesis of various autoimmune diseases, including rheumatoid arthritis, psoriasis vulgaris, multiple sclerosis, and inflammatory bowel diseases [15, 16]. In this study, the intestinal microbiota and expression of IL-17 in the intestine were explored after two weeks of IFNT supplementation in a mouse model. The hypothesis is that IFNT supplementation alters intestinal microbiota and intestinal innate immunity in mouse model.

2. Materials and Methods

2.1. Bacterial Strains. This study used the *Escherichia coli* F4-producing strain W25K (hereafter referred as ETEC; O149:K91, K88ac; LT, STb, EAST), which was originally isolated from a piglet with diarrhea [17].

2.2. IFNT Supplementation for Mice. This study was conducted according to the guidelines of the Laboratory Animal Ethical Commission of the Chinese Academy of Sciences. ICR (Institute for Cancer Research) mice (six weeks of age) were purchased from SLAC Laboratory Animal Central (Changsha, China). The mice were housed individually in a pathogen-free animal vivarium (temperature, 25°C; relative humidity, 53%; 12 h dark/12 h light) and had free access to a standard rodent diet [18] and drinking water. After three days of accommodation, mice were assigned randomly into two groups (IFNT and control; $n = 10$/group). Mice in the control group were fed the basal diet [18] and normal water, while mice in IFNT group were fed the basal diet and water containing recombinant IFNT (40 μg/L) for two weeks. The effective supplemental dosage of IFNT was established in previous study [5, 19]. At the end of the two weeks of experimental period, mice were sacrificed to collect contents of the lumens of the jejunum, ileum, and colon, as well as feces. The tissues including jejunum, ileum, and colon were also collected. Feed and water intake and body weight gain were monitored throughout the experiment. Samples were collected and stored at −80°C until processed.

2.3. ETEC Infection of Mice. After three days of accommodation to the conditions of the vivarium, ICR mice were assigned randomly into two groups (ETEC and IFNT+ETEC; $n = 10$/group). Mice in IFNT+ETEC group were fed the basal diet and recombinant IFNT-supplemented water (40 μg/L) for two weeks, while mice in ETEC group were fed the basal diet and had normal water. After two weeks of feeding, mice in both groups were inoculated with 10^8 CFUs of ETEC W25K by oral gavage. At 6 hours after infection, all active mice were sacrificed to collect the jejunum, and the samples were stored at −80°C until processed.

2.4. 16S rDNA Sequencing with Illumina MiSeq Sequencing. DNA was extracted from the luminal contents of the jejunum, ileum and colon, and feces using the Qiagen QIAamp DNA Stool Mini Kit according to the protocol for isolation of DNA. Equal amounts of DNA from six different mice were pooled to generate one common sample for each type of sample (i.e., control versus IFNT, intestinal source, and feces). The V4-V5 region of the bacterial 16S ribosomal RNA gene was amplified by PCR using primers 515F 5′-barcode-GTGCCAGCMGCCGCGG-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′, where barcode is an eight-base sequence unique to each sample. Illumina MiSeq sequencing and general data analyses were performed by a commercial company (Biotree, Shanghai, China). Miseq PE Libraries, Miseq Sequencing, and further analyses were based on previous work [20].

2.5. RT-PCR. Total RNA was isolated from liquid nitrogen frozen and ground jejunum, ileum, and colon using TRIZOL regent (Invitrogen, USA) and then treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. Synthesis of the first strand (cDNA) was performed using oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen, USA). Primers were selected according to previous references [18, 21]. β-actin was used as an internal control to normalize expression of target gene transcripts. The RT-PCR experiment was conducted according to previous studies [18, 21].

2.6. Statistical Analyses. Data shown are the means ± the standard error of the mean (SEM). All statistical analyses for data were performed using SPSS 16.0 software (Chicago, IL, USA). Data were analyzed for the two treatment groups using Student's t-test. Differences of $P < 0.05$ are considered significant.

3. Results

3.1. IFNT Treatment Increases Feed Intake. To investigate the effect of IFNT supplementation on mouse growth performance, feed intake, water intake, and body weight were monitored in IFNT-supplemented mice and control mice. With two weeks of IFNT supplementation, the averages for feed intake and water intake for IFNT-supplemented mice were significant ($P < 0.05$) higher than for control mice (Figures 1(a) and 1(b)). However, IFNT supplementation had no significant effect on body weight of mice (Figure 1(c)).

3.2. Changes in Bacterial Diversity of the Intestinal Microbiota Associated with IFNT Supplementation. To explore the influence of IFNT supplementation on the intestinal microbiota, we analyzed the intestinal microbiota at end of two weeks of IFNT supplementation with 16S rDNA sequencing (Table 1). For microbiota in the jejunum, both Shannon and Simpson indices demonstrated that the diversity of microbiota in mice with IFNT supplementation was higher than the control mice, while the richness indices (Ace and Chao) suggested that the community richness in IFNT-supplemented and control mice was similar (Table 1). For the microbiota in the

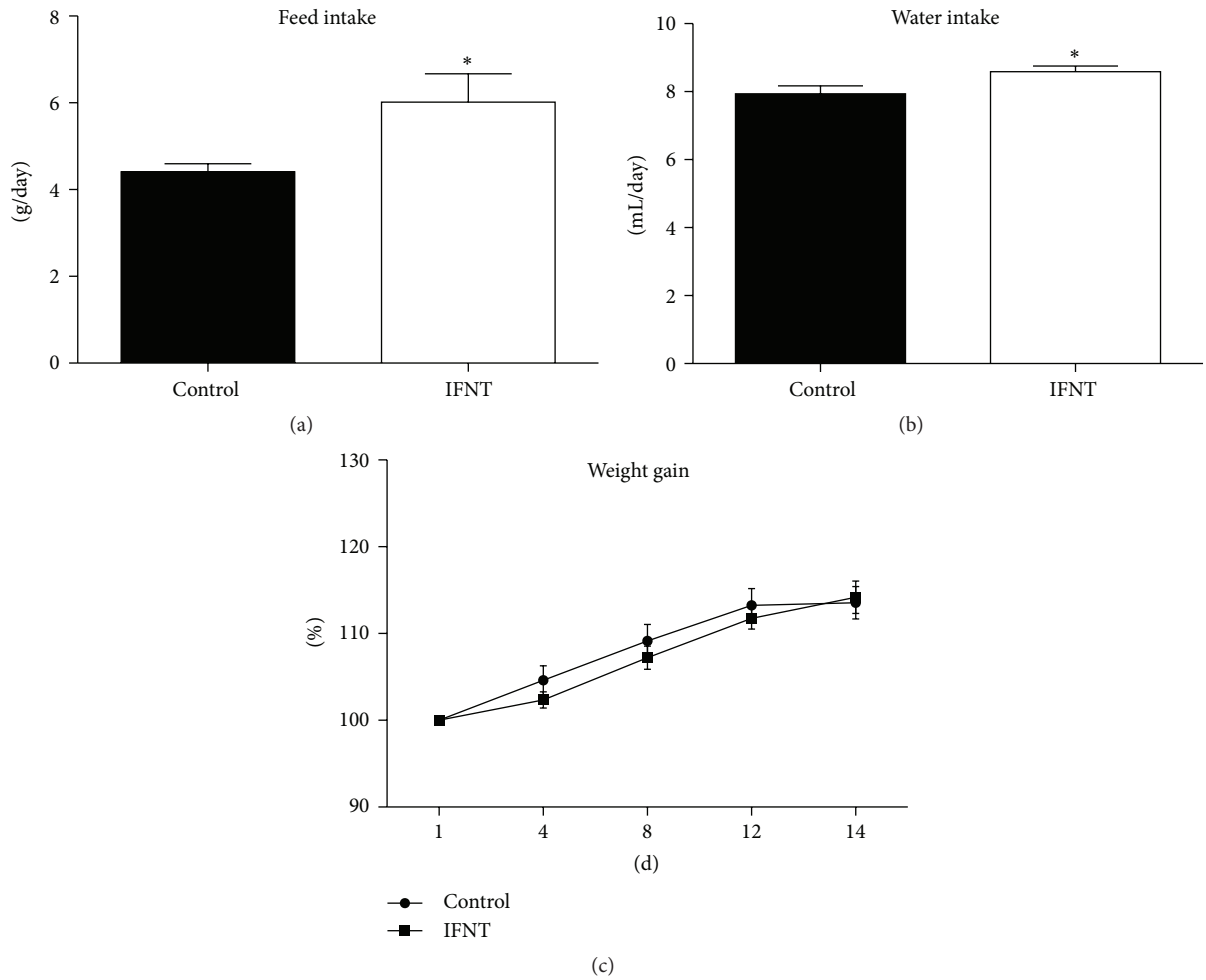

Figure 1: IFNT supplementation has little effect on mouse body weight. (a) Average feed intake in the control and IFNT-supplemented mice ($n = 10$). (b) Average water intake for control and IFNT-supplemented mice ($n = 10$). (c) Relative body weight gains for control and IFNT-supplemented mice ($n = 10$). Control mice were fed the basal diet and normal water, while mice in IFNT group were fed the basal diet and IFNT-supplemented water for two weeks. The asterisk ($*$) indicates a statistically significant difference between two treatment groups ($P < 0.05$). Data were analyzed using Student's t-test.

Table 1: Comparison of phylotype coverage and diversity estimation of the 16S rDNA gene libraries at 97% similarity from the pyrosequencing analysis.

Group	Number of readings	Number of OTU	Coverage	Richness estimator		Diversity index	
				Ace (95% CI)	Chao (95% CI)	Shannon (95% CI)	Simpson (95% CI)
Jejunum							
Control	12323	51	99.85%	78 (62–120)	70 (57–108)	1.06 (1.04–1.09)	0.52 (0.51–0.53)
IFNT	15653	64	99.95%	70 (66–83)	67 (65–78)	1.32 (1.30–1.35)	0.44 (0.43–0.45)
Ileum							
Control	12442	37	99.89%	52 (42–84)	57 (42–110)	0.73 (0.70–0.75)	0.70 (0.69–0.71)
IFNT	12264	66	99.79%	124 (99–167)	174 (101–400)	1.06 (1.03–1.08)	0.56 (0.55–0.56)
Colon							
Control	10327	298	99.58%	323 (311–343)	322 (309–349)	4.32 (4.29–4.35)	0.032 (0.030–0.034)
IFNT	11276	288	99.68%	306 (297–324)	313 (299–345)	4.32 (4.29–4.34)	0.027 (0.026–0.028)
Feces							
Control	12613	314	99.69%	336 (326–356)	339 (325–369)	4.39 (4.36–4.41)	0.026 (0.025–0.027)
IFNT	12493	312	99.58%	345 (331–371)	363 (337–414)	4.16 (4.13–4.19)	0.046 (0.044–0.049)

ileum, the diversity of microbiota (Shannon and Simpson) and richness indices (Ace) for mice with IFNT supplementation were higher than that of control mice (Table 1). For the microbiota in the colon, the diversity of microbiota (Shannon) and richness indices (Ace and Chao) were similar for IFNT-supplemented and control mice (Table 1). For the fecal microbiota, microbial diversity (Shannon and Simpson) in mice with IFNT supplementation was lower than for control mice, while the community richness (Ace and Chao) for IFNT-supplemented mice was similar to that for control mice (Table 1). Collectively, IFNT supplementation increases the diversity of microbiota in small intestine, while decreasing the diversity of microbiota in the feces.

3.3. IFNT-Associated Alterations in Intestinal Microbiota. The taxonomy of the intestinal microbiota was assessed using a taxon-dependent analysis and the RDP classifier. Seven phyla, including one candidate division (TM7), were found in the microbiota of the jejunum for all samples, including six phyla in the control mice and seven phyla in mice with IFNT supplementation. Eight phyla were found in the microbiota of the ileum of all samples, including six phyla in control mice and seven phyla in mice with IFNT supplementation. Ten phyla were found in the microbiota of the colon of all samples, including ten phyla in control mice and eight phyla in mice with IFNT supplementation. Ten phyla were found in the microbiota of the feces of all samples, including nine phyla in control mice and ten phyla in mice with IFNT supplementation.

For the jejunum, the two most abundant phyla in IFNT-supplemented mice, accounting for approximately 99% of all assigned sequence readings, were *Firmicutes* (94.5%) and *Bacteroidetes* (4.4%) (Figure 2(a)). In control mice, most abundant phyla were *Firmicutes* (97.6%) and *Bacteroidetes* (1.2%) (Figure 2(a)). For the ileum, the three most abundant phyla in IFNT-supplemented mice were *Firmicutes* (95.3%), *Candidate_division_TM7* (2.3%), and *Proteobacteria* (1.1%) (Figure 2(b)), while in control mice, they were *Firmicutes* (97.7%), *Proteobacteria* (1.5%), and *Bacteroidetes* (0.5%) (Figure 2(b)). For the microbiota in the colon, the three most abundant phyla in IFNT-supplemented mice were *Bacteroidetes* (72.2%), *Firmicutes* (23.0%), and *Proteobacteria* (4.1%) (Figure 2(c)), while they were *Bacteroidetes* (75.8%), *Firmicutes* (18.8%), and *Proteobacteria* (4.3%) in control mice (Figure 2(c)). For feces, the three most abundant phyla in IFNT-supplemented mice were *Bacteroidetes* (43.6%), *Firmicutes* (48.1%), and *Proteobacteria* (5.9%) (Figure 2(d)), while *Bacteroidetes* (53.2%), *Firmicutes* (39.2%), and *Proteobacteria* (5.3%) were most abundant for control mice (Figure 2(d)).

For the microbiota of the jejunum, the two most abundant genera in IFNT-supplemented mice, accounting for approximately 99% of all assigned sequence readings, were *Lactobacillus* (94.3%) and *S24-7_norank* (4.9%) (Figure 3(a)). In control mice, they were *Lactobacillus* (97.3%) and *S24-7_norank* (1.2%) (Figure 3(a)). For the ileum, the five most abundant genera in IFNT-supplemented mice were *Lactobacillus* (93.3%), *Candidatus-Saccharimonas* (2.3%), *Allobaculum* (1.2%), *Desulfovibrio* (1.1%), and *Enterorhabdus* (0.6%), while they were *Lactobacillus* (97.3%), *Candidatus-Saccharimonas* (0.3%), *Allobaculum* (0.1%), *Desulfovibrio* (1.5%), and *Enterorhabdus* (0.07%) in control mice (Figure 3(b)). For the microbiota in the colon, IFNT supplementation increased the percentages of *Blautia* (7.0% versus 5.1%), *Bacteroides* (6.4% versus 3.7%), *Alloprevotella* (5.2% versus 1.3%), and *Lactobacillus* (4.0% versus 2.5%), compared with control mice (Figure 3(c)). For the fecal microbiota, IFNT supplementation increased the percentages of *Lactobacillus* (30.0% versus 16.7%), *Bacteroides* (3.3% versus 1.8%), and *Allobaculum* (4.5% versus 0.4%), while decreasing the *Blautia* (2.7% versus 6.5%) compared with control mice (Figure 3(d)).

Collectively, IFNT supplementation affects the composition of intestinal microbiota in mice, especially those for the colon and feces.

3.4. IFNT Inhibits Expression IL-17 in the Intestine. The effect of IFNT supplementation on activation of intestinal innate immunity in mice was further explored, focusing on the expression of polymeric immunoglobulin receptor (*Pigr*), *Mucin-4*, *Cryptidin-1*, *Cryptidin-4*, *Cryptidin-5*, *Il-17*, interferon gamma (*Ifn-γ*), lysozyme (*Lyz*), and *J-chain* in the jejunum, ileum, and colon [18, 21]. In the jejunum, IFNT supplementation significantly decreased the expression of *Cryptidin-5*, *Il-17*, *Ifn-γ*, and *Lyz*, while it had little effect on the expression of the other transcripts (Figure 4(a)). IFNT supplementation had no significant effect on the expression of those transcripts in the ileum of mice (Figure 4(b)). In the colon, IFNT supplementation significantly lowered the expression of *Cryptidin-1* and *Il-17* but had little effect on the expression of the other transcripts (Figure 4(c)). As IFNT supplementation decreased the expression of *Il-17* in the jejunum and colon, we further validated the effect of IFNT to decrease expression of IL-17 in ETEC infected mouse model. We found that ETEC infection promotes the *Il-17* expression in the mouse jejunum at 6 hours after infection (W. Ren and Y. Yin, unpublished results). After two weeks of IFNT supplementation, expression of *Il-17* in the jejunum was significantly lower in IFNT-supplemented mice, compared to that of nonsupplemented mice during ETEC infection (Figure 4(d)). Thus, IFNT supplementation reduces the expression of the inflammatory cytokine, IL-17, in the intestine of mice.

4. Discussion

In this study, although two weeks of IFNT supplementation increases the mouse feed and water intake but has little effect on body weight of mice. Results of a previous study revealed that IFNT supplementation (8 μg/kg BW/day) reduces body weight beginning at 3 weeks after IFNT supplementation in Zucker Diabetic Fatty rats, while lower dose of IFNT supplementation (4 μg/kg BW/day) has no significant effect on body weight during 8 weeks of IFNT treatment [19], indicating that the effect of IFNT on body weight depends on dosage and duration of IFNT treatment. However, in a mouse model with high-fat or low-fat diet, 12 weeks of IFNT treatment does not significantly affect body weight

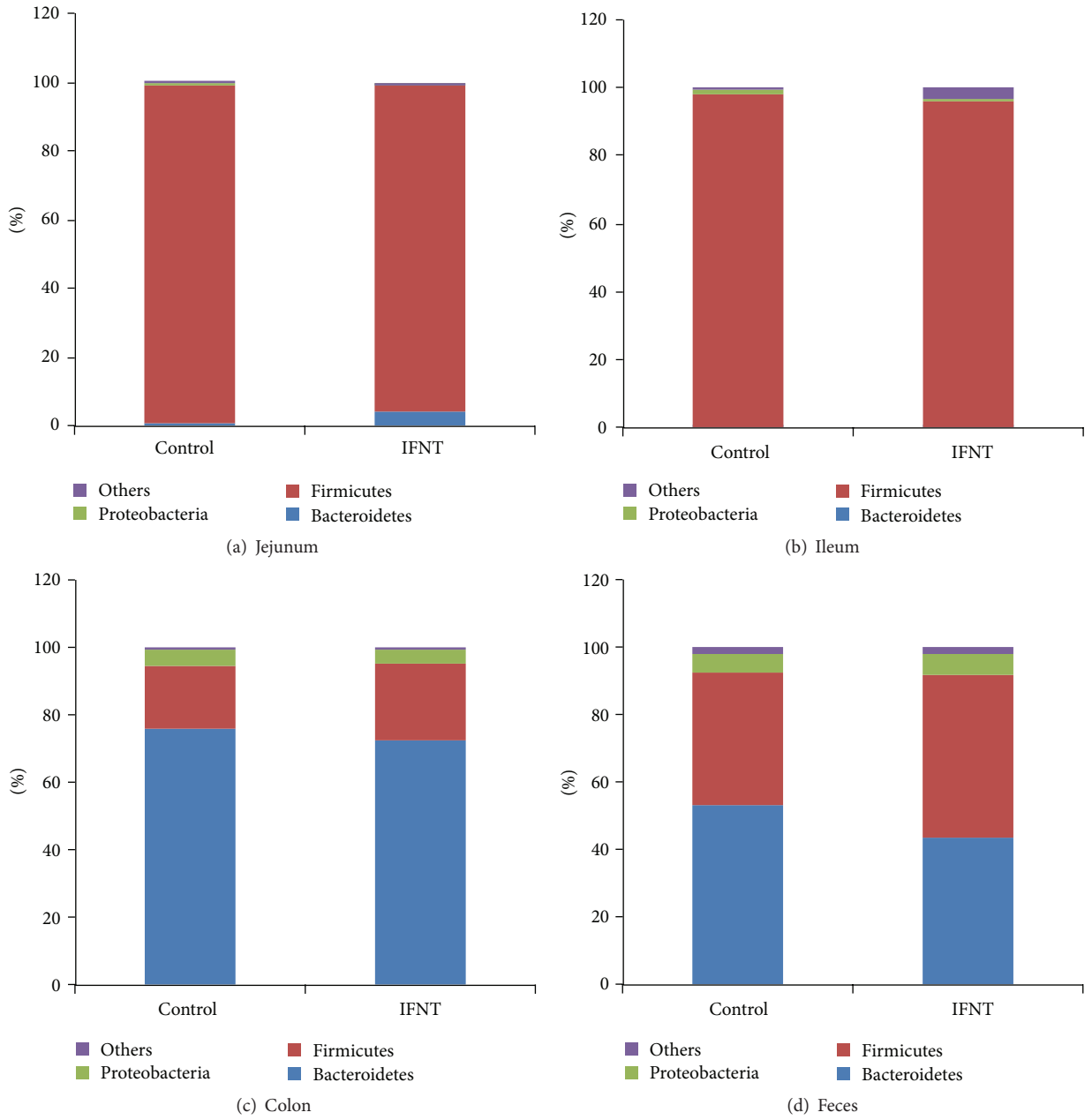

Figure 2: Composition of the intestinal microbiota at the phylum level after IFNT supplementation. (a) The microbial composition in the jejunum. (b) The microbial composition in the ileum. (c) The microbial composition in the colon. (d) The microbial composition in the feces.

[5]. However, IFNT supplementation has little effect on feed intake and water intake in those investigations [4, 5].

In the present study, IFNT supplementation increases the microbial diversity in the jejunum and ileum, while decreasing the microbial diversity in the feces of mice. The gut microbiota affects numerous biological functions [22, 23] and is linked to the pathogenesis of various diseases, such as obesity [24], cancer [25], and liver cirrhosis [26]. The influence of the gut microbiome on host physiological functions and the pathogenesis of disease in hosts may result from the activities of the microbiome and its metabolic products [22]. It is widely accepted that body weight is associated with the composition of intestinal microbiome and its metabolic capacity [27]. An increase in the relative proportion of *Firmicutes* is linked to obesity as *Firmicutes* ferments plant polysaccharides to produce short-chain fatty acids (SCFA), which provides additional energy for the host [28]. In phyla, IFNT supplementation decreases the percentage of *Firmicutes*, while increasing the *Bacteroidetes* in the jejunum and ileum. However, IFNT supplementation increases the percentage of *Firmicutes*, while decreasing the *Bacteroidetes* in the colon and feces. Thus, IFNT supplementation may regulate body weight and metabolism through effects on the intestinal microbiota. At the genus level, IFNT supplementation

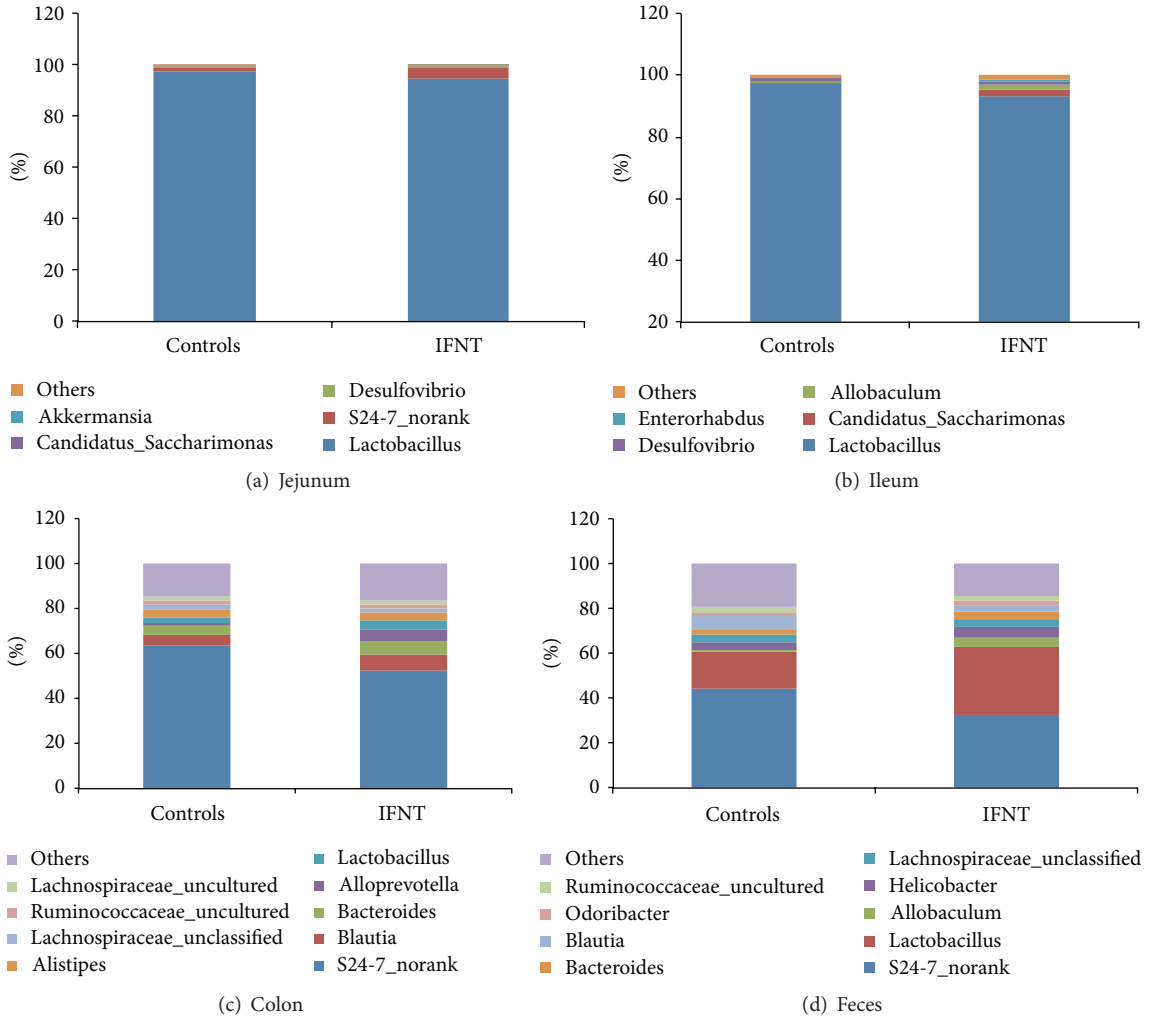

Figure 3: The composition of the intestinal microbiota at the genus level after IFNT supplementation. (a) The microbial composition in the jejunum. (b) The microbial composition in the ileum. (c) The microbial composition in the colon. (d) The microbial composition in the feces.

decreases the *Lactobacillus* in the jejunum and ileum but increases the percentage of *Lactobacillus* and *Bacteroides* in the colon and feces. *Lactobacillus* has critical roles in the intestine to combat gastrointestinal bacterial pathogens and rotaviruses through competitive metabolic interactions and the production of antimicrobial molecules [29]. *Bacteroides* are known for their capacity to metabolize a wide variety of oligosaccharides from the intestinal luminal, such as xylan, starch, and host-derived glycans [30]. Thus, results of the present study suggest that IFNT supplementation affects those functions of the intestinal microbiome in mice.

IFNT supplementation inhibits intestinal expression of IL-17, which suggests that IFNT reduces intestinal inflammation. IL-17 is produced by inducible Th17 (iTh17) cells and natural Th17 (nTh17) cells and regarded as an intestinal proinflammatory cytokine [31]. IL-17 can activate nuclear factor κB (NF-κB) transcription factors, extracellular signal-regulated protein kinase (ERK1 and ERK2), c-Jun N-terminal kinases (JNK-1 and JNK-2), and mitogen-activated protein kinases (p38 MAPKs) pathways, leading to upregulation of expression of inflammatory cytokines, such as IL-6 and IL-1 [32]. Recent investigations have revealed that mammalian target of rapamycin (mTOR) is a critical signaling pathway for Th17 responses and IL-17 expression [33–37]. The mTOR signaling regulates IL-17 expression through hypoxia-inducible factor 1 α (HIF-1α) and ribosomal protein S6 kinase (S6K: S6K1 and S6K2) [37–40]. mTOR signaling activates HIF-1α, which promotes IL-17 expression by activating RORγt (a key transcriptional regulator of Th17 cells) and mediating degradation of Foxp3 (a key transcriptional regulator of Treg cells) [40]. S6K1 promotes the expression of early growth response protein 2 (EGR2), which then inhibits growth factor independent 1 transcription repressor (GFI1), which can negatively regulate expression of IL-17 without affecting *Rorc* expression [37, 38]. S6K2 (the nuclear-localized counterpart of S6K1) binds to RORγt to promote nuclear translocation of RORγt, which can complex with HIF-α and p300 in the nucleus to promote expression of IL-17 [37–39]. Thus, the underlying mechanism by which IFNT supplementation reduces intestinal IL-17 expression is of interest. The effect of

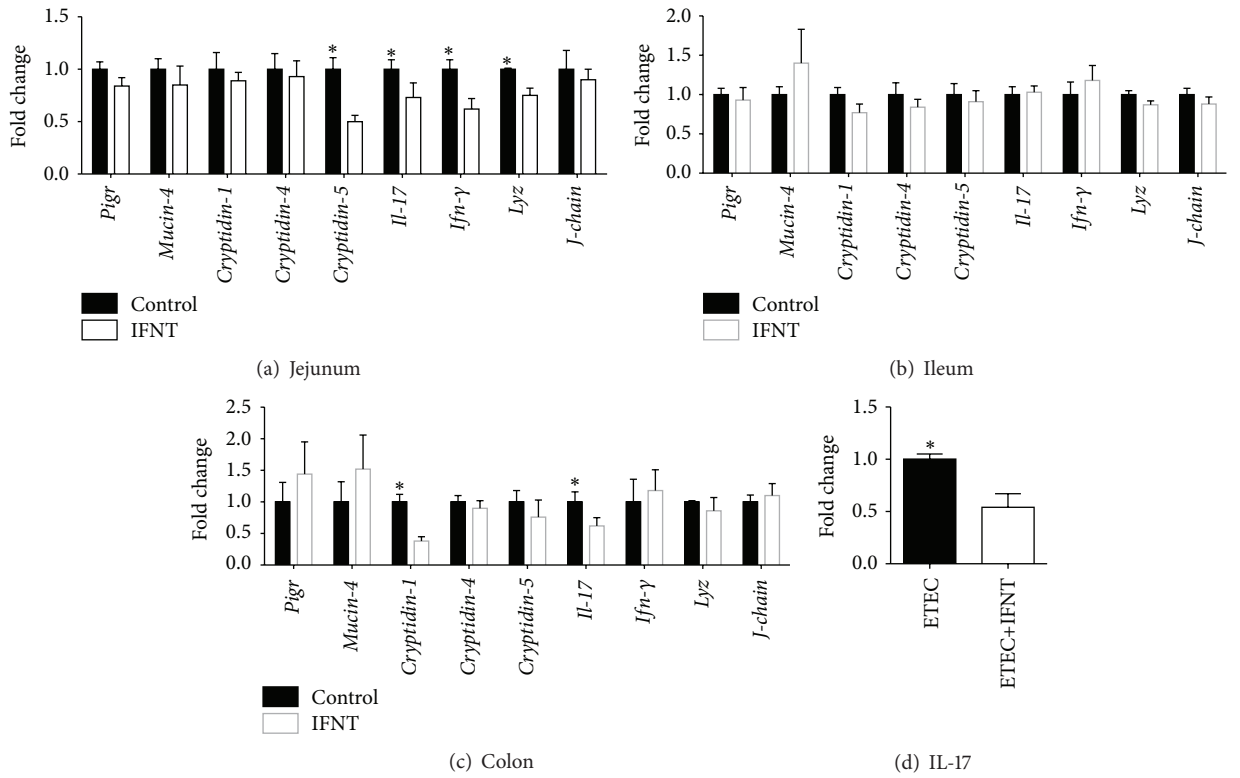

Figure 4: IFNT supplementation decreases expression of IL-17. (a) Expression of innate immune factors (*Pigr*, *Mucin-4*, *Cryptidin-1*, *Cryptidin-4*, *Cryptidin-5*, *Il-17*, *Ifn-γ*, *Lyz*, and *J-chain*) in the jejunum of mice ($n = 10$). (b) Expression of innate immune factors in the ileum of mice ($n = 10$). (c) Expression of innate immune factors in the colon of mice ($n = 10$). (d) IFNT decreases expression of IL-17 in the jejunum of mice following ETEC infection ($n = 10$). Data were analyzed using Student's t-test. An asterisk ($*$) indicates a statistically significant difference between treatment groups ($P < 0.05$).

IFNT supplementation to decrease expression of IL-17 in the intestine indicates a potential therapeutic application of IFNT to mitigate intestinal inflammatory diseases associated with expression of IL-17.

In conclusion, IFNT supplementation affects the diversity and composition of the intestinal microbiota and decreases expression of IL-17 in mice. The findings from this study are significant in understanding the physiological and immunological functions of IFNT in treatment of inflammatory diseases.

Abbreviations

EGR2: Early growth response protein 2
ERK: Extracellular signal-regulated protein kinase
ETEC: Enterotoxigenic *Escherichia coli*
FoxP3: Forkhead box P3
GFI1: Growth factor independent 1 transcription repressor
HIF-1α: Hypoxia-inducible factor 1α
mTOR: Mammalian target of rapamycin
RORγt: Retinoic acid receptor-related orphan receptor gamma t
S6K: Ribosomal protein S6 kinase.

Competing Interests

The authors have no competing interests.

Authors' Contributions

Wenkai Ren and Bie Tan designed the experiment; Wenkai Ren and Shuai Chen conducted the experiment; Wenkai Ren, Liwen Zhang, and Gang Liu analyzed the data; Tarique Hussain, Xiao Hao, Jie Yin, and Jielin Duan helped in the experiment; Wenkai Ren wrote the paper; Guoyao Wu, Fuller W. Bazer, and Yulong Yin revised the paper.

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Research Article

Conjugated Bilirubin Differentially Regulates CD4+ T Effector Cells and T Regulatory Cell Function through Outside-In and Inside-Out Mechanisms: The Effects of HAV Cell Surface Receptor and Intracellular Signaling

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We recently reported an immune-modulatory role of conjugated bilirubin (CB) in hepatitis A virus (HAV) infection. During this infection the immune response relies on CD4+ T lymphocytes (TLs) and it may be affected by the interaction of HAV with its cellular receptor (HAVCR1/TIM-1) on T cell surface. How CB might affect T cell function during HAV infection remains to be elucidated. Herein, *in vitro* stimulation of CD4+ TLs from healthy donors with CB resulted in a decrease in the degree of intracellular tyrosine phosphorylation and an increase in the activity of T regulatory cells (Tregs) expressing HAVCR1/TIM-1. A comparison between CD4+ TLs from healthy donors and HAV-infected patients revealed changes in the TCR signaling pathway relative to changes in CB levels. The proportion of CD4+CD25+ TLs increased in patients with low CB serum levels and an increase in the percentage of Tregs expressing HAVCR1/TIM-1 was found in HAV-infected patients relative to controls. A low frequency of 157insMTTTVP insertion in the viral receptor gene *HAVCR1/TIM-1* was found in patients and controls. Our data revealed that, during HAV infection, CB differentially regulates CD4+ TLs and Tregs functions by modulating intracellular pathways and by inducing changes in the proportion of Tregs expressing HAVCR1/TIM-1.

1. Introduction

Bilirubin (BR), long considered to be exclusively a toxic waste product, has recently been recognized as an immune-modulatory metabolite able to modulate CD4+ T lymphocyte (TL) function [1–3]. Particularly, we recently reported an immune-modulatory role of conjugated BR (CB) in hepatitis A virus (HAV) infection [1]. BR can suppress inflammation and increase antioxidant enzyme generation in activated neonatal neutrophils by downregulating the lipopolysaccharide- (LPS-) induced generation of IL-8 [4]. Moreover, *in vitro* models reveal that BR concentrations >25 μM modulate CD4+ T cell and neutrophil apoptosis [3, 4]. The induction of tolerance reported after the administration of BR to transplant recipients, which results from the *de novo* generation of T regulatory cells (Tregs) in murine models [5, 6], is in agreement with the ability of BR to inhibit T cell proliferation and to decrease IL-2 production in human lymphocytes [1]. Furthermore, a BR-conferred protection against autoimmune diseases has been described [3, 7, 8], presumably as a result of the capacity of BR to bind to the peptide binding groove of human leukocyte antigen (HLA) molecules, blocking the antigenic peptide presentation to T cell receptor (TCR) and hence suppressing autoimmune responses [9]. This is consistent with the fact that BR treatment results in downregulation of inducible MHC class II expression, affecting Ag presentation to CD4+ T cells [3], and supports that, under pathological circumstances, changes in normal BR concentration may modulate specific immune responses through specific receptors.

Infections with hepatotropic viruses cause an elevation of serum aminotransferase activity and of serum-associated BR [1, 10, 11]. Viral hepatitis A is a major health concern worldwide, with a higher incidence in developing countries. Although improved hygiene and vaccination have reduced the HAV infection rate, the virus remains widespread and the infection is generally acquired in early childhood [12]. HAV is also considered a foodborne pathogen, based on the documented outbreaks of infection caused by the consumption of frozen fruits in developed countries [13–15], as well as a major cause of acute liver failure and transplant in pediatric patients [16]. The progression of HAV infection is restricted by the host immune response [10], which may also be affected by host-metabolic components. Particularly, during the final stages of HAV infection, heme degradation is interrupted, leading to the deregulation of BR internalization and excretion by hepatocytes, which results in increased CB values (0.3 to 6 mg/dL) [11].

We recently reported that CB plays a role in adjusting STAT-1 and STAT-5 function and in determining cytokine profiles during HAV infection [17, 18]. The fact that a TGF-beta-associated anti-inflammatory cytokine profile is observed in HAV-seropositive pediatric patients with low serum values of CB (<2 mg/dL) suggests a potential role for Tregs in the clinical courses induced by HAV. Conversely, a proinflammatory profile is found in patients with higher serum CB levels (>2 mg/dL) [18, 19]. This is consistent with the temporary inhibition of Tregs function described during infection, which has been explained in terms of a specific interaction between HAV and its cellular receptor HAVCR-1/TIM-1 on the T cell surface [20]. Interestingly, a six-amino-acid insertion in the *HAVCR1/TIM-1* (157insMTTTVP) gene is associated with the development of severe HAV infection [21]. Although data from several reports indicate that this receptor, together with TCR and costimulatory signals, regulate the expansion and effector functions of T helper cells [22], the functions and mechanisms through which HAVCR1/TIM-1 may be regulating T cell activity are poorly understood. Particularly, whether specific metabolic products, such as BR, might affect these mechanisms during the course of HAV infection remains to be elucidated.

2. Materials and Methods

2.1. Reagents. CB was from Merck-Millipore, Darmstadt, Germany. Anti-CD3 mAb, anti-CD28 mAb, anti-phosphotyrosine (anti-pTyr) PY20-Alexa 488, anti-CD4-Alexa 488, anti-CD25-PercP, anti-FOXP3-Alexa 488, and anti-TIM-1-PE were from Biolegend, San Diego, CA, United States. Rabbit anti-mouse-IgG1 and IgG2a were from Fisher Biotec, Hampton, New Hampshire, United States. Carboxyfluorescein diacetate succimidylester (CFSE) was from Biolegend. The 7-Plex T-Cell Receptor Th17 and IL-17 Magnetic beads MAGPIX Kits were from Merck-Millipore. Treg ($CD4+CD25+CD127^{low}$) and CD4+ T cell isolation kit were from Miltenyi Biotec, Bergisch Gladbach, Germany. The primers were from IDT, Coralville, Iowa, United States. The DNA Extraction and Purification Kit and Gel and PCR Clean-up System were from Promega, Madison, Wisconsin, United States. Recombinant Taq DNA Polymerase was from Thermo Fisher Scientific, Waltham, Massachusetts, United States, and QIA Quick PCR purification kit was from Qiagen, Hilden, Germany.

2.2. In Vitro Analysis

2.2.1. Cell Purification. Ficoll-Paque PLUS (Healthcare, Uppsala, Sweden) gradient centrifugation was used to isolate peripheral blood lymphoid cells (PBLCs) from anticoagulated blood samples of pediatric healthy donors as described previously [17]. The buffy coat of each sample was washed three times with PBS (300 ×g; 10 min; room temperature) and resuspended in autoMACS Running Buffer (Miltenyi Biotec). CD4+ TLs were purified by negative magnetic selection with the CD8, CD19, CD123, and CD127 antibodies followed by Treg (CD4+CD25+) positive selection with anti-CD25 Micro Beads (Miltenyi Biotec). Before experimentation, CD4+ TLs and Tregs were arrested for 2 h in RPMI 1640 (HyClone, Logan, UT) supplemented with 2% (v/v) fetal calf serum (FCS) with 2 mM L-glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 50 μM β-mercaptoethanol (Sigma, St Louis, MO).

2.2.2. Phosphotyrosine Intracellular Staining. Purified CD4+ TLs (5×10^6) from pediatric healthy donors were incubated according to different protocols. Cells were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies

(Biolegend) with or without various doses of CB (0.3, 2 or 15 mg/dL) in 1 mL RPMI 1640 medium supplemented with 10% FCS and 5% CO_2 at 37°C for 30 minutes. Nonstimulated cells were included as a negative control. After the incubation, the cell pellet was obtained by centrifugation (300 ×g; 10 min; room temperature) and resuspended in 100 μL of 1x fixation/permeabilization buffer (Biolegend Fix/Perm Buffer). Cell permeabilization was performed for 30 min at 4°C under dark conditions. The cells were then washed twice by centrifugation and permeabilized by adding 50 μL of 1x permeabilization buffer (Biolegend Perm Buffer). Then, cells (1×10^6) were resuspended in 100 μL of assay buffer (Biolegend) and incubated with 2.0 μL of anti-phosphotyrosine PY20-Alexa 488 antibody (30 min; 4°C) while being protected from light. The cells were then washed twice by centrifugation (300 ×g; 10 min), resuspended in 250 μL PBS, and analyzed using a GUAVA EASYCYTE 6 with INCYTE 2.0 software (Merck-Millipore). The median fluorescence intensity (MFI) was obtained from the acquisition of 10,000 events of triplicate counts of 1×10^6 cells.

2.2.3. Treg Cell Suppression Assay. A total of 1×10^6 Tregs (CD4+CD25+FOXP3+) per condition purified from pediatric healthy donors were preincubated with increasing concentrations of CB (0, 0.3 and 2 mg/dL), in a final volume of 300 μL of supplemented RPMI (5% fetal calf serum (FCS) + 5% bovine calf serum (BCS)) for 72 h at 5% CO_2. Following which, Tregs were recovered and cocultured with CD4+CD25− effector T cells (Teff) previously labeled with 2 μM CFSE at 37°C for 10 min. Cocultures of 1 : 2 ratio of Teff : Tregs (preincubated with or without CB), at 37°C and 5% CO_2, were performed in final volume of 300 μL/well, in RPMI 1640 supplemented with 5% fetal calf serum (FCS) + 5% bovine calf serum (BCS) and stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL). After 7 days of coculture, the cells were harvested and cell proliferation was evaluated by flow cytometry. The proliferation of Teff stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies in the absence of Tregs was used as a positive control. The percent of suppression was determined with the following formula: (mean final Teff cell number − mean final Teff cell number incubated with Treg cells)/(mean final Teff cells number) × 100.

2.2.4. Percentage of Tregs Expressing TIM-1. A total of 400,000 Tregs per condition from pediatric healthy donors were stimulated with anti-CD3 (1 μg/mL), anti-CD28 (1 μg/mL), and different doses of CB (0.3, 2, or 15 mg/dL) in a total volume of 1 mL of RPMI 1640 (Hyclone) (10% FCS) for 72 h at 37°C and 5% CO_2. Nonstimulated cells were included as a negative control. Cells were recovered and stained with 2.0 μL of TIM-1-PE antibody for 20 min at 2–8°C under dark conditions. Finally, the percentage of Tregs TIM-1+ cells was determined by flow cytometry. Three independent experiments were performed.

2.2.5. IL-17 Cytokine Profile. Purified CD4+ TLs (1×10^6) from pediatric healthy donors were incubated according to different protocols. Cells were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies in absence of presence of various doses of CB (0.3, 2 or 15 mg/dL) in 1 mL RPMI 1640 medium supplemented with 10% FCS and 5% CO_2 at 37°C for 72 hours. Nonstimulated cells were included as a negative control. After the incubation, cell supernatant (100 μL) was recovered and clarified by high-speed centrifugation. We used a 1-plex kit (IL-17) from Merck-Millipore, and cytokine analysis was performed using a MAGPIX system powered by xMAP Luminex Technology with the xPONENT® software of EMD (Merck-Millipore). The assay was performed according to the supplier's instructions. Briefly, following the prewetting of each plate, 50 precombined beads of IL-17 were added and washed twice. Cell supernatants (25 μL) from distinct stimulation conditions were diluted 1 : 2 with the assay buffer and added to the plate. The plate was shaken for 30 s at 300 g and then incubated overnight on a plate shaker at 300 g at room temperature. The plate was washed twice, 25 μL of detection antibody was added per well, and the plate was then incubated for one hour on a plate shaker. Subsequently, 50 μL of a streptavidin-PE conjugate was added per well and incubated for 30 min at room temperature. Finally, the plate was washed three times, 150 μL of sheath fluid was added to each well, and the plate was read by MAGPIX machine (Merck-Millipore).

2.3. Ex Vivo Analysis

2.3.1. Study Population. A total of 315 unrelated subjects from South and West México were included in this study, 156 of which were pediatric patients (<15 years old) with acute HAV infection (85 sera samples from South México pediatric patients recruited during 2011 and 71 blood samples from West México pediatric patients recruited during May 2015 to February 2016). The remaining subjects included 60 pediatric healthy controls and 99 healthy adult donors (18–50 years old) from West México. The study was conducted at the Centro de Referencia de Hepatitis Virales del Occidente de México and the Unidad de Inmunovirologia in the Servicio de Biologia Molecular, Hospital Civil de Guadalajara, Fray Antonio Alcalde (HCFAA), in Guadalajara, Jalisco, México.

Patients and healthy donors with liver disease who were undergoing treatment with a hepatotoxic drug, those with acute or chronic hepatitis E virus (HEV), hepatitis B virus (HBV), or hepatitis C virus (HCV) infections, and those diagnosed with autoimmune hepatitis were excluded from the study. None of the pediatric patients and pediatric healthy controls included in the study had been vaccinated against HAV and HBV. After the healthy donors and children's parents had provided informed consent, blood samples were obtained by venipuncture. The Ethical Committees of the HCFAA and the Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, approved this study.

2.3.2. Clinical and Demographic Data. Hepatitis was defined as hepatomegaly, fever (>38°), and/or jaundice with elevated values of serum AST (>38 IU/L) and ALT (>35 IU/L), as previously described [18]. Additionally, CB (>0.3 mg/dL) and albumin values were measured and demographic and clinical

Table 1: Demographic and clinical characteristics of HAV-infected pediatric patients and controls.

Characteristic	Healthy controls ($n = 60$)	Patients		P value
		HAV+ CB: >0.3–2 mg/dL ($n = 70$)	HAV+ CB: >2 mg/dL ($n = 86$)	
Gender (% female)	41	58	56	NS
Mean age (years ± SD)	6.5 ± 2.5	6.5 ± 3.425	8.05 ± 3.85	NS
Mean ALT (IU/L ± SD)	21.58 ± 13.72	733.39 ± 629.50	1471.06 ± 1238.43	NS
Mean AST (IU/L ± SD)	13.87 ± 9.99	513.94 ± 493.8	1041.06 ± 935.33	<0.05
Mean CB (mg/dL ± SD)	0.20 ± 0.087	1.15 ± 0.70	5.33 ± 2.65	—
Anti-HAV IgM	–	+	+	—
Anti-HAV IgG	–	–	–	—

ALT: alanine aminotransferase; AST: aspartate aminotransferase; SD: standard deviation; NS: not significant.

features were recorded using a structured questionnaire, as previously reported [18].

2.3.3. Serological Tests. To detect acute hepatitis A infection, serum samples from pediatric patients diagnosed with hepatitis were screened for the presence of anti-HAV IgM and the absence of anti-HAV IgG. All samples were negative for antibodies to HBV, HCV, and HEV. The presence of anti-HAV IgM and the absence of anti-HAV IgG, the surface antigen of HBV (HBsAg), and anti-HCV antibodies were tested by using a third-generation microparticle immunoenzymatic assay (AxSYM HAVAB-M 2_0, AxSYM HBsAg (V2), and AxSYM HCV 3.0; Abbott Laboratories, Chicago, IL) with an AxSYM analyzer (Abbott Laboratories). Total anti-hepatitis B core antigen anti-HBc (total IgM and IgG) and anti-HEV antibodies were measured by using immunoenzymatic assays (Monolisa Anti-HBc PLUS, Bio-Rad Laboratories, Chicago, IL, MP Diagnostics, Geneva, Switzerland and MyBiosource, San Diego, CA, USA, resp.) with a PR 3100 TSC analyzer (Bio-Rad). The levels of albumin/globulin, ALT, AST, alkaline phosphatase, total protein, total BR, and CB were measured in the serum samples, following routine clinical laboratory procedures.

2.3.4. Liver Injury Categorization in HAV-Infected Children. Pediatric patients who tested positive for acute HAV infection (anti-HAV IgM^+ and anti-HAV IgG^-) and negative for antibodies to HBV, HCV, and HEV and who exhibited abnormal levels of ALT and AST (>38 IU/L and/or >35 IU/L, resp.) were categorized as follows and detailed in Table 1:

Patients who exhibited CB levels > 0.3 mg/dL to 2 mg/dL.

Patients who exhibited CB levels > 2 mg/dL.

Healthy Controls (H). Children with normal hepatic enzymatic activity in the absence of HAV, HEV, HBV, and HCV serological markers.

2.3.5. TCR Signaling Pathway. The cell lysates of purified CD4+ T cells (5×10^6) from 16 healthy pediatric donors, 11 pediatric patients infected with HAV with serum CB levels between 0.3 and <2 mg/dL, and 7 pediatric patients infected with HAV with serum CB levels > 2 mg/dL were analyzed to detect TCR pathway proteins phosphorylation. Cells were lysed with 100 μL of lysis buffer (100 mM Hepes of pH 7.5, 1 M $MgCl_2$, 3 M NaCl, 1 mM EDTA, Triton X-100 and a protease inhibitor cocktail of PMSF, BGP, NAF, $NaVO_4$, leupeptin, antipain, aprotinin, and DTT). Then, cell lysates were incubated for 30 min at 4°C with stirring, and finally, supernatant was obtained by centrifugation (300 ×g; 10 min; 4°C). The protein supernatant content was estimated by a microwell plate version of the Bradford method. The cell lysates were analyzed with a 7-plex T cell receptor kit (CD3 epsilon, CREB, ERK MAP 1/2, LAT, LCK, SYK, and ZAP-70 phosphoproteins) with a MAGPIX system powered by xMAP Luminex Technology with the xPONENT® software. Briefly, following the prewetting of each plate for 10 min with assay buffer, 25 μL of cell lysates (20 μg of protein) and 25 μL of precombined beads of all the 7 individual proteins were added to the plates. The plates were shaken and incubated overnight at 4°C while protected from light. Then, the plates were washed twice, 25 μL of detection antibody was added to each well, and the plates were further incubated for one hour on a plate shaker while protected from light. Detection antibody was removed and subsequently, 50 μL of a streptavidin-PE conjugate was added per well and incubated for 15 min at room temperature while protected from light. A total of 25 μL of Amplification buffer was added per well, and plates were incubated 15 min at room temperature while protected from light. Finally, the plate was washed three times to remove streptavidin-PE and Amplification buffer and, beads in each well were resuspended with 150 μL of assay buffer. The plate was read by MAGPIX machine. HeLa and stimulated Jurkat cell lysates were used as negative and positive controls, respectively. At least 50 events per bead were read for each sample in triplicate wells.

2.3.6. Th17 Cytokine Profile Analysis. Before cytokine evaluation, sera from pediatric healthy donors and HAV-seropositive patients were first clarified by high-speed centrifugation. We used a 5-plex kit (IL-6, IL-21, IL-22, and macrophage inflammatory protein 3α (MIP-3α (CCL20)) and IL-17F) from Merck-Millipore, and multicytokine analysis

was performed using a MAGPIX system. The assays were performed as described before.

2.3.7. CD4-CD25 Staining. PBLCs of 32 pediatric patients coursing the acute phase of HAV infection and 17 pediatric healthy donors were costained with anti-CD25+ and anti-CD4+ antibodies. Briefly, 1×10^6 cells were resuspended in 100 μL of assay buffer (Merck-Millipore) and incubated with 2.0 μL of anti-CD4-Alexa 488, anti-CD25-PercP antibodies (30 min; 2–8°C) while protected from light. The cells were then recovered by centrifugation (300 ×g; 5 min), resuspended in assay buffer, and evaluated by flow cytometry. The percentage of positive cells was obtained from the acquisition of 10,000 events. Triplicate counts from the 1×10^6 cells resuspended in assay buffer were conducted.

2.3.8. CD4-CD25-TIM-1 Staining. A total of 1×10^6 PBLCs per condition from 17 HAV-seropositive pediatric patients and 25 pediatric healthy donors were incubated with 2.0 μL of Alexa Fluor anti-CD4 antibody, 2.0 μL of anti-CD25-PE, and 2.0 μL of anti-PerCP TIM-1 in dark conditions at 2–8°C for 30 min. The cells were then recovered by centrifugation at 300 ×g for 5 min, resuspended in assay buffer, and analyzed by flow cytometry. The percentage of positive cells was obtained from the acquisition of 10,000 events. Triplicate counts from the 1×10^6 cells resuspended in assay buffer were conducted.

2.3.9. Evaluation of Polymorphism 157insMTTTVP on HAVCR1/TIM-1 Gene Locus. Total genomic DNA was purified from PBLC samples obtained from 99 unrelated donors and 21 HAV-infected pediatric patients. PCR and subsequent DNA sequencing of 294 bp covering exon 4 of gene encoding *HAVCR1/TIM-1* HAV receptor allowed detecting the 157insMTTTVP polymorphism as previously described [23]. Briefly, total genomic DNA was extracted using Wizard Genomic DNA Purification Kit following manufacturer instructions and used as template for PCR. Oligonucleotides used were Forward 5′-GGG CAA TGA CCA AGA TTG AC-3′ and Reverse 5′-ACC TTG ATA CAA TGC CCT GG-3′ [24]. PCR reactions were performed in a 50 μL reaction volume, containing 20–50 ng DNA, 2 mM $MgCl_2$, 1x reaction buffer (Promega, MA), 0.2 mM dNTPs (Invitrogen, CA), 0.4 mM of each oligonucleotide, and 0.4 U of Taq DNA Polymerase. PCR reactions were incubated in a Techne Endurance Tc-300 system (Staffordshire, UKA) by 35 cycles of 94°C/1 min, 55°C/1 min and 72°C/1 min. Amplified DNA products were separated in 1.5% agarose gels and visualized by ethidium bromide staining. DNA fragments of the expected length were extracted from gel using Wizard PCR Clean-Up system, quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific), and sequenced bidirectionally using BigDye Terminators v2.0 Cycle Sequencing Kit (Applied Biosystems ABI, Foster City, CA) following manufacturer instructions. Sequencing reactions were run on an automated DNA sequencer ABI PRISM 3700 (ABI). Electropherograms obtained from sequencing files were edited with the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html. Carlsbad, CA) and converted into fasta files. Sequences were assembled and compared with reference sequences retrieved from GenBank by using MEGA software version 6.0 (Molecular Biology and Evolution 30: 2725–2729). A multisequence alignment file was obtained as final dataset using ClustalW algorithm with default settings, and allowed detecting the presence of the 18-nt gene insertion based on comparison with the sequences downloaded from GenBank. The reference sequences from GenBank were AF043724 (wild-type: no insertion) and CR457114 as an example of the 18-nt gene insertion as reported [23].

2.4. Statistical Analysis. Data are presented as the mean or medians and standard deviation (SD). Statistical comparisons were performed by using Graph Pad Prism software version 5.01 (Graph Pad Software, Inc., San Diego, CA). Nonparametric Kruskal-Wallis and Mann-Whitney *U* tests for comparisons between groups were used to calculate the statistical significance of the assay results. A P value < 0.05 was considered statistically significant. Post hoc methods were used to ensure that there were differences between the compared groups. To study associations between variables, the Spearman correlation coefficients were calculated.

3. Results

3.1. CB Reduces the Degree of Tyrosine Phosphorylation in CD4+ TLs. CD4+ TLs were treated with CB, and the degree of tyrosine phosphorylation was assessed to determine the effect of this metabolite on the overall signaling capacity of the cells. As expected [25, 26], we observed an increased MFI of phosphorylated tyrosine in CD4+ TLs stimulated with anti-CD3 and anti-CD28 antibodies (157.2 ± 58.23) as compared with cells that were not stimulated (54.90 ± 32.03) (Figures 1(a) and 1(b)). However, treating the cells with clinically relevant concentrations of CB in conjunction with CD3 and CD28 engagement resulted in decreased intracellular tyrosine phosphorylation as compared to CD3- and CD28-stimulated cells (Figures 1(c)–1(f)). Together, these data suggest that CB causes changes in the intracellular signaling pathways of TLs.

3.2. CB Modifies the Degree of CD3-Epsilon, SYK, and CREB Phosphorylation in CD4+ TLs of HAV-Seropositive Pediatric Patients. The TCR signaling pathway is an intracellular signaling pathway specifically related to the activity and function of T cells. The MFIs of phosphorylated CREB, CD3-epsilon, SYK, ERK, LCK, LAT, and ZAP-70 were evaluated in CD4+ TL lysates from pediatric healthy donors, HAV-seropositive pediatric patients with CB levels between 0.3 and 2 mg/dL, and HAV-seropositive pediatric patients with CB > 2 mg/dL under basal conditions (Figure 2(a)). No changes in the degree of phosphorylation for ERK, LCK, LAT, and ZAP-70 were found. In contrast, a significant increase in the MFI of phosphorylated CREB in patients with CB between 0.3 and 2 mg/dL (77.40 ± 60.44) compared with patients with CB > 2 mg/dL (28.79 ± 3.414) and healthy controls (29.75 ± 4.665) was observed, Figure 2(d). Interestingly, a

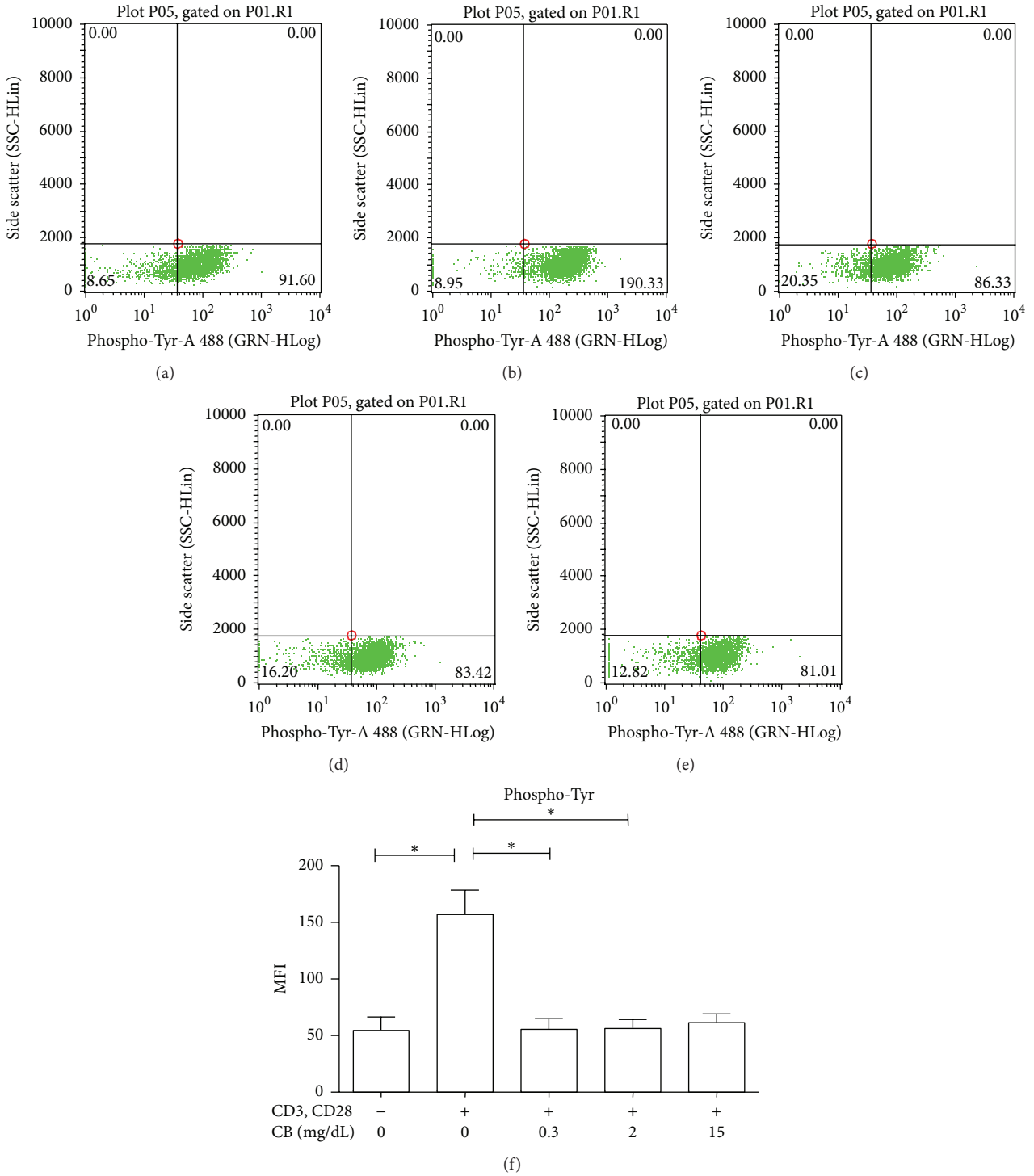


Figure 1: CB causes a decrease in the degree of tyrosine phosphorylation in human CD4+ TLs *in vitro*. Purified CD4+ TLs from pediatric healthy donors were incubated at 37°C, 5% CO_2, 30 min, under different conditions: (a) TLs without stimulus. (b) TLs with anti-CD3 and anti-CD28. (c) TLs with anti-CD3, anti-CD28, and 0.3 mg/dL CB. (d) TLs with anti-CD3, anti-CD28, and 2 mg/dL CB. (e) TLs with anti-CD3, anti-CD28, and 15 mg/dL CB. Cells were subsequently recovered and stained with an anti-pTyr mAb and then analyzed by using flow cytometry. Representative dot plots are shown (a–e). (f) The medians and standard deviations of three repetitions are presented. Nonparametric Kruskal-Wallis for comparison between groups was used to calculate statistical significance. $P < 0.05$ was considered statically significant. $^{*}P < 0.05$.

trend toward a reduction in the MFI of phosphorylated CD3-epsilon and SYK and a significant reduction in the MFI of phosphorylated CREB was found in patients with CB levels > 2 mg/dL compared with patients with CB levels between 0.3 and 2 mg/dL (Figures 2(b)–2(d)). These results suggest that, in the context of HAV infection, an augment in phosphorylation of the TCR signaling pathway occurs and it is not affected by discrete CB levels in the microenvironment, whereas CB levels greater than 2 mg/dL result in a reduced phosphorylation of the TCR signaling pathway.

3.3. The Percentage of CD4+CD25+ T Cells in HAV+ Patients Increased with Low Serum CB Concentration. Previous data show differences relative to the intracellular activity of CD4+ TLs based on the CB concentrations present in the medium. To have an overview of the potential role of CB on Tregs subpopulation in HAV+ patients, anti-CD4 and anti-CD25 staining were performed in pediatric patients and pediatric healthy controls. Patients diagnosed with acute HAV infection and presenting CB values below 2 mg/dL show a significantly increased percentage of CD4+CD25+ T cells (8.995 ± 3.006) compared with patients with CB levels > 2 mg/dL (3.068 ± 1.992) and healthy controls (3.546 ± 1.928) (Figures 3(a), 3(b), and 3(c)). This observation agrees with the analysis of CD4+ cells from HAV+ patients with distinct CB levels and stained with anti-CD25 and anti-FOXP3, where we found an increase in the percentage of CD4+CD25+FOXP3+ cells in those HAV+ patients with serum CB levels < 2 mg/dL (data not shown). Moreover, a trend toward a negative correlation between the percentage of CD4+CD25+ T cells and CB levels in HAV+ patients was found (Figure 3(d)). A Th17 profile characterized for increased levels of IL-6, IL-21, IL-22, CCL20, and IL-17F was found in patients with CB > 2 mg/dL (Figure 3(e)). Altogether, these data suggest that the proportion of Tregs is essential in the modulation of the inflammatory process activated during HAV infection and subsequently that Tregs proportion may be related to the serum CB concentration.

3.4. Tregs Activity Is Augmented after CB Treatment In Vitro. To determine the potential role of CB in modulating the activity of Treg cells, coculture assays were performed with Tregs from pediatric healthy donors that were pretreated with and without CB and their autologous CFSE-labeled Teffs in the presence of anti-CD3 and anti-CD28 mAbs. Efficient Teff proliferation (nonsuppressive activity) was observed after stimulation with anti-CD3 and anti-CD28 in the absence of Tregs (data not shown). In contrast, a trend toward increased suppressive activity of Tregs was observed when pretreated with 0.3 mg/dL CB relative to Tregs without CB pretreatment (Figures 4(a), 4(b) and 4(d)). A significant increase in Treg suppressive activity was observed after treatment with 2 mg/dL CB relative to Tregs without CB pretreatment (Figures 4(c) and 4(d)). Moreover, in patients with low CB levels (0.3–2 mg/dL), Tregs appear to be less active, supporting that CB values greater than 2 mg/dL result in increased Treg activity. No differences in IL-17 levels were found in supernatants from TLs treated with anti-CD3, anti-CD28, and different doses of CB relative to TLs treated with anti-CD3 and anti-CD28 without CB (Figure 4(e)), suggesting that CB does not induce a Th17 profile. Thus, our data support that the effect of CB is on Tregs function and this may be related to the efficient control of the inflammatory process activated during HAV infection.

3.5. Following CB Treatment In Vitro, TIM-1 Expression in Tregs Is Augmented. As our data pointed out that CB upregulates the numbers and the activity of Treg cells we investigated the possible mechanism responsible for this; particularly, we assessed whether changes in TIM-1 expression on the Tregs surface may influence the interaction between the virus and cell and the consequent functionality/activity of these cells during a specific period of the infection. Herein, the effect of CB on the relative proportion of TIM-1 on Tregs was evaluated. Tregs purified from pediatric healthy donors were treated with varying concentrations of CB and stimulated with anti-CD3 and anti-CD28 mAbs. Then, cells were stained to determine possible changes in the expression of TIM-1 because of CB addition. A significant increase in the percentage of TIM-1 expression in Tregs treated with CB (2 mg/dL) (4.783 ± 1.341) and CB (15 mg/dL) (5.333 ± 0.3803) (Figure 5(f)) compared with control (unstimulated cells without CB) (1.620 ± 0.7238) (Figure 5(a)) was found. Additionally, the percentage of cells expressing TIM-1 increased as the concentration of CB increased (Figures 5(c)–5(f)). These data suggest that CB levels have an effect on the expression of TIM-1 in Tregs.

3.6. HAV Infection Leads to an Increase in the Number of CD4+CD25+ T Cells Expressing TIM-1. To determine whether the proportion of CD4+CD25+ T cells expressing TIM-1 changes during the course of HAV infection, samples from healthy donors and HAV+ patients were analyzed *ex vivo* as outlined in Figure 6. The percentage of CD4+CD25+ TIM-1 positive cells was significantly higher in HAV-infected pediatric patients (1.658 ± 0.25) compared with healthy controls (0.6628 ± 0.07482) Figure 6(c). Given the acute status of the infection in these patients (all of them had CB > 0.3 mg/dL), these data are consistent with the *in vitro* results: CB modulates the proportion of CD4+CD25+TIM-1+ T cells, even in the presence of the virus.

3.7. In Healthy Donors and HAV-Seropositive Patients the 157insMTTTVP Insertion in the HAVCR1/TIM-1 Gene Is Found at a Low Frequency. The 157insMTTTVP polymorphism located in exon 4 of the gene *HAVCR1/TIM-1* has recently been associated with the development of fulminant hepatitis A in an Argentinean population [23]. To determine if the presence of this polymorphism and the activity of Treg cells during HAV infection could be related, the frequency of this polymorphism in a control group and a group of HAV+ pediatric patients was estimated. Gene sequencing of exon 4 was performed and aligned against reference sequences from GenBank in a cohort, which included a total of 99 control samples (98 sequences obtained with primer

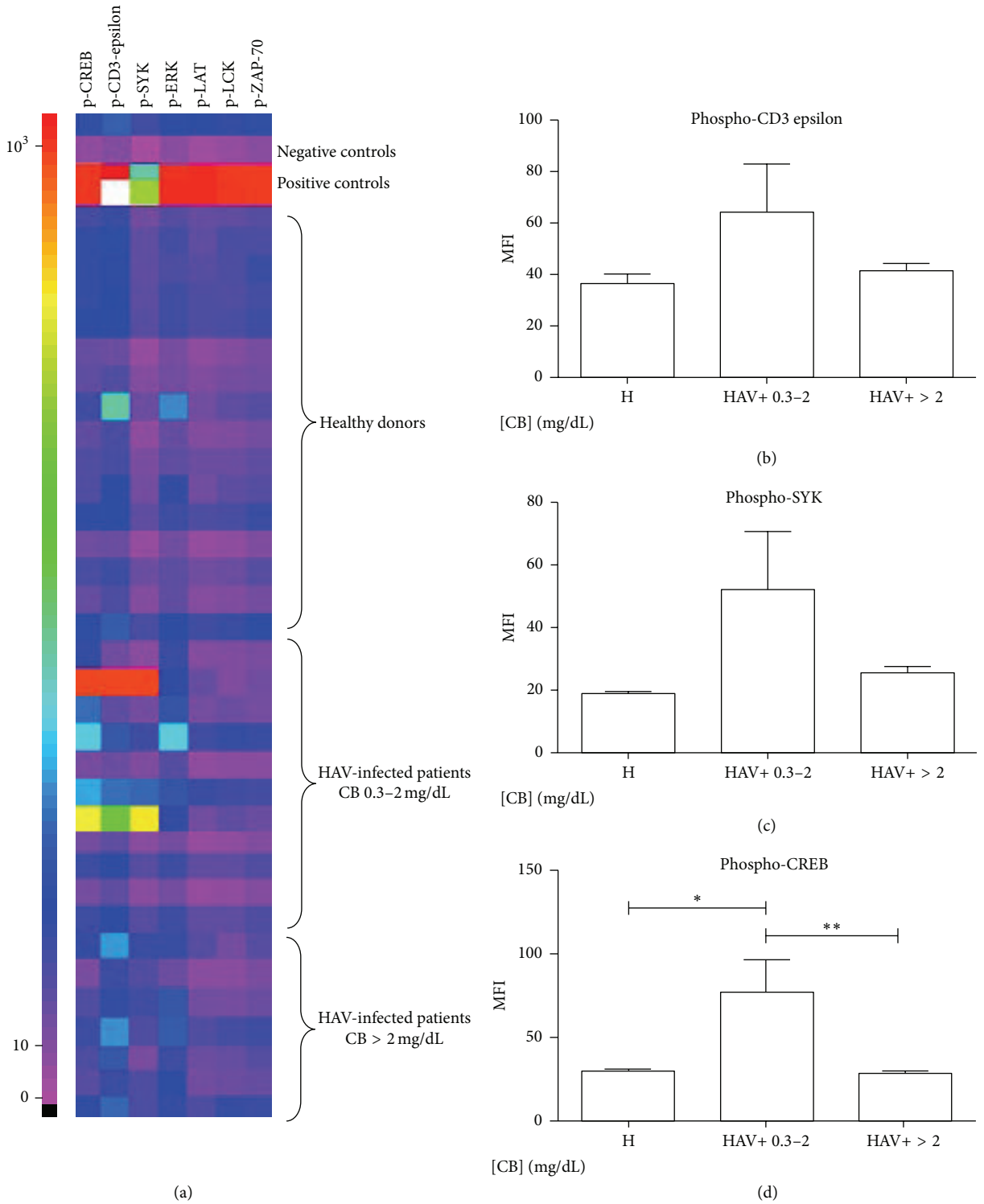


Figure 2: CD4+ TLs from HAV-seropositive patients with CB levels greater than 2 mg/dL have a lower degree of CD3 epsilon, CREB, and SYK phosphorylation. TCR-related intracellular signals were evaluated in CD4+ TLs lysates from pediatric patients and healthy donors by MAGPIX Technology. Representative Heat map (MFI) is shown in (a). The medians of the MFI and SD of phosphorylated CD3 epsilon (b), phosphorylated SYK (c), and phosphorylated CREB (d) from HAV+ patients with CB 0.3–2 mg/dL ($n = 11$), HAV+ patients with CB > 2 mg/dL ($n = 7$), and healthy (H) donors ($n = 16$) are shown. Nonparametric Kruskal-Wallis for comparison between groups was used to calculate statistical significance. $P < 0.05$ was considered statically significant. $^{*}P < 0.05$ and $^{**}P < 0.001$.

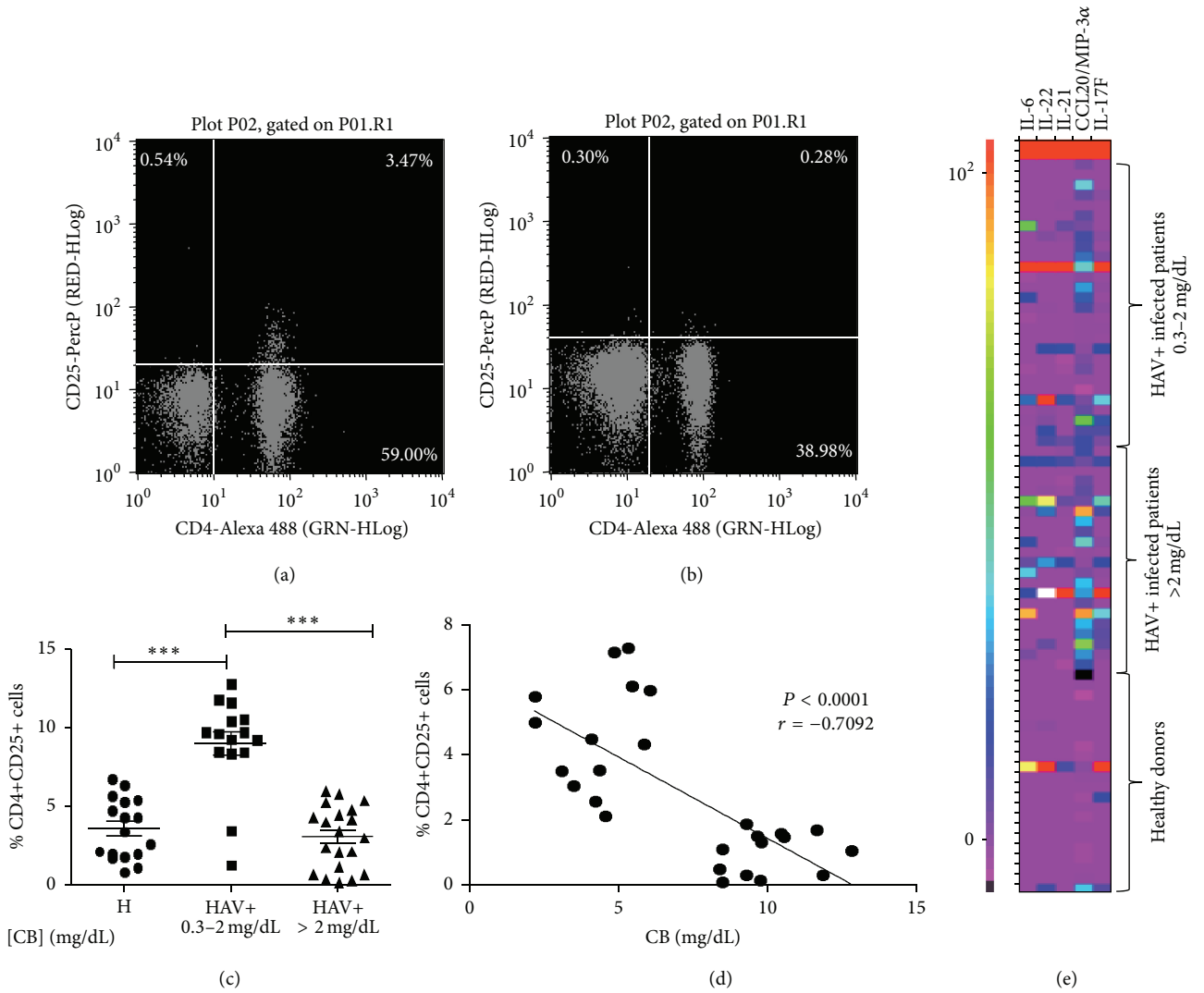
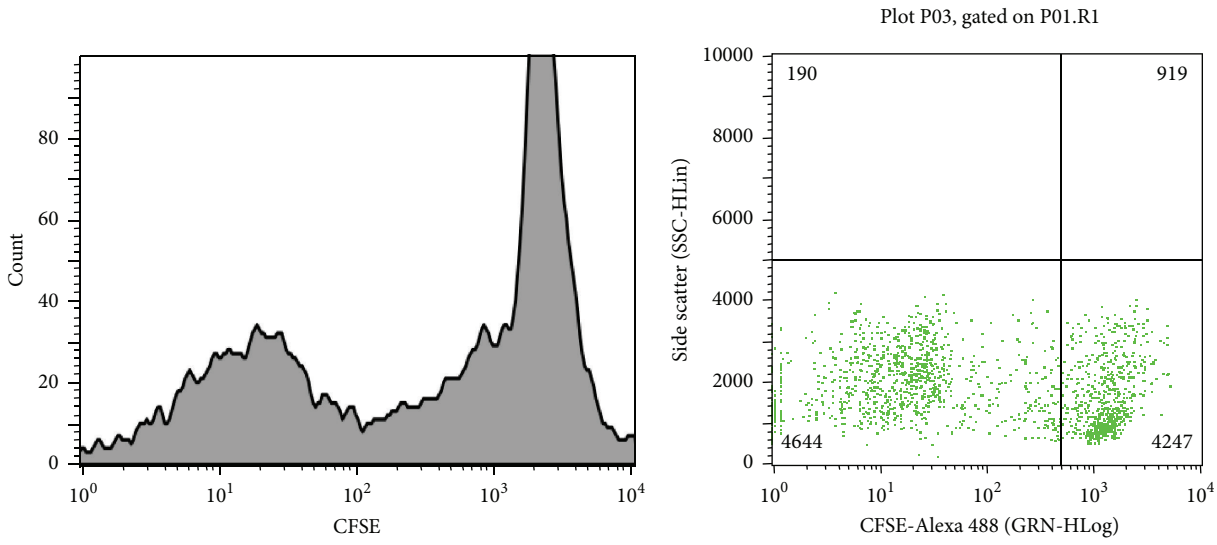


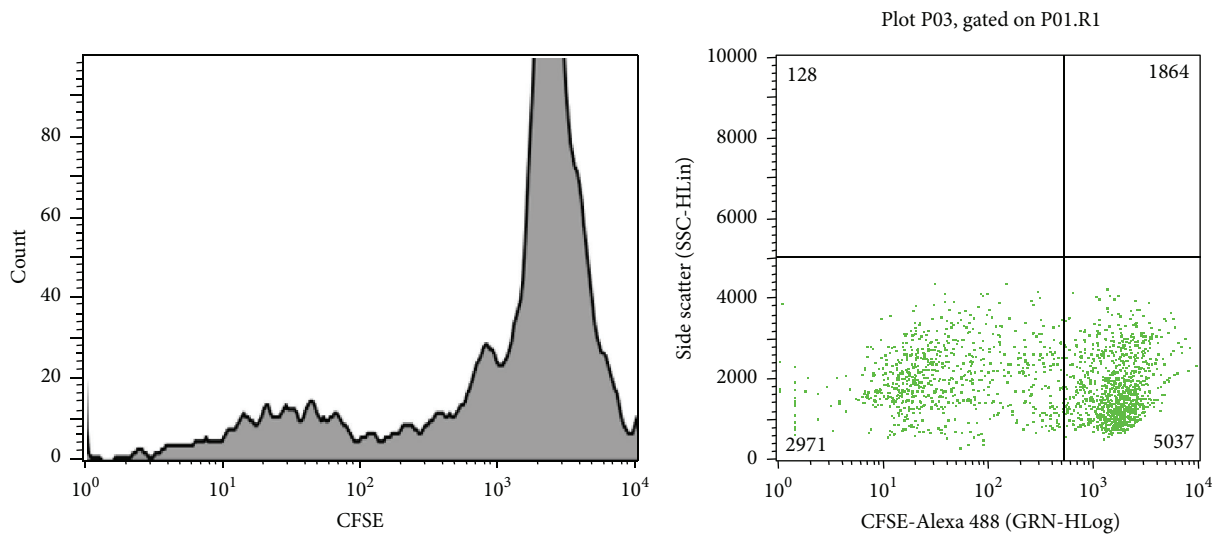
Figure 3: HAV-seropositive patients with low CB levels show an increase in the percentage of CD4+CD25+ TLs. PBLCs were separated from patients and controls. Cells were stained with anti-CD4-Alexa 488 and anti-CD25-PercP and then analyzed by using flow cytometry. Representative dot plot from (a) HAV+ patients with CB 0.3–2 mg/dL and (b) HAV+ patients with CB greater than 2 mg/dL are shown. (c) The results are displayed as the percentage of double positive cells for CD4 and CD25. The medians and the SD from 17 healthy donors, 17 patients with CB 0.3–2 mg/dL, and 15 patients with CB greater than 2 mg/dL are presented. Nonparametric Kruskal-Wallis for comparison between groups was used to calculate statistical significance. $P < 0.05$ was considered statistically significant. $^{***}P < 0.0001$. The Spearman correlation coefficient for the percentage of CD4+CD25+ T cells and the CB levels in HAV+ patients was calculated in (d). (e) Th17 cytokines were evaluated in serum from patients with CB 0.3–2 mg/dL ($n = 44$), patients with CB greater than 2 mg/dL ($n = 45$) and healthy donors ($n = 40$) by MAGPIX Technology. Representative Heat map (pg/mL) of 40 controls and 71 patients is shown.

Forward, 93 sequences obtained with primer Reverse) and 21 HAV-seropositive patients (20 sequences Forward, 21 Reverse sequences).

Two insertional events in the same position, one of 15 nucleotides (ATG ACG ACT GTT CCA) and one of 18 nucleotides (ATG ACA ACG ACT GTT CCA), encoding the amino acids sequences MTTVP and MTTTVP, respectively, were found. In the case of the controls, two samples had the 18-nucleotide insertion, both confirmed for each DNA strand. In the case of HAV-seropositive patients, one sample was found with the 18-nucleotide insertion. The proportions of samples with the insertion are shown in Table 2. Overall, a frequency of 8.08% for the control group and of 9.56% for HAV-seropositive patients was found (Table 2). The odds ratio was 1.19, (with a confidence interval of 0.2340–6.0515). This low frequency of 157insMTTTVP insertion in the *HAVCR1/TIM-1* gene in healthy donors and HAV-seropositive patients suggests that no association is observed between this polymorphism in the viral receptor and symptoms of HAV-related disease. Thus, this gene polymorphism is present in Mexican population but does not seem to be playing a role in the susceptibility to HAV infection.



(a)



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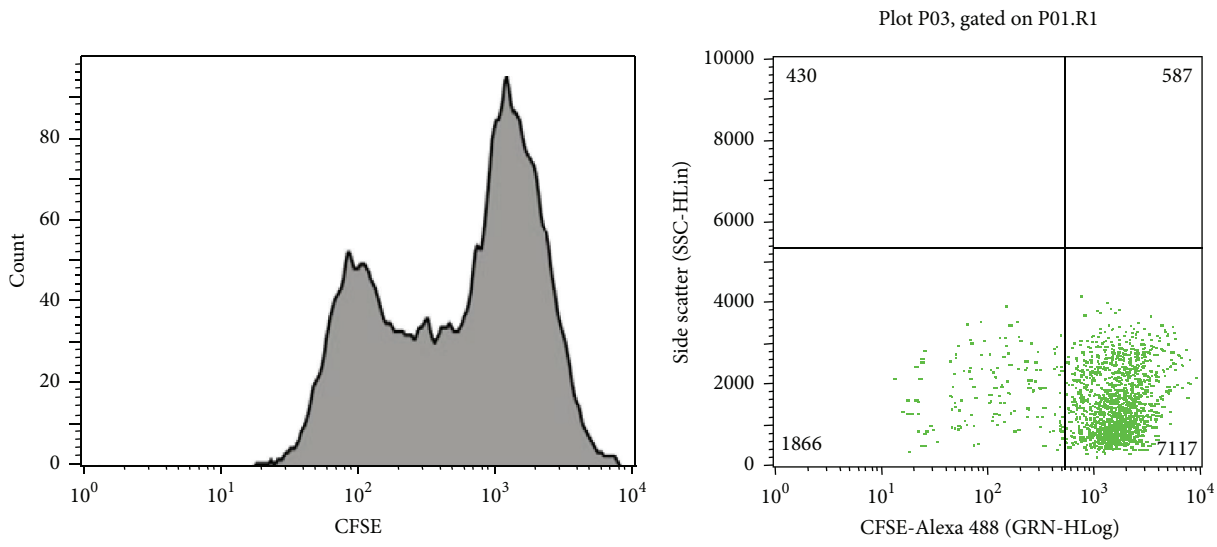

(c)

Figure 4: Continued.

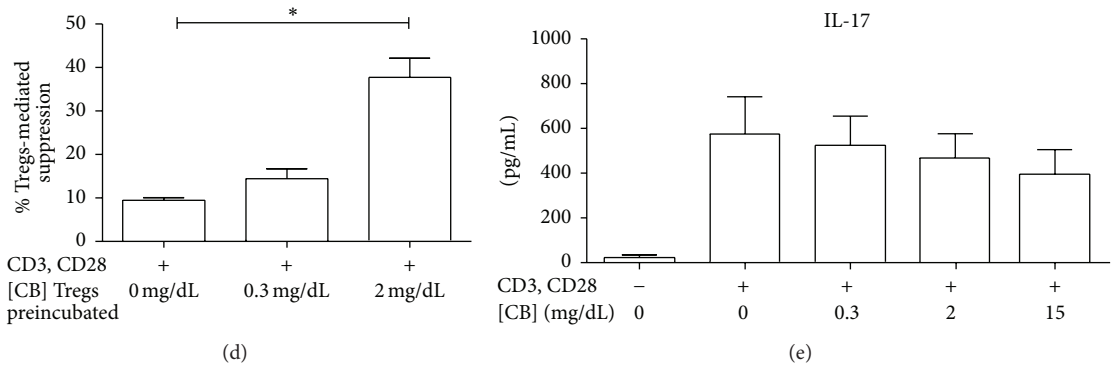

Figure 4: Treatment with CB *in vitro* induces an augment in the activity of Tregs. Purified CD4+CD25+FOXP3+ Tregs from pediatric healthy donors were incubated for 72 hours with and without different doses of CB. Then, Tregs were cocultured with CFSE-labeled Teff (TCD4+CD25−) for 7 days, and cells were stimulated with anti-CD3 and anti-CD28. (a) Dilution of fluorescence and cell count because of the proliferation of Teff stimulated with anti-CD3 and anti-CD28 cocultured with Tregs in the absence of CB. (b) Dilution fluorescence and cell count because of the proliferation of Teff when cocultured with Tregs with CB 0.3 mg/dL. (c) Dilution fluorescence and cell count because of the proliferation of Teff when cocultured with Tregs preincubated with CB 2 mg/dL. Data shown are representative of three independent experiments. (d) The results are displayed as the percentage of Tregs suppression by each condition. (e) IL-17 concentration was evaluated by MAGPIX Technology in cell supernatants from CD4+ TLs from pediatric healthy donors stimulated with anti-CD3 and anti-CD28 in presence or absence of CB. The medians and standard deviations of three repetitions are presented. Nonparametric Kruskal-Wallis for comparison between groups was used to calculate statistical significance. $P < 0.05$ was considered statically significant. $^{*}P < 0.05$.

Table 2: The frequency of the 157insMTTTVP insertion in *HAVCR1/TIM1* gene in healthy donors and HAV-infected patients.

	Control group	HAV-infected pediatric patients
Total samples	99	21
Samples with insertion (2 alleles)	2	1
Samples with insertion (1 allele)	6	1
Percentage	8.06	9.52

4. Discussion

The results obtained in this study support the hypothesis that CB plays an important role in modulating the functionality of immune system cells during the infectious process mediated by HAV. The resolution of the infection caused by HAV is influenced by the dynamics of T lymphocytes, particularly CD4+ TLs and Tregs [27]. During HAV infection, CD4+ TLs participate in cytokine secretion and helper functions to eradicate the virus [28]; these functions are directly related to the activation of the TCR signaling pathway [25]. Signals transduced by CD3 epsilon contribute to the survival of T cells [26]. In these cells, CREB activation promotes proliferation, survival, and differentiation by regulating the Th1, Th2, and Th17 responses, in addition to the signaling cascades required for the generation and maintenance of Tregs [29]. Moreover, SYK efficiently phosphorylate components of the TCR signaling cascade, acting as a positive regulator [30]. Our results support the idea that CB acts directly on this pathway, modulating the functionality of CD4+ TLs. Different responses were noticed when *in vitro* and *ex vivo* data were compared regarding the dose-response effect of CB on intracellular signals. This may be because overall phosphorylation was detected with an anti-pTyr antibody for the analysis *in vitro* whereas specific signaling components were evaluated *ex vivo*. The *in vitro* analysis of CD4+ TLs showed a lower degree of tyrosine phosphorylation in cells treated with CB (between 0.3 mg/dL to 15 mg/dL) than in controls (Figure 1). This is in agreement with previous studies [31], which indicate that BR inhibits the activity of the catalytic domain of protein kinases via a noncompetitive mechanism. In contrast, in patients with low levels of CB the degree of phosphorylation was not affected in our study, as patients showed a rise in the degree of phosphorylation of CREB. This suggests that, in the context of HAV infection, an augment in phosphorylation of the TCR signaling pathway occurs and discrete CB levels in the microenvironment do not affect it. Interestingly, our *ex vivo* analysis showed that patients with CB > 2 mg/dL had a lower degree of phosphorylation of proteins involved in the TCR pathway such as CD3 epsilon, SYK, and CREB (Figure 2). This suggests that the activity of these particular proteins is diminished during the course of HAV infection and CB levels may be related to this process.

To have an overview of the potential role of CB on Tregs subpopulation, we investigated the percentage of CD4+CD25+ TLs in HAV-seropositive patients with various serum CB levels. The results indicated an inverse relationship between the level of CB and the percentage of CD4+CD25+ T cells (Figure 3). This could be explained by the mechanism proposed by Sakaguchi et al. [32], who identified a specific demethylated region in the FOXP3 locus of Tregs, which contains a binding site for the CREB transcription factor, indicating that CREB stabilizes FOXP3 expression and thus promotes and maintains the Treg populations. In our study, increased CREB phosphorylation was observed only in CD4+ TLs from patients with low CB levels. This finding suggests that the concentration of this bile metabolite in the medium is crucial for the development of CD4+ TLs and potentially regulates Treg population size and/or function. In addition,

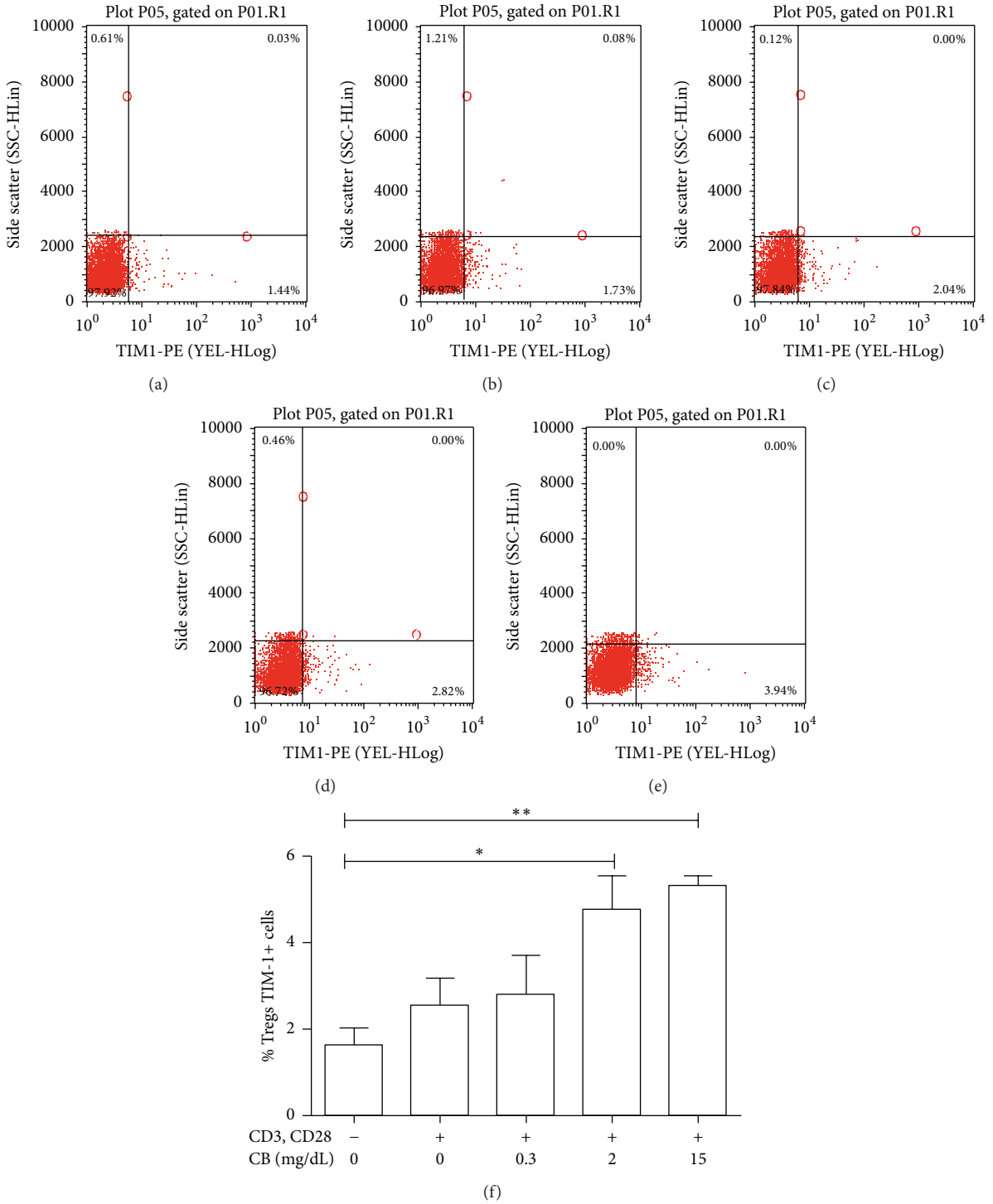

FIGURE 5: CB *in vitro* induces an augment in the percentage of Tregs expressing TIM-1. Purified Tregs from pediatric healthy donors were incubated at 37°C, 5% CO_2 with different concentrations of CB for 72 h. Then, cells were recovered and stained with anti-TIM-1-PE and the percentage of Tregs expressing TIM-1 was analyzed by using flow cytometry. Representative dot plots are shown. (a) Tregs without stimulus. (b) Tregs stimulated with anti-CD3 and anti-CD28. (c) Tregs stimulated with anti-CD3, anti-CD28 and CB (0.3 mg/dL). (d) Tregs stimulated with anti-CD3, anti-CD28, and CB (2 mg/dL). (e) Tregs stimulated with anti-CD3, anti-CD28, and CB (15 mg/dL). (f) The results are presented as TIM-1 percentage. The medians and SD of three independent experiments are shown. Nonparametric Kruskal-Wallis for comparison between groups was used to calculate statistical significance. $^{*}P < 0.05$ was considered statistically significant. $^{**}P < 0.001$.

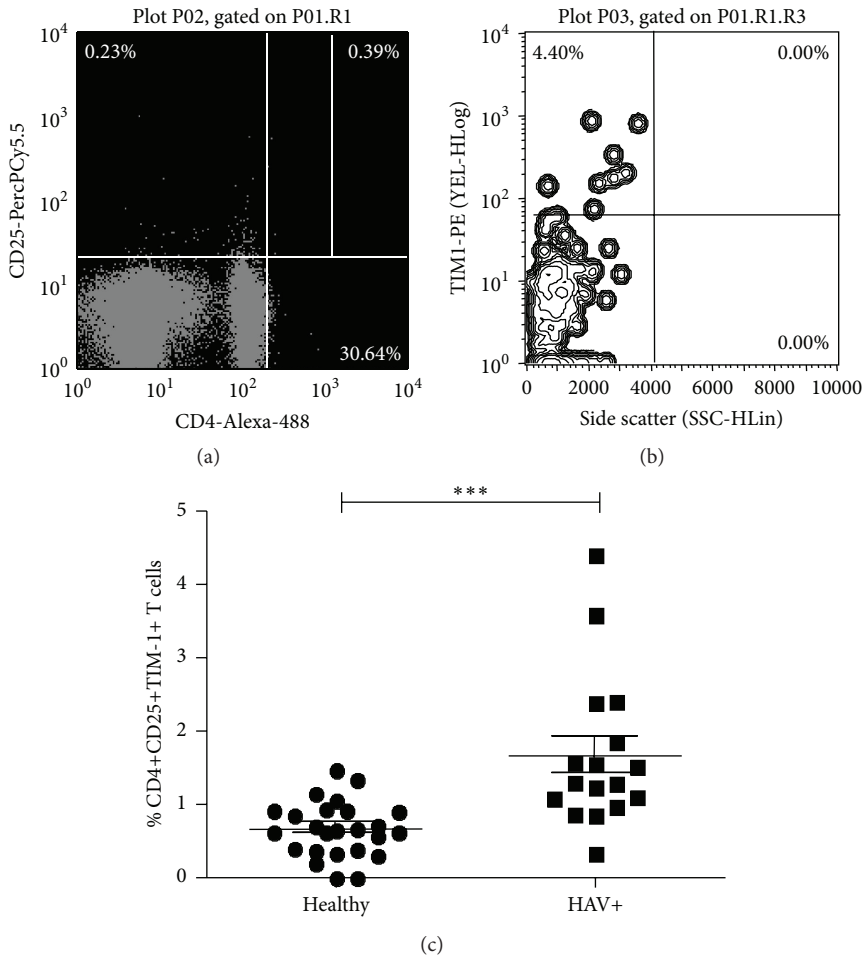

Figure 6: HAV infection leads to an increase in the relative proportion of TIM-1 receptor on CD4+CD25+ TLs. PBLCs from HAV+ pediatric patients were stained with anti-CD4-Alexa 488, anti-CD25-PerCP and anti-TIM1-PE antibodies and evaluated by using flow cytometry. (a) Representative dot plot of CD4+CD25+ staining and selection of the right upper quadrant. (b) Representative dot plot of TIM-1 versus Side Scatter linked to the right upper quadrant on (a), which represents TIM-1 percentage on CD4+CD25+ cells. (c) The results are displayed as the percentage of TIM-1+CD4+CD25+ T cells. The medians and standard deviations from 25 healthy donors and 17 HAV-infected pediatric patients are presented. Nonparametric Mann-Whitney U test for comparison between groups was used to calculate statistical significance. $P < 0.05$ was considered statically significant. $^{***}P < 0.0001$.

several authors have reported the relationship of BR and Tregs, but the reports are contradictory. First, Liu et al., 2008, reported that *in vitro* BR treatment of CD4+ T cells did not induce an expansion of Tregs [3]. In contrast, Rocuts et al., 2010, established that treatment with BR administered to murine allograft pancreatic islets recipients promotes *de novo* generation of Tregs [6], which leads to tolerization after the administration of BR in transplant recipients [33]. Moreover, Huang et al., 2015, reported that an increase in the proportion of Tregs with memory phenotype and TNFRII high expression in cirrhotic patients correlated with hyperbilirubinemia [34]. The discrepancy in results is most likely due to the differences in methodologies and models used in each of the studies. Although our data support the notion that CB may promote *de novo* generation of Tregs by modulating intracellular signals, specifically CREB phosphorylation, preliminary data from *in vitro* CB treatment of naïve TLs revealed no differences in FOXP3 expression (data not shown), suggesting that, under these particular experimental conditions, CB does not promote *de novo* generation of Tregs. Large-scale studies are necessary to dissect the functional significance of various concentrations of CB in Tregs differentiation in the context of HAV infection. Particularly, a detailed study of the intracellular signals modulated in Tregs because of CB treatment deserves detailed analysis. Altogether, our *in vitro* data strongly suggest that CB > 2 mg/dL increases the suppressive capacity of Tregs (Figure 4), thus contributing to the nonoptimal functional status of CD4+ TLs present during the HAV infection. This is in agreement with the anti-inflammatory properties of BR previously described and with the fact that, under our experimental conditions, CB did not induce IL-17 secretion in TLs (Figure 4). Of interest is the fact that Th17 differentiation requires TCR activation signaling pathway. In our study, a Th17 profile is found in HAV-infected patients with CB greater than 2 mg/dL (Figure 3). Thus, it is plausible that Th17 profile results of ERK, LAT,

LCK, and ZAP-70 signals activated in T cells in the presence of the virus, given no differences relative to CB levels, were found in these particular pathways (Figure 2(a)). In addition, Th17 differentiation requires IL-6 and we recently reported an augment in this cytokine in HAV-infected patients with greater CB levels [17]. Given Th17 profile was assessed in serum from patients, this particular profile could result from IL-6 secretion from macrophages.

There is evidence that the antiproliferative activity of Tregs is regulated during the HAV infection. This has been mainly attributed to the interaction generated between the virus and the HAVCR1/TIM-1 receptor expressed on Tregs [20]. Changes in the proportion of TIM-1 may modulate the degree of the activation of this cell subtype. Discrete concentrations of BR suppress the reactivity of CD4+ T cells through mechanisms, including inhibition of CD28, B7-1, and B7-2, resulting in a reduction of costimulatory signals [3]. Interestingly, intraperitoneal administration of BR in mice influences the expression of Fc receptors in macrophages [35]. These data support the hypothesis that BR could modulate immune functions, due to its lipophilic character and direct interaction with cell membranes, suggesting that BR is associated with membrane receptors. The results of these studies coincide with the results obtained in the present study, which indicate that CB is able to modulate the proportion of Tregs expressing the HAVCR1/TIM-1 receptor *in vitro* (Figure 5), as well as the data obtained by the *ex vivo* analysis in the presence of HAV infection (Figure 6). Higher levels of CB result in a greater proportion of HAVCR1/TIM1-positive Tregs. This coincides with increased suppressive function of these Tregs, and thus, it is plausible that CB modulates the function of Tregs via the expression of HAVCR1/TIM-1. Moreover, based on a small population, the 157insMTTTVP insertion in the *HAVCR1/TIM-1* gene found in low frequency did not support a functional association between genetic differences at the host level and the presence of HAV infection. A larger population is required in order to better assess the contribution of gene polymorphisms in the viral receptor to the development of clinical outcomes during the HAV infectious process.

In conclusion, our data strongly suggest that, during HAV infection, CB plays a role in determining T cell function by modulating intracellular pathways and by inducing changes in the function of Tregs in mechanisms related to the expression of HAVCR1/TIM-1 on the cell surface. The CB levels found to play a role in viral hepatitis infection might provide insights for other infectious diseases eventually affected by host metabolites at the level of regulating immune responses. HAV and its interaction with the immune system represent a field for future investigation. In particular, exploring the nature of these interactions may contribute to understand why this virus does not persist in the infected host, whereas other viruses, including hepatitis B and hepatitis C, do.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Negative Impact of Hypoxia on Tryptophan 2,3-Dioxygenase Function

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Tryptophan is an essential amino acid for hosts and pathogens. The liver enzyme tryptophan 2,3-dioxygenase (TDO) provokes, by its ability to degrade tryptophan to N-formylkynurenine, the precursor of the immune-relevant kynurenines, direct and indirect antimicrobial and immunoregulatory states. Up to now these TDO-mediated broad-spectrum effector functions have never been observed under hypoxia *in vitro*, although physiologic oxygen concentrations in liver tissue are low, especially in case of infection. Here we analysed recombinant expressed human TDO and *ex vivo* murine TDO functions under different oxygen conditions and show that TDO-induced restrictions of clinically relevant pathogens (bacteria, parasites) and of T cell proliferation are abrogated under hypoxic conditions. We pinpointed the loss of TDO efficiency to the reduction of TDO activity, since cell survival and TDO protein levels were unaffected. In conclusion, the potent antimicrobial as well as immunoregulatory effects of TDO were substantially impaired under hypoxic conditions that pathophysiologically occur *in vivo*. This might be detrimental for the appropriate host immune response towards relevant pathogens.

1. Introduction

There is great interest in understanding the composition of the tissue microenvironment and its consequence for immune responses. One of the most important microenvironmental factors is the oxygenation status of tissues, since oxygen affects a plethora of cellular processes including innate and adaptive cellular immune defence mechanisms. Cells in different tissues are exposed to a wide range of oxygen concentrations, and local oxygen concentrations between 1 and 12% O_2 are physiologic [1]. In particular the liver has a unique anatomical structure that creates an oxygen gradient between 8% O_2 and 4% O_2 within the liver compartments and the pO_2 is even more reduced during infection to ≤1% O_2 [2, 3].

Human tryptophan 2,3-dioxygenase (TDO) is a liver enzyme with a well-described function in tryptophan homeostasis and crucial immunoregulatory features. The latter was shown *in vitro* [4] and *in vivo*. Bessede et al. nicely demonstrated that $TDO^{-/-}$ mice exhibit an increased sensitivity to endotoxin-induced shock, indicating the potential relevance of TDO in anti-inflammatory reactions [5]. Further hints towards an immunoregulatory function of TDO derive from the facts that TDO is expressed in hepatocarcinomas and other malignancies and TDO-mediated production of tryptophan metabolites protects tumor cells against immune rejection [6–9]. Interestingly, hypoxia is frequently observed in tumoral tissue and influences host defence [10–12]. Hence, hypoxia is a microenvironmental factor that might have an impact also on TDO-mediated functions.

It was shown by us that recombinantly expressed human TDO is able to inhibit the growth of bacteria, parasites, and viruses [4] and also infected tissue displays low oxygen levels or hypoxia [12]. Several factors contribute to these infection-

or inflammation-associated hypoxic states, for example, increased oxygen consumption by inflamed resident cells, infiltrating inflammatory cells, and proliferating pathogens as well as a decreased oxygen supply due to vascular pathology and microthrombosis [13].

Here we were interested in TDO functions under hypoxia. Using stably transfected HeLa T-REx™ cells expressing recombinant human TDO [4] and liver homogenates from WT and $TDO^{-/-}$ mice we found that the TDO-mediated degradation of tryptophan to kynurenine was inhibited under low oxygen concentrations. Consequently the antimicrobial functions of TDO against tryptophan-auxotroph bacteria and parasites were abrogated *in vitro*. In summary our studies revealed that low oxygen levels might be detrimental for antimicrobial effector molecules (IDO, TDO), inhibiting appropriate immune reactions during infections, which possibly leads to inadequate microbial clearance and subsequent overwhelming or chronic infections.

2. Methods

2.1. Cells, Media, and Reagents. HeLa T-REx cells were purchased from Invitrogen (Karlsruhe, Germany) and stably transfected with pcDNA4-hTDO vector (Invitrogen, Karlsruhe, Germany) containing human liver TDO cDNA to produce HeLa-hTDO cells as described by us [4]. The expression of recombinant hTDO in HeLa-hTDO cells was induced by stimulation with tetracycline.

The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, USA), supplied with 5% heat-inactivated fetal calf serum (FCS) in culture flasks (Costar, Cambridge, USA) and split weekly in 1 : 10 ratios by using trypsin/EDTA (Gibco, Grand Island, USA). Mycoplasma contamination was regularly excluded via PCR. Hypoxia growth experiments were carried out using alternatively a HERAcell 150 I CO_2 incubator (Thermo Fisher Scientific, Langenselbold, Germany) or the Anoxomat™ system (Mart Microbiology B.V., Drachten, Netherlands) with 1–10% O_2 and 10% CO_2. The IMDM was buffered with sodium bicarbonate and therefore had an optimal buffering capacity at the 10% CO_2 environment to maintain the physiological pH. Consequently the pH values of the cell culture medium with or without cells under normoxia and hypoxia were in the range of 7.15–7.48.

2.2. Cell Proliferation Assay. 1×10^3 HeLa-hTDO cells were seeded per cm^2 in 25 cm^2 cell culture flask (Corning, NY, USA), ^{3}H-thymidine was added at day 0, and the cells were incubated under normoxia (20% O_2) or hypoxia (1% O_2) for 1–7 days. The incorporation of ^{3}H-thymidine was detected using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Rodgau Jügesheim, Germany).

2.3. Cell Viability Assay. 3×10^4 HeLa-hTDO cells per well were incubated in 96-well Costar microtiter plates (Corning, NY, USA) for 24 h under normoxia (20% O_2) or hypoxia (1% O_2). Then the cells were washed with PBS and stained with Calcein AM (1 : 2000) or Ethidium-Homodimer-1 (Eth-D-1) (1 : 500) (live/dead viability/cytotoxicity kit, Invitrogen, Karlsruhe, Germany). After incubation time of 45 minutes the fluorescence was detected using the plate reader Synergy Mx (Winooski, VT, USA). Calcein was excited using a fluorescein optical filter (485 ± 10 nm) and Eth-D-1 using a rhodamine optical filter (530 ± 12 nm). The fluorescence emissions were acquired separately as well, Calcein at 530 ± 12 nm and Eth-D-1 at 645 ± 12 nm.

2.4. Enzyme Activity Assays

2.4.1. Detection of Kynurenine in Cell Supernatants. TDO activity was determined by analysing kynurenine concentration in supernatants of stimulated or unstimulated HeLa-hTDO, using Ehrlich's reagent as described before [14]. Defined kynurenine samples served as control.

2.4.2. Assessment of mTDO Activity in Liver Homogenates. Assessment of mTDO activity in liver homogenates was done according to the method of Moreau et al. [15], with some modifications: livers were homogenized in three times the weight using a tissue homogenizer (Precellys, VWR International, Erlangen, Germany). 400 μL liver homogenisate was added to 1 mL TDO-buffer (200 mM potassium phosphate buffer (pH 7.0), 10 mM ascorbic acid, 5 μM hematin, and 10 mM L-tryptophan) in 48-well microtiter plates and was incubated without lid under normoxia (20% O_2) or hypoxia (1% O_2) for 4 h at 37°C. Then the reaction was stopped using 1/10 v/v 30% trichloroacetic acid, again incubated for 30 min at 60°C, and centrifuged. Supernatants were mixed with an equal volume of Ehrlich's reagent and absorbance was detected at 492 nm with a microplate reader (SLT Lab Instruments, Crailsheim, Germany).

Kynurenine (Sigma-Aldrich, St. Louis, USA) was diluted in culture medium or buffer and used as standard. For the calculation of the kynurenine content, linear regression and GraphPad Prism software were used.

2.5. In Vitro Infection Experiments. For *in vitro* infection studies 3×10^4 HeLa-hTDO were incubated under normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of tetracycline for 72 h. Thereafter they were infected with different bacteria or parasites.

2.5.1. Bacterial Infections and Read-Out. For bacterial infections *Enterococcus faecalis* (ATCC 29212) was used. Tryptophan auxotrophy was tested before starting infection experiments. Bacteria were grown on brain heart infusion agar (Difco, Hamburg, Germany), containing 5% sheep blood and incubated at 37°C in 5% CO_2-enriched atmosphere or in cell culture medium in the absence or presence of cells. Tetracycline-sensitivity was tested and a half-maximal bacterial growth was observed in the presence of 40 μg/mL tetracycline under normoxia and under hypoxia, which is thousandfold more than the concentration we used in the experiments. For use in experiments, a 24 h old single bacterial colony was picked, resuspended in tryptophan-free

RPMI 1640 (Gibco, Life Technologies, Darmstadt, Germany), and diluted serially. Ten μL of the bacterial suspension corresponding to 10–100 CFU was added to each well of the 96-well microtiter plates containing preincubated HeLa-hTDO cells. After 18 h bacterial growth was monitored using a microplate photometer (SLT Lab Instruments, Crailsheim, Germany) by measuring the optical density at 620 nm. In some experiments, the bacterial population present in the cultures was enumerated by counting colony forming units (CFU) after plating 10 μL aliquots of serially diluted culture supernatants on blood agar [16].

2.5.2. Parasite Infections and Read-Out. *Toxoplasma gondii* (RH strain, ATCC, Wesel, Germany) or *Neospora caninum* (Nc-1 strain, kind gift of G. Schares, Greifswald-Insel Riems, Germany) tachyzoites were maintained in human foreskin fibroblasts (ATCC, Wesel, Germany) in IMDM containing 5% FCS. Tachyzoites were harvested after 5 days of incubation, resuspended in PBS, and counted. Preincubated HeLa-hTDO cells were infected with 3×10^4 toxoplasma or 4×10^4 neospora per well. Parasite growth was determined by the ^{3}H-uracil incorporation method as described before [17]. In brief 48 h after infection 0.33 μCi ^{3}H-uracil was added and after additional 24 h host cells were lysed by freeze and thaw cycles. The incorporation of ^{3}H-uracil was detected using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Rodgau Jügesheim, Germany).

2.6. Protein Analysis. 3×10^6 HeLa-TDO cells were left unstimulated or stimulated with tetracycline (10 ng/mL) for 72 h under normoxia (20% O_2) or hypoxia (1% O_2). Then the cells were harvested and lysed by three freeze and thaw cycles in a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Proteins were separated by electrophoresis using 10% NuPAGE Novex *Bis*-Tris Mini Gels in the appropriate electrophoresis system (Invitrogen, Karlsruhe, Germany) and semidry blotted on nitrocellulose membranes (CarboGlas, Schleicher & Schuell, Dassel, Germany). After blocking of the membranes with 3% (w/v) skim milk powder in TBS for 1 h at room temperature, they were incubated in the respective primary antibodies overnight at 4°C. Anti-β-actin antibody (1 : 10000, Sigma, St. Louis, USA) or anti-human-TDO2 antibody (GTX 40401, GeneTex, Irvine, USA) was diluted in 3% (w/v) skim milk powder in TBS. After washing the membranes were incubated with goat-anti-mouse HRP-conjugated or goat-anti-rabbit HRP-conjugated IgG (1 : 10000, Jackson ImmunoResearch Lab., Dianova, Hamburg, Germany), diluted in 3% (w/v) skim milk powder in TBS, for 2 h at room temperature. After additional washing steps proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). Densitometric analysis was carried out with ImageJ software.

2.7. T Cell Proliferation Assay. 3×10^6 HeLa-hTDO cells were incubated with or without tetracycline (10 ng/mL) for 72 h under normoxia (20% O_2) or hypoxia (1% O_2) in 20 mL cell culture medium in culture flasks. Then the supernatants were harvested and used as cell culture medium for freshly isolated 1.5×10^5 Ficoll-separated peripheral blood lymphocytes (PBL)/well. PBL were activated using the monoclonal anti-CD3 antibody OKT3; unstimulated PBL and tryptophan-supplemented PBL served as control group. T cell proliferation was determined after three days by adding ^{3}H-thymidine for 24 h. The incorporation of ^{3}H-thymidine was detected using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Rodgau Jügesheim, Germany).

2.8. Animals. This study was carried out in strict accordance with the German Animal Welfare Act and a protocol approved by the local authorities. TDO-deficient mice were generated as described previously [18]. WT littermates were used as controls. Mice were housed under SPF conditions in the animal facility and were 8–12 weeks old.

2.9. Data Analysis and Statistical Tests. All experiments were done in triplicate and data are given as mean ± standard deviation (SD; Figures 1(a), 4(a), 4(c), 5(a), and 6(a)) of a representative experiment or as mean ± standard error of the mean (SEM; Figures 1(b), 2(a)–2(c), 3, 4(b), 4(d), 5(b), 6(b), and 7) of three to eight independent experiments. For statistical analysis the two-tailed paired t-test (Figure 3) or the two-tailed unpaired t-test (all other data) was used and significant differences were marked with asterisks ($^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$). The analysis was performed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. HeLa-hTDO Survives Incubation under Hypoxia. We have shown before that the tryptophan-degrading enzyme human tryptophan 2,3-dioxygenase (hTDO), expressed in a tetracycline-inducible HeLa-cell based system, has antibacterial, antiparasitic, and antiviral capacities *in vitro* [4]. These antimicrobial effects are the result of the hTDO-induced degradation of tryptophan, which is an essential amino acid for tryptophan-auxotroph organisms, since the supplementation with additional tryptophan abrogated the effects.

In order to analyse hTDO-mediated antimicrobial effects under hypoxic conditions, we first monitored HeLa-hTDO cell survival under hypoxia. Proliferation studies revealed no significant differences in cell growth, when HeLa-hTDO cells were cultured under normoxia or hypoxia (1% O_2) for 7 days (Figure 1(a)). Additionally we observed no significant alterations in cell proliferation under normoxia or hypoxia when the cells were stimulated with tetracycline (data not shown). Therefore we concluded that the HeLa-hTDO cells thoroughly survive the hypoxic environment, at least beyond the three-day incubation phases in the subsequent analyses. This cell survival was also confirmed using a fluorescence-based method of assessing cell viability and cell death (Figure 1(b)). For these experiments HeLa-hTDO cells were incubated for 24 h under normoxia (20% O_2) or hypoxia (1% O_2) and afterwards stained with Calcein AM to detect intracellular esterase activity as indication for living cells

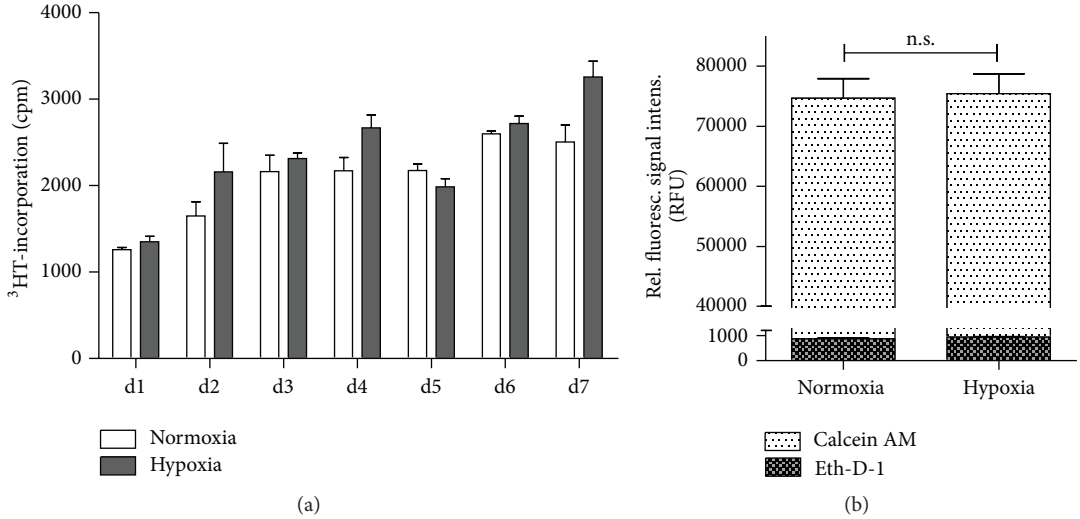

Figure 1: HeLa-hTDO cells survive incubation under hypoxic conditions. (a) Proliferation assay: 1×10^3 HeLa-hTDO cells were seeded per cm^2 in 25 cm^2 cell culture flask (Corning, NY, USA), ^{3}H-thymidine was added at day 0, and the cells were incubated under normoxia (20% O_2) or hypoxia (1% O_2) for 1–7 days. The incorporation of ^{3}H-thymidine was detected using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Rodgau Jügesheim, Germany). (b) Fluorescence-based cell viability/cytotoxicity assays: 3×10^4 HeLa-hTDO cells per well were incubated for 24 h under normoxia (20% O_2) or hypoxia (1% O_2). Then the cells were stained with Calcein AM (white bars) or Ethidium-Homodimer-1 (Eth-D-1) (plaid bars) indicating living or dead cells, respectively. After incubation the fluorescence was detected using a fluorescein optical filter (excitation 485 ± 10 nm; emission 530 ± 12 nm) for Calcein or using a rhodamine optical filter (excitation 530 ± 12 nm; emission 645 ± 12 nm) to detect Eth-D-1. For both assays a two-tailed unpaired t-test was used to compare the groups (n.s. = not significant), $n = 3$ independent experiments with three replicates each. The bars indicate the mean value ± SEM.

or Ethidium-Homodimer-1 (Eth-D-1) that enters nonintact plasma membranes of dead cells. Figure 1(b) shows that the relative fluorescence signal intensity after staining with Calcein AM does not significantly differ in normoxia- or hypoxia-treated HeLa-hTDO cells (white bars). Furthermore Eth-D-1 treatment revealed no enhanced cell death under hypoxia (plaid bars).

3.2. Enzymatic Activity of Human TDO Is Reduced upon Hypoxia. The enzymatic activity of hTDO under hypoxia was analysed by determination of the tetracycline-induced hTDO-mediated conversion of tryptophan to kynurenine in cell culture supernatants after 72 h of incubation. Tetracycline stimulated HeLa-hTDO cells produced kynurenine dose-dependently, when they were incubated under normoxia in the presence of tryptophan (Figure 2(a)). Interestingly the cells generated significantly lower amounts of kynurenine under hypoxic conditions (1% O_2) (Figure 2(a)). When tetracycline stimulated cells were incubated under different oxygen concentrations, the kynurenine amounts positively correlated with the amounts of oxygen present (Figure 2(b)). Therefore oxygen was required for the production of kynurenine. Reoxygenation studies confirmed survival of HeLa-hTDO cells and preservation of enzymatic function within these cells under hypoxic conditions (Figure 2(c)). In these experiments the tetracycline-induced TDO-mediated kynurenine production was determined after 72 h of incubation under normoxia (20% O_2) or hypoxia (1% O_2) and compared to the kynurenine production within cell supernatants after subsequent 48 h incubation under normoxia. The TDO-mediated conversion of tryptophan to kynurenine was drastically inhibited by hypoxia, but the cells were able to produce high amounts of kynurenine in the following normoxic phase, demonstrating cell survival and preservation of enzymatic activity. Interestingly, normoxia-pretreated and hypoxia-pretreated cells showed no significant differences in their tryptophan-degrading capacity in the reoxygenation phase *ab initio* (Figure 2(d)).

3.3. Expression of Human TDO under Hypoxic Conditions. Next, Western Blot analyses were performed to get quantitative information about hTDO protein amounts in HeLa-hTDO cells that were stimulated under normoxia (20% O_2) and hypoxia (1% O_2). Figure 3(a) depicts an exemplary Western Blot. The protein amount of hTDO, induced by tetracycline stimulation of HeLa-hTDO cells, was not altered upon hypoxia as compared to the normoxia control. The stimulation of the cells with the proinflammatory cytokine IFN-γ did not induce a TDO expression in the cells, as expected. The summary of eight densitometric evaluations of independent Western Blot analyses is shown in Figure 3(b). Since β-actin protein amounts are also reduced upon hypoxia [19], the ratio of hTDO protein to β-actin protein was calculated. There were no significant differences detectable in hTDO protein amounts under normoxic and hypoxic conditions detectable.

3.4. Inhibition of hTDO-Mediated Antimicrobial Effects by Hypoxia. Although hTDO protein levels were unaltered under hypoxia (Figures 3), the enzymatic activity of hTDO

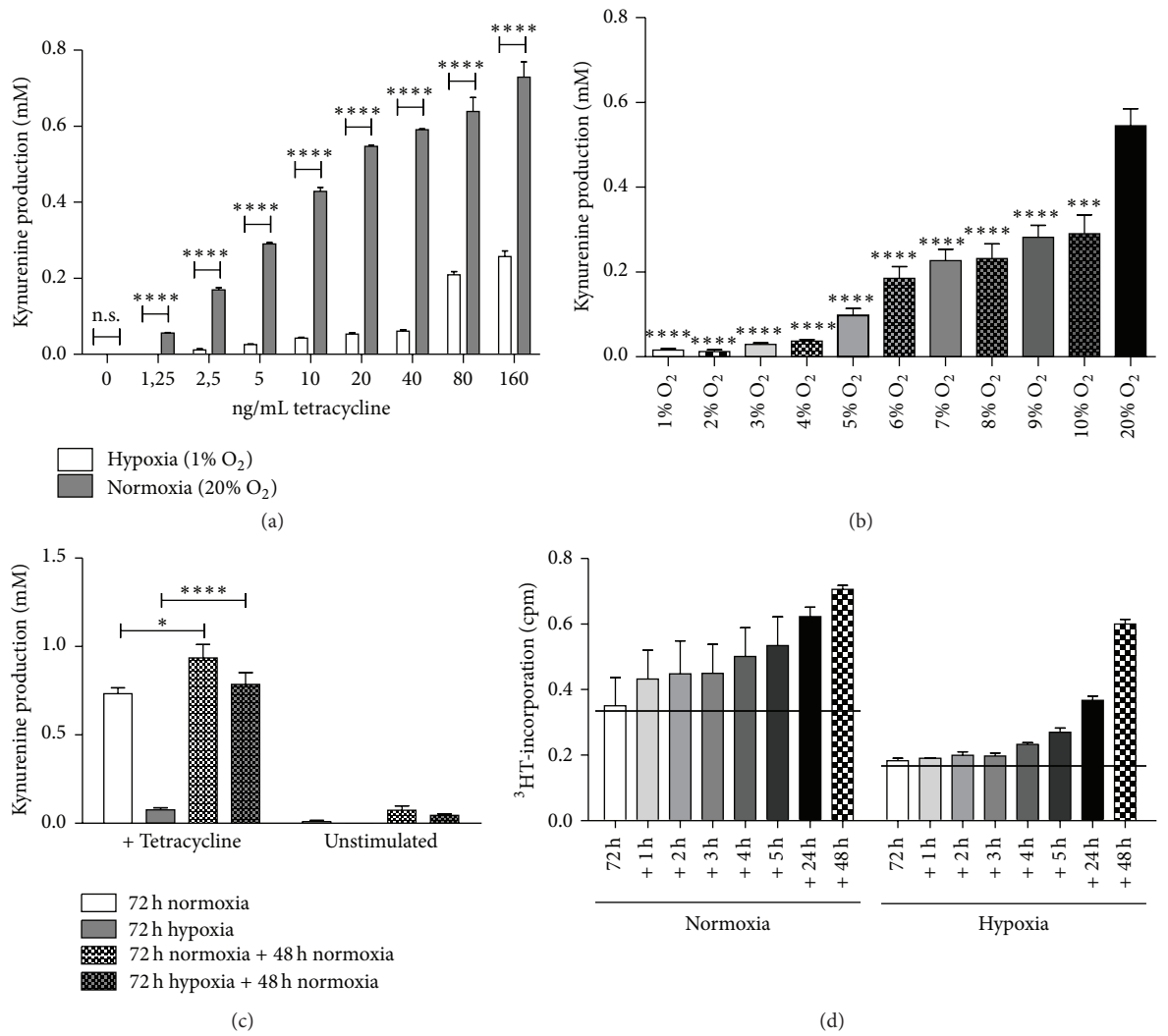

Figure 2: Loss of TDO activity in HeLa-hTDO cells under hypoxic conditions. (a) Kynurenine detection in cell culture supernatants of tetracycline (0–160 ng/mL) stimulated HeLa-hTDO cells incubated under hypoxia (white bars) or normoxia (grey bars). (b) Kynurenine detection in cell culture supernatants in tetracycline stimulated (160 ng/mL) HeLa-hTDO cells that have been incubated under different oxygen conditions (1–20% O_2) for 72 h. (c + d) Reoxygenation study: HeLa-hTDO cells were incubated under normoxia (20% O_2) or hypoxia (1% O_2) with or without tetracycline (40 ng/mL) for 72 h. Then the kynurenine amount produced by TDO was detected in cell culture supernatants. A second experimental group was subsequently transferred to normoxia and after incubation of additional 48 h (c) or 1–5 h, 24 h, and 48 h (d) the kynurenine amount was also detected in cell culture supernatants. In all experiments a significant alteration of kynurenine production under hypoxia as compared to the normoxia control is marked with asterisks ($^{*}p \leq 0.05$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$, n.s. = not significant), two-tailed unpaired t-test; $n = 3$ independent experiments with three replicates each. The bars indicate the mean value ± SEM.

was reduced up to nearly 90%, as determined by measurement of kynurenine in HeLa-hTDO cell supernatants after 72 h of incubation under normoxia (20% O_2) or hypoxia (1% O_2) (Figure 2). In order to analyse hTDO-mediated antimicrobial functions under hypoxia, we infected unstimulated or tetracycline prestimulated HeLa-hTDO cells with different tryptophan-auxotroph pathogens. Figure 4(a) shows the result of infection experiments with the facultative anaerobe, gram-positive bacterium *Enterococcus faecalis*. Enterococci grew in the presence of unstimulated HeLa-hTDO cells, whereas bacterial growth was inhibited by TDO-positive cells, which have been stimulated with ≥5 ng/mL tetracycline (white bars) under normoxic conditions. Interestingly, this TDO-mediated antibacterial effect was lost under hypoxic conditions (grey bars). The same result was observed in infection experiments using other tryptophan-auxotroph bacteria, such as group B streptococci and staphylococci (data not shown). Figure 4(b) shows that moreover the growth of the obligate intracellular apicomplexan parasite *Neospora caninum* (nc-1 strain) was inhibited within activated HeLa-hTDO cells under normoxia and that this antiparasitic effect was abolished under hypoxic conditions.

A more detailed analysis of the hTDO-mediated antibacterial effect under different low oxygen conditions

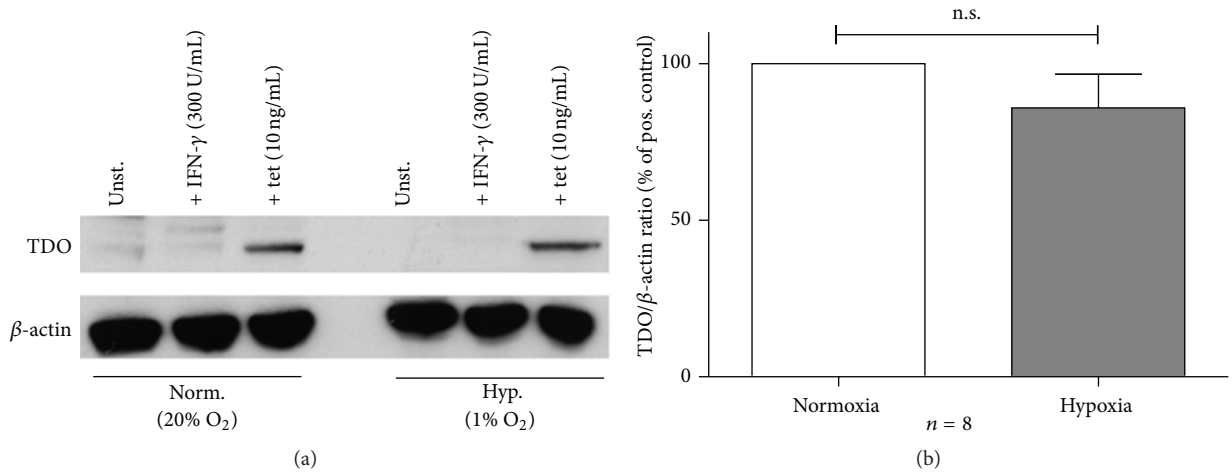

Figure 3: Unaltered hTDO expression in HeLa-hTDO cells under hypoxic conditions. (a) Exemplary Western Blot protein analysis of hTDO and β-actin protein expression in HeLa-hTDO cells after 72 h of incubation under normoxia (20% O_2) or hypoxia (1% O_2). (b) Densitometric evaluation of Western Blot protein analyses: ratio of relative hTDO protein expression to β-actin protein expression as % of positive control ± SEM, $n = 8$ independent experiments. Comparison of hTDO/β-actin protein ratio under normoxia or hypoxia via a two-tailed, paired t-test; n.s. = not significant.

(1–10% O_2) in comparison to normoxia is shown in Figure 5. The antibacterial efficiency of hTDO correlated with the presence of oxygen and low oxygen conditions significantly inhibited the antibacterial effect as determined by the optical density (Figure 5(a)) or by counting the colony forming units (Figure 5(b)).

3.5. Inhibition of hTDO-Mediated Immunoregulatory Effects by Hypoxia. We have mentioned before that TDO regulates immune reactions *in vitro* and *in vivo*, for example, by creating tolerance towards the rejection of tumor cells [6]. Furthermore tumoral tissues are often poorly vascularized and inefficiently supplied with blood and contain only low oxygen amounts [10, 11]. Hence, we tested the immunoregulatory property of hTDO in HeLa-hTDO cells under hypoxia *in vitro*. In order to avoid IFN-γ production by allogeneic T cells which would result in IDO induction in HeLa cells by coculture, supernatants of tetracycline-activated or unstimulated and hypoxia- or normoxia-treated HeLa-hTDO served as culture medium for freshly isolated peripheral blood lymphocytes (PBL). T cell growth was triggered by the addition of the monoclonal anti-CD3 antibody OKT3 and monitored by the ^{3}H-thymidine incorporation method [4]. This OKT3 stimulation induced strong T cell proliferation, which is illustrated in a single experiment in Figure 6(a). The T cell proliferation was inhibited by the TDO-mediated depletion of tryptophan, since it could be restored by the supplementation of tryptophan. However, such T cell inhibition did not occur in conditioned medium that has been harvested from hypoxia-treated HeLa-hTDO cells, which demonstrates the loss of immunoregulatory hTDO functions under hypoxia. Figure 6(b) shows the summary obtained from three different experiments.

3.6. Ex Vivo TDO Activity under Hypoxic Conditions. Although stably transfected HeLa cells are a useful tool to efficiently examine hTDO functions *in vitro*, we extended our studies and confirmed the data by more physiological *ex vivo* liver homogenate experiments. In these experiments freshly isolated liver tissue of WT or TDO-deficient mice was homogenized in PBS and incubated in the presence of 20% O_2 or 1–9% O_2 in a buffer that allows the determination of TDO protein activity by the production of kynurenine [15]. Since the normoxic and the hypoxic groups contained the same amounts of murine TDO protein, the direct effect of normoxia and hypoxia on TDO enzyme activity could be revealed (Figure 7). Under hypoxia murine TDO produced significantly lower kynurenine amounts as compared to the normoxia control group. Furthermore, no kynurenine was detectable in liver homogenates of TDO-deficient mice, as expected.

4. Discussion

In this study we investigated the influence of hypoxia on the activity of the tryptophan-degrading enzyme human tryptophan 2,3-dioxygenase (hTDO) by using a the tetracycline-inducible HeLa T-REx system together with *ex vivo* studies analysing murine TDO [4]. Under normoxic conditions (20% O_2) hTDO activity reduced tryptophan amounts in tetracycline stimulated HeLa-hTDO cells and cell culture supernatants [4]. However, such high oxygen concentrations do not occur within liver tissue physiologically. The liver has a unique anatomical structure that creates an oxygen gradient within the liver compartments. Incoming highly oxygenated blood via the hepatic artery is subsequently mixed with oxygen-depleted blood in the hepatic portal vein. Then the blood flows towards the central vein of the lobule and is depleted of oxygen, resulting in an oxygen pressure (pO_2) of about 8% O_2 in the periportal area and of about 4% O_2 in parenchymatic perivenous areas [2]. Additionally, oxygen concentrations in the liver are even more reduced

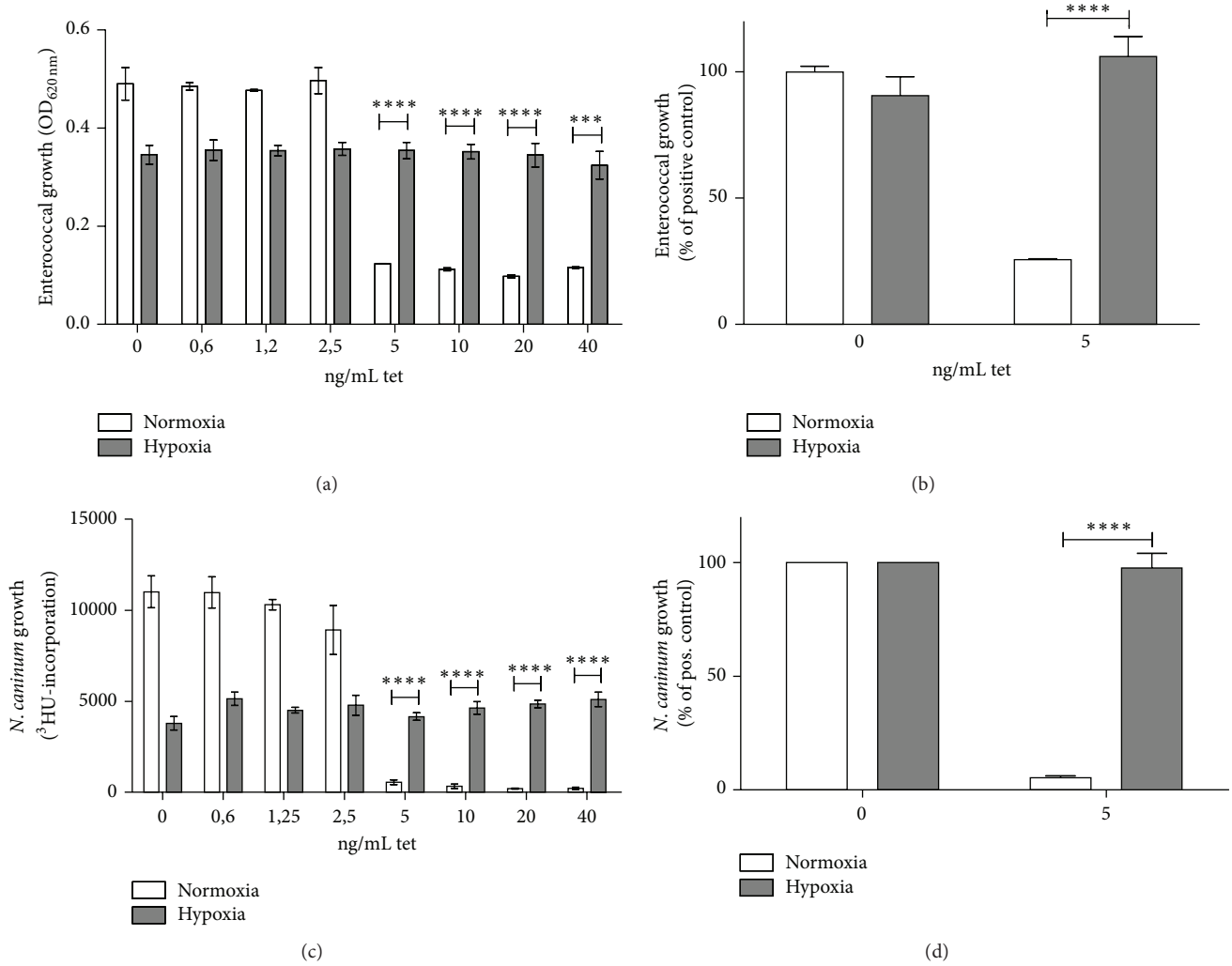

Figure 4: Loss of TDO-mediated antimicrobial functions in HeLa-hTDO cells under hypoxic conditions. Infection experiments: following 72 h prestimulation with denoted amounts of tetracycline, HeLa-hTDO cells infected with *Enterococcus faecalis* (a and b) or *Neospora caninum* (Nc-1 strain) (c and d). After 24 h the bacterial growth or after 48 h the parasite growth was determined by measurement of $OD_{620\,nm}$ or by the ^{3}H-uracil incorporation method, respectively. (a and c) Single experiment with three replicates and (b and d) summary of three independent experiments with three replicates each. A significant decrease of microbial growth under hypoxia as compared to the normoxia control is marked with asterisks ($^{***}P \leq 0.001$; $^{****}P \leq 0.0001$) and was calculated by a two-tailed, unpaired *t*-test. The bars indicate the mean value ± SD (a and c) or ± SEM (b and d).

upon infection. For example, pO_2 of ~1.3% was detected within the liver tissue of *Schistosoma mansoni*-infected mice [3]. Furthermore a high expression rate of the hypoxia inducible factor-1α (HIF-1α) was detected in the infiltrative belt surrounding hepatic alveolar echinococcosis lesions within rat livers [20]. Overexpression of HIF-1α in the actively multiplying infiltrative region of these lesions was closely related to angiogenesis and microvasculature [20]. Similar pO_2 levels of approximately 1 to 3% O_2 were detected in other inflamed and infected tissues in the skin, the lungs, and the gut [12]. For example, *Leishmania major* infected mice display low oxygen levels of about 2.8% O_2 in pronounced skin lesions, while the resolution of the wound was accompanied by an increase of lesional oxygen levels [21]. Hence there is a correlation between infected and inflamed tissues and low oxygen amounts within these tissues. Given the fact that hTDO has antimicrobial properties under normoxic conditions *in vitro* and that a microbial-induced hypoxic state is detected within liver tissue, we checked whether putative hTDO-mediated antimicrobial effects might persist under low oxygen conditions. Therefore the expression and activity of recombinant human TDO in transfected HeLa cells as well as *ex vivo* murine TDO were analysed under normoxic and hypoxic conditions. In first step the survival of HeLa-hTDO cells within a hypoxic microenvironment of 1% O_2 was confirmed in cell proliferation tests, in fluorescence-based cell viability/cytotoxicity assays and in reoxygenation-based enzyme activity studies. All of these tests provided no indication for an enhanced hypoxia-induced cell death of HeLa-hTDO cells. Then the enzymatic activity of hTDO

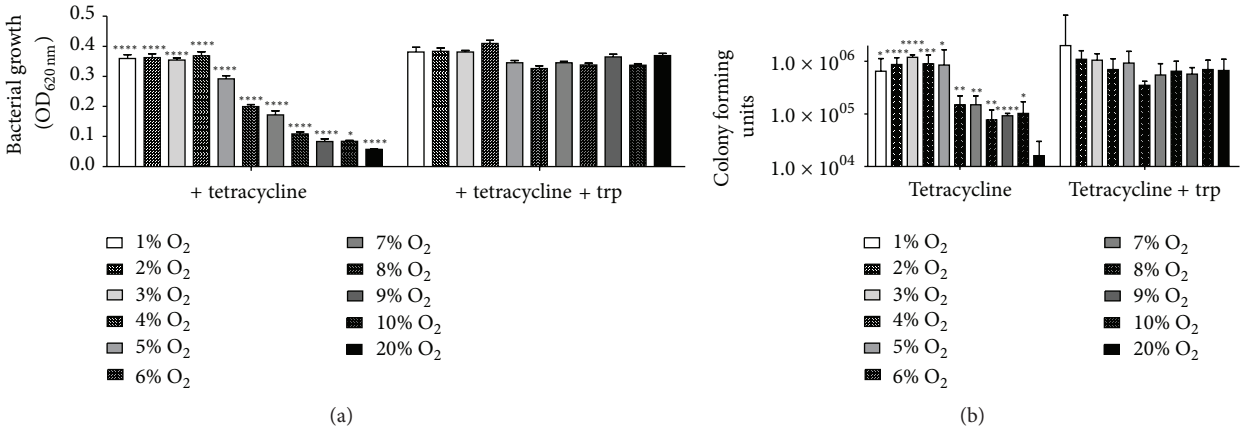

Figure 5: Loss of TDO-mediated antibacterial functions in HeLa-hTDO cells under low oxygen conditions. Infection experiments: HeLa-hTDO cells were incubated under different oxygen conditions (1–20% O_2) for 72 h with or without tetracycline (160 ng/mL) and afterwards infected with *Enterococcus faecalis* in the presence or absence of additional tryptophan. (a) After 24 h the bacterial growth was determined by measurement of $OD_{620\,nm}$, in 3 independent experiments with three replicates each. (b) Determination of colony forming units by serial dilution and summary of 7 independent experiments. Significant alterations of microbial growth as compared to the normoxia control are marked with asterisks ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$) and were calculated by a two-tailed, unpaired *t*-test. The bars indicate the mean value ± SEM (a) or SD (b).

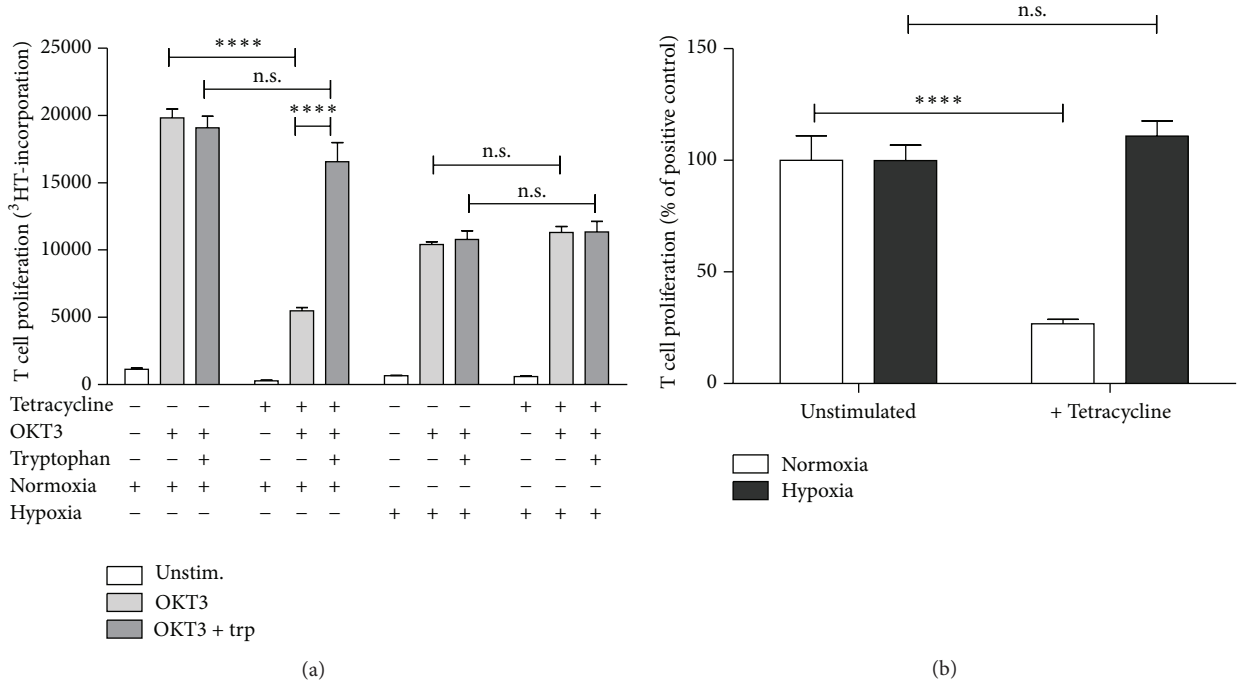

Figure 6: Loss of TDO immunoregulatory function in HeLa-hTDO cells under hypoxic conditions. T cell proliferation experiments: HeLa-hTDO cells were prestimulated with or without tetracycline (10 ng/mL) for 72 h under normoxia (20% O_2) or hypoxia (1% O_2). Then the supernatants were harvested and served as cell culture medium for freshly isolated peripheral blood lymphocytes (PBL). 1.5×10^5 PBL/well were activated in 96-well plates with a monoclonal anti-CD3 antibody (OKT3) and T cell proliferation was determined after three days by adding ^{3}H-thymidine for 24 h. The incorporation of ^{3}H-thymidine was detected using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Rodgau Jügesheim, Germany). A significant alteration of T cell proliferation as compared to the respective control group is marked with asterisks ($^{****}p \leq 0.0001$; n.s. = not significant) and was calculated via a two-tailed, unpaired *t*-test. (a) Single experiment with three replicates and (b) summary of three independent experiments with three replicates each. The bars indicate the mean value ± SD (a) or ± SEM (b).

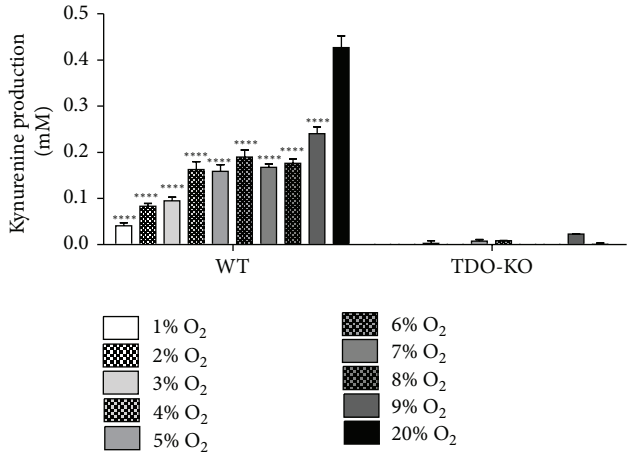

Figure 7: Loss of TDO activity in liver homogenates under hypoxic conditions. Determination of murine TDO *ex vivo* activity. Livers of WT or TDO-deficient C57BL/6 mice were homogenized in PBS and liver homogenisates were added to TDO-buffer in 48-well microtiter plates, followed by an incubation under normoxia (20% O_2) or other oxygen amounts (1–9% O_2) for 4 h at 37°C. Then the reaction was stopped using 30% trichloroacetic acid and the kynurenine amount was determined in the supernatants via supplementation of Ehrlich's reagent and absorbance at 492 nm. $n = 3$ independent experiments with three replicates each. A significant alteration of kynurenine levels as compared to the normoxia control is marked with asterisks ($^{****}p \leq 0.0001$), as determined via a two-tailed, unpaired t-test. The bars indicate the mean value ± SEM.

was determined by measurement of kynurenine, which is the product of the TDO-mediated tryptophan degradation. Human TDO efficiently catalysed the formation of kynurenine under normoxic conditions (20% O_2), whereas significantly lower levels of kynurenine were generated under oxygen concentrations detected in liver tissue physiologically (1–10% O_2). Our *ex vivo* studies using liver homogenates from wildtype and TDO-deficient mice clearly show that also the mTDO-dependent degradation of tryptophan is significantly reduced under low oxygen conditions (<9% O_2). In particular the low pO_2 of 1–3% O_2, which has been detected in infected liver tissue significantly restricted the enzymatic activity of human and murine TDO enzymes. The reason for the lower tryptophan conversion rate under hypoxic conditions *in vitro* could be general downregulation of TDO protein levels or a reduced enzymatic function. The analysis of human TDO protein amounts within normoxia- and hypoxia-treated HeLa-hTDO cells via Western Blot analyses showed no decrease of hTDO protein amounts under hypoxia. HTDO protein levels were correlated with the respective β-actin band, since it is known that hypoxia caused general alterations in protein expression levels [18]. Therefore a decrease in hTDO expression could not account for the decrease of hTDO activity under hypoxia. Hence the enzymatic activity of hTDO must be altered, matching the fact that TDO is a protoheme-containing enzyme that catalyses the insertion of O_2 into the pyrrole ring of L-tryptophan and is therefore dependent on cellular oxygen portions [22]. In line with that we observed that normoxia-pretreated and hypoxia-pretreated cells showed no significant differences in their tryptophan-degrading capacity in the reoxygenation phase *ab initio*.

A second dioxygenase that likewise catalyses the degradation of tryptophan is the enzyme indoleamine 2,3-dioxygenase (IDO). Already in the 1980s it was shown that the intracellular degradation of tryptophan, induced by IFN-γ, restricted the growth of the intracellular parasite *Toxoplasma gondii* in human fibroblasts [23]. Since then IDO crystallized as broad-spectrum antimicrobial effector molecule that is effective against a variety of tryptophan-auxotroph pathogens *in vitro* [24]. Interestingly, IDO-mediated tryptophan depletion is also inhibited under low oxygen concentrations (≤3% O_2) which results in a loss of IDO-mediated antimicrobial effects *in vitro* [19, 25, 26]. This was first shown in infection experiments using the intracellular bacterium *Chlamydia trachomatis* in human fallopian tube cells and pinpointed to the hypoxia-dependent inhibition of IFN-γ signalling [26]. This loss of IDO-mediated antimicrobial effects under hypoxia raised the question to what extent TDO might lose its antimicrobial properties upon hypoxic circumstances.

Here we show that the antibacterial and antiparasitic effects of hTDO were lost in HeLa-hTDO cells under hypoxia. Human TDO was no longer able to inhibit the growth of *Enterococcus faecalis* and other tryptophan-auxotroph bacteria such as *Staphylococcus aureus* or group B streptococci (data not shown). Furthermore the hTDO-mediated defence against the intracellular parasite *Neospora caninum* was lost under hypoxic conditions. Since hTDO is expressed within HeLa-hTDO cells by the addition of tetracycline and not by IFN-γ stimulation, perturbations in the IFN-γ signalling pathway cannot account for these observations, but only the lack of molecular oxygen.

Unfortunately it is impossible to analyse potential antimicrobial functions of mTDO or hTDO in isolated primary hepatocytes, since these cells readily undergo dedifferentiation and lose hepatocyte function [27]. Therefore further studies need to be performed analysing such TDO functions in *ex vivo* induced stem cells (e.g., embryonic stem cells, pluripotent stem cells, and hepatic progenitor cells) that are differentiated into hepatocyte-like cells with potential TDO function.

Since there are also no data claiming for an antimicrobial function of mTDO *in vivo* up to now, the immunoregulatory function of TDO is in the focus of research. Here we show that also the T cell inhibitory function of hTDO was lost in HeLa-hTDO cells upon hypoxic conditions. Again hypoxia prevented hTDO-mediated tryptophan depletion and kynurenine production which led to unhindered OKT3-driven T cell proliferation in supernatants of hTDO-positive HeLa-hTDO cells. In consistence with findings from other groups we detected reduced overall T cell proliferation under hypoxia. For example, Atkuri et al. clearly demonstrated that the influence of oxygen levels on the T cell proliferation depends on the stimulus used to activate T cells. While the proliferation in response to phytohemagglutinin was not altered under different oxygen conditions, the CD3/CD28 crosslinking and the stimulation with Con A lead to significant higher

proliferation under atmospheric oxygen levels than under physiologic oxygen levels (5% and 10% O_2), with the latter being comparable to our experimental setting [28]. This observation might be of relevance *in vivo*, since hypoxia is frequently observed in tumoural tissue [10, 11]. Tumor cells, as well as healthy cells, adapt to hypoxia by various appropriate physiologic responses, for example, by altered expression of genes that switch from oxidative to glycolytic metabolism [29, 30]. These cellular responses are caused by the induction of the hypoxia-inducible factor (HIF) protein complex due to hypoxia. The HIF complex regulates the expression of more than 100 genes included in metabolism, angiogenesis, vascular tone, cell differentiation, and apoptosis, among them various enzymes [31]. TDO mRNA is frequently expressed within human hepatocarcinomas, but information about TDO activity is missing and the cellular source is still unknown [6, 7]. TDO-mediated production of tryptophan metabolites protects TDO-transfected tumour cells against immune rejection [8, 9]. Therefore the role of TDO in immunoregulation is crucial.

Our data indicate hypoxia as an environmental factor which is present in tumoural tissue that strongly impacts TDO activities and might therefore be beneficial for tumour growth.

5. Conclusions

The strong influence of low oxygen amounts on innate and adaptive immunity in both inflamed resident cells and infiltrating immune cells was described before [32–34]. Since several antimicrobial and immunoregulatory effector molecules in addition to TDO and IDO, as, for example, the phagocyte NADPH oxidase (PHOX), type 2 nitric oxide synthase (NOS2), and mitochondria rely on molecular oxygen as a substrate, hypoxia impairs their activity, which might promote the survival of pathogens [12]. Therefore low oxygen levels might lead to an inadequate control of microorganisms and to subsequent overwhelming or chronic infections. The role of TDO in this context has still to be identified.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Inflammation Thread Runs across Medical Laboratory Specialities

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We work on the assumption that four major specialities or sectors of medical laboratory assays, comprising clinical chemistry, haematology, immunology, and microbiology, embraced by genome sequencing techniques, are routinely in use. Medical laboratory markers for inflammation serve as model: they are allotted to most fields of medical lab assays including genomics. Incessant coding of assays aligns each of them in the long lists of big data. As exemplified with the complement gene family, containing C2, C3, C8A, C8B, CFH, CFI, and ITGB2, heritability patterns/risk factors associated with diseases with genetic glitch of complement components are unfolding. The C4 component serum levels depend on sufficient vitamin D whilst low vitamin D is inversely related to IgG1, IgA, and C3 linking vitamin sufficiency to innate immunity. Whole genome sequencing of microbial organisms may distinguish virulent from nonvirulent and antibiotic resistant from nonresistant varieties of the same species and thus can be listed in personal big data banks including microbiological pathology; the big data warehouse continues to grow.

1. Introduction

Biologic inflammation in its multifaceted subsistence lends itself to delineation by big laboratory data IT—in this age of data warehouse extension. Many life maintaining biological interactions function as multicomponent weight balance, equilibrium standing for health. Settling on one or the other side to vacate fine adjustment may go on to result in final targeting be it for health maintenance or to develop pathological transformation. The paths to excess are paved with stop- and go-signaling: activation signals can be held back with the hazard to cause overshoot in the other direction of the balance. To such intrinsically complex regulatory framework of a single system adds up the interaction between different systems involving health maintaining cross-reactivities or expanding pathological effects.

Thus a large amount of metabolites, proteins, intermediate and terminal enzymes interact simultaneously to maintain physiological wellbeing or they will thwart equilibrium. When attempting diagnosis, medical laboratories test for single disease-related leading analytes/markers and they go for an appropriate choice to give patients and physicians a representative picture to tailor therapy. We here describe some approaches to sort out the relevant results for patient care in precision medicine. In this analysis we exclude the doctors' choice on categories on order sheets of the appropriate lab assays at the outset, assuming that big data accumulate over time during medical checkups unrelated to a single morbus.

2. Metabotyping May Circumscribe Inflammation in the Lab

Metabotyping and high-resolution omics data has the promise to picture diseases based on metabolite's profile or ratios of selected analytes and might develop into a relevant component of diagnosis and treatment of single nosological entities. Mechatronic engineering designs, testing and operation of machinery and equipment, in which there is a high level of functional integration of mechanical systems with electronics and computer control in laboratory equipment brings information gain from metabotyping upfront. Biologists of the Swiss Federal Institute of Technology work on

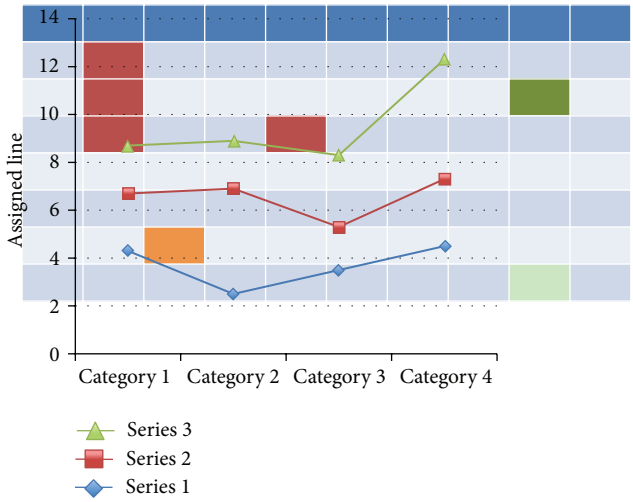

Figure 1: Metabolome profiling. Real-time metabolome profiling by injection of living bacteria, yeast, or mammalian cells into a high-resolution mass spectrometer enabling automated monitoring of several hundred metabolites can be simultaneously quantitatively estimated within minutes in a circuitry displayed in the work published by the Swiss Federal Institute of Technology. Output-fluxes of ~300 compounds using automated monitoring in 15–30 s cycles over several hours are possible. The figure is a simplified transposition of single data points from hundreds of possible analyses (squares, randomly highlighted with colors) becoming linked using bioinformatics into series and categories meaningful for exploration [29].

real-time simultaneous analysis of hundreds of analytes measured with the same instrument [1], results of which might be funneled into biocomputing circuits. Mathematical models are then used to quantitatively relate metabolomics, expression, and proteomics data to the functional network output related to fluxes (Figure 1). The usability of such waves of IT based information, particularly if used in health-monitoring systems, will need original/innovative approaches for secure storage [2].

We here attempt to envision the inflammation portion of the whole body metabolism as an envelope containing interactive signal circuits which interact at the frontend of genetic, transcriptional, and proteomic backgrounds and react to inflammation inducing forces: the current view of senescence being brought forward, at least in part, by inflammatory mechanisms has coined the neologistic term of inflammaging, none the least of these being senescence (inflammaging) [3–6]. Our update can be read as a background to discern digital memories eventually leading to biological computer science [7].

3. Medical Laboratory Copes with Big Data

Each single patient produces data in the long run with her or his standard data entry description: accession number, sample number, patient ID, sex, birthday, clinic, ward, doctor, order comment collection source, (repeat) collection date, and sample comment. Generation of big data cannot be circumvented since long. Dialog boxes upon receipt of samples in the lab may help to reduce data flow but cannot ban it. Analysis of most disease-related metabolites, including glycoproteidic biomarkers for disease diagnosis, is based on ELISA, ECLIA, and enzyme-substrate colorimetric/light extinction/electrochemoluminescence (ECL) technology.

Mass spectrometry with improved resolution is now often preceded by multidimensional chromatographic separation schemes which enlarges the spectrum of possible analytes. A uniform distribution of the number of acquired MS/MS, protein, and peptide identifications undergo proteomic runs which allow protein identification on large scales estimated up to as high as ~14 000 proteins and ~250 000 unique peptides [8].

A large spectrum glycoprotein profiling in plasma, serum, other bodily fluids or tissues is thus possible. Transplantation of donor organs into patients in need has exceeded HLA-compatibility requirements since ABO blood type system incompatible donor/recipient pairs are becoming routine [9]. Infectious and inflammatory complications remain within limits in such settings and their prophylaxis includes tight lab test controls. Subclinical inflammation [10], reliably diagnosed using C-reactive protein (CRP) serum level cutoff ≥ 10 mg/L, constitutes a risk factor for the development of interstitial fibrosis and seems to reflect not only inflammation but general well being: CRP is a subtle separator for hand grip strength, physical performance, and decline in older populations [11].

Serum pattern recognition compounds, among them CRP, bind to apoptotic cells and nucleoprotein autoantigens and Fcγ receptors to ultimately inhibit plasmacytoid dendritic cell interferon responses which are elicited by autoantibody immune complexes [12]. The monomeric form of CRP ALS inhibits renal cell-directed complement activation mediated by properdin [13]. Transplantation medicine largely contributing to big data is going to include ABO-glycan microarray results which now allow detailed characterization of donor-specific antibodies necessary for effective transplant management of solid organs [14–16].

In health care, the use of "big data," meant to use the large number of digitalized single analyses accumulated daily [17], a computational medicine, that is, eHealth or Electronic Health or Medical Record (HER and EMR), expands at the expense of health care professionals to read its message. Data warehouses containing big data need continuous validation, data management for updates, and most importantly an analysis to convert such resource into clinically relevant information for medical care [18]. Mass spectrometry and bioinformatics IT solutions may establish profiles which help to diagnose frequent and rare disease alike. The designation of given lab analyses as biomarker is currently moving towards genome sequencing to reveal genetic risks for future illness (Figure 2) [19]—obviously a component of personal big data; patients on their own will thus access to their data bank. Multibiomarker diagnosis and disease activity scoring, often a difficult curse to work, is being bundled by the clinician intending to focus on patients' complaint and clinical signs

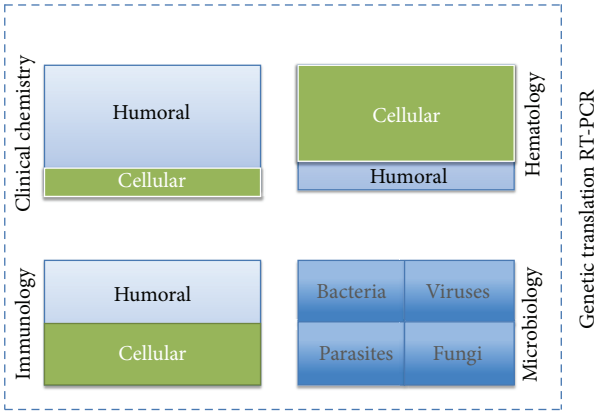

Figure 2: The four main specialities of laboratory medical analyses. The four major sections of medical laboratory analyses are shown using a four-box pattern, that is, clinical chemistry, haematology, immunology, and microbiology. Approximate quota of humoral and cellular assays is given. The big data data warehouse is substantially enlarged if each assay is completed/translated using DNA testing by real-time polymerase chain reaction (RT-PCR). Genetic embracement is drawn as broken line.

and symptoms pattern clustered together by cross-reactive algorithms [20, 21].

4. An Attempt to Categorize

For practical reason, we here lend the four major fields of laboratory medicine in Switzerland, under the auspices of a federal organization, Foederatio Medicorum Analyticorum Helveticorum (FAMH): clinical chemistry, immunology, haematology, and microbiology with genetics wrapping up each of these (Figure 2). To a large extent, this distinction of laboratory analyses by category corresponds to international habits defined by the International Federation of Clinical Chemistry (IFCC). The substantial expansion of the analyses performed boosts each of these specialities alike, enriching the respective fields with unprecedented wealth of data. This brings medical doctors to dilemmas on the appropriate choice of information for patient care—in the present context focusing on inflammation. To turn big data into appropriate data has become a topic of many fields endowed with data warehouses. Thus computational methods may throw a bridge from bench to bedside and vice versa [7, 25] and web-based platforms would allow seamless interaction between warehouse and patient-centered information as recently put forward through an EU project [26]. Academic as well as business intelligence literature counsels are available for assisting transformation of big data into a selection of data assembled for care of single disease entities (http://www.sas.com/ and http://www.aacc.org/). Structuring of such information for particular disease states is crucial [27] and platforms for gene sets might improve understanding different biological data types to reach meaningful outputs [28]. Web mapping and power-grid including care for people on a medical device that depends on electricity may be included.

Software solutions for medical laboratories, that is, laboratory data management systems provide for a several decenny old experience on a local, intralaboratory scale. Data access strategies orient themselves on priority, storage options, and delivery methods. Long-term archival of data implies repeat migration from one media/storage environment to updated systems on regular schedule to prevent hacking.

5. Calibration, Steps, Hierarchy, and How to Scale

Laboratory data management and categorizing stand or fall with nomenclature of single analyses.

Whilst encryption is useful for privacy/intimacy, our golden age of surveillance likes coding as a (secrete) language system. The multilayer dimensionality of big data warehouses needs data-driven algorithms necessary to reach their basic goal: to translate big data into clinically useful evidence. Logistic regression models, Cox analysis, and Kaplan Meier curves can sort out analytes which would predict clinical evolution, for example, kidney-associated morbidity [30–32]; our own studies are being in line with researchers in Scandinavia. Platelet count and ICU survival, actually completely unrelated parameters, can be used for predictive modelling purpose [33]. One of the possibilities to unify assignments of terms for distinct analyses currently successful on an international level are the Logical Observation Identifiers Names and Codes (LOINC) which have been created under the auspices of the Regenstrief Inc. Institute, Indianapolis, IN, USA (http://search.loinc.org/) [34]. At present, the usage of LOINC codes remains subject to variations in the way they are used and semantic, taxonomic interoperability might turn out to be contradictory in some places [34]. Therefore, LOINC committees enforce detailed guidance on best practices for mapping from local to international LOINC codes and for using LOINC codes in data exchange [35]. Experiences using data warehouse produced collaboratively between academic medical centers and private practice throwing bridges to EHR do reveal potential to improve utilization of clinical pathology testing [36]. Thus, in the US, objective electronic laboratory reporting has now been promoted as a public health priority with two coding systems endorsed: LOINC for lab test orders and Systemized Nomenclature of Medicine-Clinical Terms (SNOMED CT) for test results, the former being in use more commonly [37]. LOINC is now *de rigueur* in France: Assistance Public des Hôpitaux de Paris, APHP, has created a biomedical observation dictionary mapped to LOINC which is bound to integrate this language into the entire biomedical production chain. Since its outset in 2010, participation of 120 laboratories including 50.000 codes now ensures interoperability in the entire French EHR system [38]. LOINC codes comprise categories to inform the data storage software on (i) the analyte, (ii) measured property, that is, enzyme activity or concentration, (iii) time (span) of sample collection, (iv) system used, for example, urine plasma serum, liquor, and (v) scale, that is, nominal and ordinal. One single analyte, for

Table 1: Contribution of one single analysis, complement total hemolytic activity, CH50, to big data exemplified by LOINC coding. Seven different codes are attributed to the single CH50 analysis derived from difference in the long name, property, and the different body fluid systems in which CH50 can be measured.

LOINC code	Long name	Component	Property	System
55448-5	C total hemolytic CH50 in serum or plasma	C total hemolytic CH50	—	Ser/Plas
4511-2	C total hemolytic CH50 (units/volume) in body fluid	C total hemolytic CH50	ACnc	Body fluid
21218-3	C total hemolytic CH50 (units/volume) in cerebral spinal fluid	C total hemolytic CH50	ACnc	CSF
4532-8	C total hemolytic CH50 (units/volume) in serum or plasma	C total hemolytic CH50	ACnc	Ser/Plas
30131-7	C total hemolytic CH50 (mass/volume) in serum or plasma	C total hemolytic CH50	MCnc	Ser/Plas
48071-5	C total hemolytic CH50 (titer) in serum or plasma	C total hemolytic CH50	Titr	Ser/Plas
48496-4	C total hemolytic CH50 actual/normal in serum or plasma	C total hemolytic CH50 actual/normal	RelCCnc	Ser/Plas

Table 2: Big data in electronic health records (EHR). A 2016 update with special emphasis on medical laboratory medicine.

Advantages	Drawbacks	Measures	Ref
Real time health profile	Hacker friendliness	Encryption	[22]
Ubiquitous access to electronic health record EHR	Code readability not yet universal	National health offices regulation in progress	See http://www.medicalrecords.com/
Patients' own medical record	Patient-driven medical updates necessary	Transmit medical terminology to patient	Dragon Medical software
Patient record download by hospital W-LAN	Hacker friendliness	Limit time of accessibility	SOARIAN Health Archive (Switzerland) HIPAA Space (USA)
Distribution pattern of virulence of the same bacterial strain	Exchange of DNA: most strains have overlapping genome	(i) Update bioinformatic resource (ii) Use hybridization of identification	[23]
Postmarketing surveillance of medical devices	Criteria selection	LOINC™ coding	[24]

example, complement hemolytic activity activated through the classical pathway, CH50, comprises as much as 7 different LOINC codes (Table 1), depending on which starting material was employed for CH50 analysis.

Newborn screening, including such analyses with high-stake health implications necessitates rapid/effective communication between many people and organizations and increasingly depend on big data registries which can aggregate results from national programs [39] and help harmonize inherited metabolic disorder to an international level [40]. The March of Dimes recommends screening newborns for 29 conditions, such as phenylketonuria, hypothyroidism, galactosemia, and sickle-cell anemia (http://www.marchofdimes.com/). A prenatal screening program is taking shape and involves pregnancy-associated plasma protein A (PAPP-A) and pregnancy hormone hCG and pregnancies with hypertensive disorders might be detected early on using analysis of cell-free fetal DNA, cell-free total DNA, and biochemical markers [41].

A conjugate prior distribution belonging to the same parametric family may be chosen. Such a Bayes estimator for single analyses with their variance, confidence interval can be derived from a posterior distribution; the minimum square error also called squared error risk is defined by MSE $= E[(\theta(x) - \theta^2)]$. Some of the key features of a Bayesian analysis as a powerful package for molecular sequence variation have been delineated 10 years ago [42].

This novel task of doctors on scooping the right selection of relevant data requires a minimum understanding of what IT can and cannot do for the benefit of patient care (Table 2). The European Informatics Institute (EMBL-EBI) based on the consolidated Apache Lucene technology might inspire search engine development to direct scalable search paths towards medical diagnosis [43].

5.1. Big Data in Clinical Chemistry. The number of clinically relevant chemical analyses offered for diagnostic purpose at Swiss University Hospitals and private industry amounts up to roughly 180. Henceforward, such overseeable data built up during the last century currently expands to big data produced by automated workflow using intelligent robotics with throughputs of 3–15 million clinical chemistry assays/year. As learned from internet searches, Unilabs™ processes 40.000 medical analyses/day and Synlab™ offers > 4000 different analyses to its clients. A laboratory automate, for example, the Cobas machine (Roche Diagnostics, Rotkreuz, Switzerland) with its large panel of possible analyses, may serve as an example of ever growing lab service function. Competing industries, for example, Abbott (Abbott Diagnostics, Abbott Park, IL, USA), Siemens (Siemens Healthcare, Erlangen,

Germany), Hitachi (Hitachi, Mountain View, USA), Capillarys Sebia (Paris, France), Kiestra (BD, Franklin Lakes, NJ, USA), Bruker (Bruker, Billerica, MA, USA), BioMérieux (Lyon, France), are continuously updating their offers in order to increase capacity, that is, number of analyses/time throughput. The analytes of the clinical chemistry lab section can be subdivided into provision of information for whole body pathology and into organ-specific lab assays. Selected groups of analyses are assembled as suggestion to clinicians in order to investigate single organs: endocrinology, liver function, gastroenterology, nephrology, vitamins. Each of these specialities of medicine sees its own lab definition increased in number of different analyses such as we have recently used cystatin C and its ratio to creatinine to improve significance of interpretation in kidney insufficiency [30]. The inflammation parameters in clinical chemistry, occasionally with biomarker status, are acute phase proteins, that is, CRP, serum amyloid A, fibrinogen, tryptase [44], haptoglobin, procalcitonin [45], interleukin-6, and again CRP used to classify disease stage of rheumatoid arthritis [20] and now even to estimate extent of fitness and senescence [11].

5.2. Big Data in Haematology. Two lines to enriching haematological patient findings emerged recently: (i) intelligent picture readings of blood and bone marrow films making possible telehaematology [46–48] and continuous flow analysis of single cells sorted according to their clonal origin. Picturing blood films does not lend itself to electronic storage in big data banks in contrast to findings coming out from forward and sideward scatter beamer flow cytometer cell analysers, such as Sysmex XE-5000 (Kobe, Japan), Abbot Sapphire (Abbott Diagnostics Division, Santa Clara, CA, USA), Siemens Advia (Siemens Healthcare, Erlangen, Germany), Beckman Coulter (Beckman Coulter Eurocenter, Geneva, Switzerland), and Amnis FlowSight (Seattle, WA, USA) [49].

Complete blood counts (CBC), hemostasis assays now forming part of Sysmex™ machines, bone marrow, and progress in stem cell therapeutics are all prone to be integrated into big data banks.

The diagnostic value of both microscopic and automated neutrophil left-shift parameters as indicators of inflammatory disease is limited [50] but confirmed routine automated coagulation assays and pharmacomonitoring of many drugs with LC MS/MS machines are fit for integration into big data banks and miniaturization of assay principles is contributing to this trend [51].

Blood group typing, the classical way, (still) uses haemagglutination systems with monoclonal antibodies by and large on automated platforms [52]. Results from haemagglutination are now fully completed and soon will be replaced, at least in part, by genotyping procedures [53–56]. Multiple data release prone to be sections in big data registries are now being making precision medicine even more precise. In fact, inexpensive molecular typing of histoblood types paired with powerful bioinformatics has enabled mass-scale information but bears the risk that personalized red blood cell matching for transfusion becomes less precise [55]. Small- and large-order haemogram blocks for blood microarray technology can be used to more precisely delineate anti-ABH antibodies, a progress which will make solid organ transplantation across ABO barriers more successful thanks to using bioinformatics [14].

Stem cell transfusion and cord blood based therapy are making inclusion of HLA types and GWAS whole genome typing into registries which are based on big data informatics (http://www.hpscreg.eu/).

5.3. Big Data in Immunology. Analytical approaches of the immune system branches into cellular and humoral patient samples which are tested frequently (Figure 2). The order form of our institution lists up to 200 analyses offered to the clinician, not including the large field of tests to rule out allergic diseases. Care for patients suffering from allergic diseases recognizes the usefulness of a systemwide profiling approach, which associates big lab data with the biological approach to asthma and allergy [57]. Cellular immunological analysis overlaps with hematological tests of the myeloid compartment, but they make their own data box with lymphocyte subsets and their CD marker pattern. Close to 400 CD markers have been identified up to now and their number might grow.

Contribution of the complement system to big data is considerable; with three activation pathways ~48 proteins and their fragments, 9 protein complexes and ~12 receptors [58], measured w/v and/or by functional activity, the complement system contributes to warehouses on its own. Extraction of a selection of relevant data for patient care, data from data warehouses to which complement levels contribute involves interdisciplinary algorithm flow charts. These are focusing on diagnostic and therapeutic needs in precision medicine and so far are based on care for patients with immunological and microbial diseases. However, complement analysis should also be seen in perspective with other types of analyses. With the SENIORLAB study (ISRCTN registry number 53778569) we put w/v concentrations of C4 and C3 and immunoglobulin (Ig) levels into perspective with serum vitamin D levels and senescence. Immunoassays were used to quantitate C4 and C3 and Ig in 1470 apparently healthy subjects >60 yrs. Low levels of 25(OH)D were positively associated with IgG2 and C4 (the lower vitamin D, the lower C4, Figure 3) yet inversely related to levels of IgG1 and IgA and C3 [59, 60]. As can be seen in Table 1, a single one and the very same analysis can account for 7 different LOINC codes depending on the material in which measurements are made and depending on w/v versus functional performance measured. Acute phase complement proteins related to C-reactive protein (CRP) evolve in parallel during inflammatory states and are now known to play a role in type 2 diabetes, lipid metabolism, and atherosclerosis [61] (Figure 4).

These insights suggest that complement system-related algorithms destined to extract significant patient data from big data must be seen related to noncomplement analyses performed in the routine laboratory.

On the immune cell level, high-throughput sequencing has sparked information on TCR repertoire diversity informative on functional capacity of the adaptive immune system. Diagnostic applications have been limited to measuring inflammatory markers or identifying antibodies. Nothing but

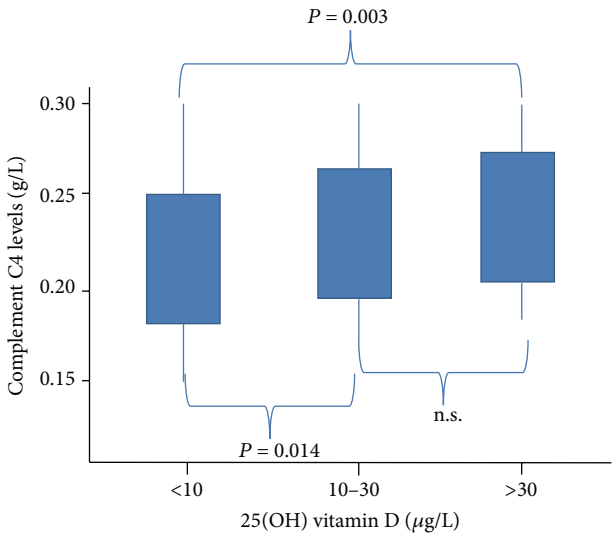

Figure 3: Complement component C4 levels depend on vitamin D sufficiency. With increasing vitamin D levels up to sufficiency (abscissa), the C4 serum levels statistically significantly increase in this cohort of healthy senior study participants > 60 years of age. From Sakem et al. 2013 [60]. A cohort of 1,470 healthy Swiss men and women, 60 years or older, were recruited for this study. A total of 179 subjects dropped out of the study because of elevated serum concentrations of C-reactive protein (>5 mg/L) making occult inflammation suspicious. 25(OH) vitamin D was measured using HPLC and levels were corroborated by parathyroid hormone measurements (not shown). The C4 levels were measured using immunonephelometry.

the diversity of $\alpha\beta$ TCRs is mirrored by a receptors' dispersity based on different peptide-sequence which might reach > 1000 [62]. Given these large numbers, high-throughput sequencing is required to achieve sufficient sequencing depth to estimate clonal abundance. TCR $\alpha\beta$ pairing can be assessed only at the single-cell level [63].

Big data warehouses will certainly have to make reference to the age group of study subjects. Thus, we have seen that IL-6 levels were lower and TNF-alpha reference intervals were higher in healthy newborns and toddlers than the adult reference intervals [64].

Biomarkers relating to particular disease states, for example, prostate-specific antigen (PSA) [65] or those of oxidized lipoproteins, genetically determined come to increase the size of big data in personalized medicine, their heritability being under scrutiny with twin pair studies [66].

Personal EHR health profiles captured by individuals themselves (e.g., from smart phones and wearable devices) will contribute to the next wave in big data—upload to the cloud and propagation across social networks make encryption prevent access to information by insurance companies, ransomware hackers, and state writ guardians [22].

5.4. Big Data in Medical Microbiology. Host, microbiomes, and pathogenic microbes are analyzable with a big data array of laboratory criteria making bioinformatics an indispensable pillar of big data in inflammation exploration. Bacterial, viral, fungal, and parasitic infections and diseases due to microbial toxins are among the most common and medically important causes of inflammation with different pathogens eliciting varied responses ranging from mild and short-term to severe and long-term [67]. Foreign bodies, catheters [68], splinters, sutures, and dirt may elicit inflammation and inflammation tissue and laboratory data may circumscribe hypersensitivity and autoimmune disease induced inflammation under the control of cytokines produced by T lymphocytes mainly. On hosts' side the predisposition to provide for a favourable environment for infectious agents, an array of receptor molecules (Fy a/b for malaria, CHO recognition on PMNL, fibronectin on catheters, Figure 4) can be appreciated to then enter BMLD (Biosafety in Microbiological and Biomedical Laboratories) banking. Nothing but the human gut containing a myriad of different bacteria and other microorganisms such as Archaea, viruses, and fungi, making the microbiome expand to big data sets, an enter-system [69]. On the side of microbes, the BMLD provides important information on the clinical presentation the infectious agent will cause if successfully attacking the host. As an example, the strain K 157 of *E. coli* is predictive of HUS and other strains of *E. coli*, such as O157:H7, O104:H4, O121, O26, O103, O111, O145, and O104:H21, produce potentially lethal toxins. Most *E. coli* are being innocuous or form part of the microbiome component. This is but one example of the enormous extent of data which microbiology occupies the space of a data warehouse. With the MALDI TOF system, fast typing has entered the practice since a decade [70] which facilitates updates of BMLD boxes. Virulence factors can be spotted in *Staphylococcus aureus* using whole genome sequencing combined with DNA microarray hybridization prone to increased big data informatics [23]. A large EHR data accumulation is in progress for diagnostic testing. For viral diseases as well laboratory assays differ between specimen analyses in acute disease and the assay approach used for specimens taken during convalesce. The current example here is guideline updates considering the ongoing Zika virus and other flavivirus epidemics (e.g., dengue, yellow fever, St. Louis encephalitis, and West Nile virus) which enforce usage of RT-PCR, also for chikungunya viruses. Proposed test algorithms start out with both molecular and antibody testing to minimize the risk for cross-reactivity vulnerable meandering (memorandum CDC) (http://www.cdc.gov/zika/pdfs/denvchikvzikv-testing-algorithm.pdf).

5.5. Big Data in Genetics. Whilst big genetic data validation overlaps and completes each of the preceding subchapters (Figure 2), genetic studies of many diseases are now allowing closer insights into human pathology. Next generation sequencing (NGS) creates challenges for validation of results. NGSs can be used to detect genetic anomalies of essentially any size scale, from SNPs to very large rearrangements; all of todays' genetic diagnostic tests could in principle be supplanted by NGS [71], including RNA analysis, because transcriptome (RNA-seq) sequencing is possible and now boosts with CRISP-Cas9 technology [72]. On the leading edge of a revolution in medicine the complete DNA sequence,

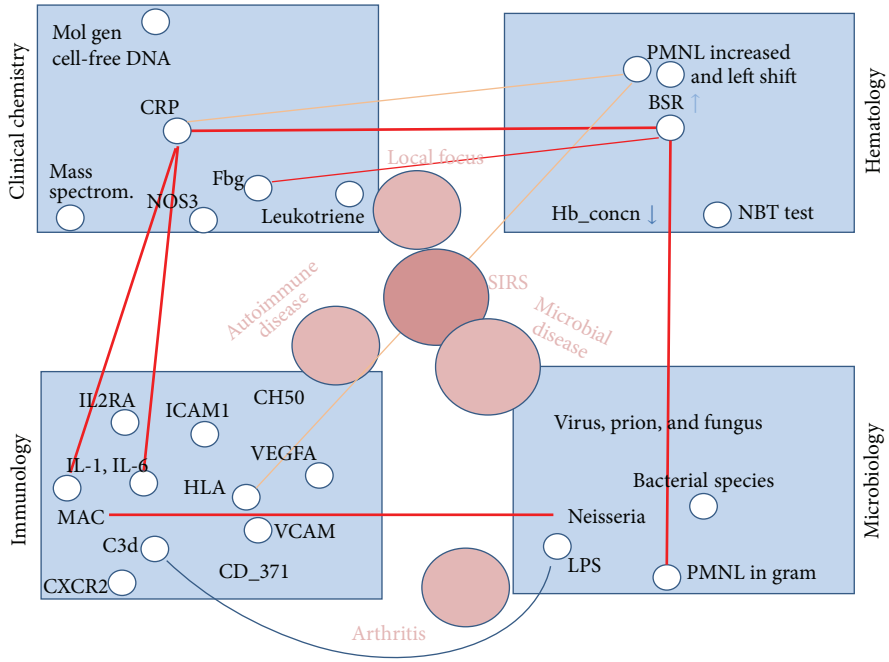

Figure 4: Analyses from different sections of the medical laboratory must be pulled together to establish evidence for inflammation. Similar to the display in Figure 2, the 4 major specialities, clinical chemistry, haematology, immunology, and microbiology, each encompass a whole array of inflammation markers. Networking across lab specialities obtrudes to pin down inflammatory disturbance. Complement analytics placed in the "immunology" box. Red bars connect strong evidence, while orange bars show medium evidence for denoted disease states. The insinuated disease states are placed arbitrarily.

properly encrypted, will become a permanent part of individual's EHR utilized by health care professionals to make decisions about drug prescriptions, diagnostics, and disease prevention [73]. Molecular geneticist validation reviews all variants called on a 10-gene panel with 30 variants per case without additional IT support, but it will be swamped when this would be have to be done for a 100-gene panel [17].

As an example phenotypes are now closely linkable to genetic findings and to the age of the patient, younger age at diagnosis associated with extensive/aggressive Crohn's disease and ulcerative colitis. Dissection of genotype-phenotype relations using Immunochip array designed to capture up to 200 different loci associated with common autoimmune diseases can be focused on NOD2, MHC, and MST1 3p21 to allow establishment of a genetic risk score and predictive modelling [74]. The genetic diseases encountered in medical practice are the tip of the iceberg, that is, those with less extreme genotypic glitches that permit full embryonic development and live birth. Genetic variants of complement genes, for example, CFIGly119Arg, such as recently evaluated in the EUGENDA cohort may be associated with age-related macular degeneration [75]. How many mutations remain hidden? With the determination of the complete sequence of the human genome, DNA imprints not only for disease but also for the gray zone between health and disease are under scrutiny. As an example, training of legasthenics could use genomic insights to improve its efficacy. As Dr. Collins puts it "it will take decades, if not centuries to understand the instructions of the genetic language, and, one might add, improve and make big data information more meaningful" [76].

6. Attempts to Constrain Big Data in Clinical Settings

Ransomware attacks hackers blocking hospital and private practice computers have now been reported here and there. Computer systems, including those needed for lab work, can be set out of function relatively easily which makes big data clouds vulnerable to an extent to which some workers keep copies on separate hardware aside; desktop virtualization systems, for example, Citrix™, are improved for hacker risk reduction and saving data apart on separate servers might do the rest to prevent big data hacking.

Two motive forces are thriving attempts to reduce the number of health parameters (i) to bring customers of the medical lab to a reasonable and patient driven block of analyses asked for [77] and (ii) to optimize financial sources doctors often ignoring the financial consequences of their test-ordering behavior.

Study results from attempts to reduce the number of ordered tests become known [78] and an expert panel may bring some good ideas but will never be able to brake medical revolution continuing to creep on us, especially in the field of laboratory analyses.

In conclusion, the current expansion of medical lab assay number expands big data warehouses. Multidisciplinary

efforts are required to master the mighty offer of data and to make the information improve patient care.

Abbreviations

Mol gen: Molecular genetic analyses
Fbg: Fibrinogen
NOS3: Nitric oxide synthase
PMNL: Polymorphonuclear neutrophilic leukocytes
Hb: Haemoglobin
NBT: Nitroblue tetrazolium
IL2RA: Interleukin-2 receptor subunit alpha
ICAM: Intercellular adhesion molecule
IL: Interleukin
HLA: Histocompatibility locus
CXCR2: C-X-C motif chemokine receptor 2
VEGFA: Vascular endothelial growth factor A
VCAM: Vascular cell adhesion molecule
LPS: Lipopolysaccharide
SIRS: Systemic inflammatory reaction syndrome
CD_371: CD marker currently coming up to 371 varieties
BSR: Blood sedimentation rate
MAC: Membrane attack complex.

Disclosure

This paper is the companion paper to an ePoster entitled *Clinical Significance of Complement System Analytics Extracted From Big Data Banks* by Nydegger et al. at the 10th International Congress on Autoimmunity, Leipzig, Germany, 2016 listed in session EPD32: *IVIG, Innate Immunity: How to Restore the Immune System to Normal* (http://www.autoimmunity.kenes.com/).

Competing Interests

The authors have no conflict of interests to declare.

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Review Article

Sickness-Associated Anorexia: Mother Nature's Idea of Immunonutrition?

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During an infection, expansion of immune cells, assembly of antibodies, and the induction of a febrile response collectively place continual metabolic strain on the host. These considerations also provide a rationale for nutritional support in critically ill patients. Yet, results from clinical and preclinical studies indicate that aggressive nutritional support does not always benefit patients and may occasionally be detrimental. Moreover, both vertebrates and invertebrates exhibit a decrease in appetite during an infection, indicating that such sickness-associated anorexia (SAA) is evolutionarily conserved. It also suggests that SAA performs a vital function during an infection. We review evidence signifying that SAA may present a mechanism by which autophagic flux is upregulated systemically. A decrease in serum amino acids during an infection promotes autophagy not only in immune cells, but also in nonimmune cells. Similarly, bile acids reabsorbed postprandially inhibit hepatic autophagy by binding to farnesoid X receptors, indicating that SAA may be an attempt to conserve autophagy. In addition, augmented autophagic responses may play a critical role in clearing pathogens (xenophagy), in the presentation of epitopes in nonprovisional antigen presenting cells and the removal of damaged proteins and organelles. Collectively, these observations suggest that some patients might benefit from permissive underfeeding.

1. Introduction

Infection or tissue trauma is known to induce a range of behavioural modifications collectively referred to as sickness behaviour. Of all these behavioural changes, sickness-associated anorexia (SAA) represents a paradox. Mobilisation of an immune response is metabolically costly [1]. The production of antibodies as well as other signalling peptides (e.g., cytokines and initial phase proteins), expansion of immune cell populations, and the induction of a febrile response all contribute towards a dramatic increase in the demand for metabolic substrates. Yet, despite the cost associated with mobilising an immune response, a decrease in appetite manifests as one of the most cardinal symptoms of an established infection.

Three observations suggest that such SAA represents an adaptive response. Firstly, noninfectious elements such as lipopolysaccharides (LPS) or certain cytokines (e.g., Il-1β and TNF-α) induce appetite loss [2]. The fact that SAA is engaged by the same canonical inflammatory mediators released in response to infection suggests that SAA forms part of the immune response. Secondly, an array of animals, ranging from vertebrates [3–6] to invertebrates [7–10], all exhibit SAA. Indeed, even primitive animals such as sea anemones retract tentacles and stop feeding after a pathogen challenge [11], indicating that SAA may indeed be evolutionary ancient and conserved across numerous species. In turn, such prevalence amongst animals suggests that SAA must impart a significant fitness advantage. Finally, SAA is consistently observed in a variety of contexts. Only African horse sickness (a lethal vector-borne equine viral infection) is known to induce a fever without inducing an anorexic response [12]. Similarly, a recent review highlighted a number of social factors (e.g., maternal care for the young or guarding behaviour against intruding males) which may attenuate aspects of sickness behaviour [13]. Yet, strikingly, no examples where anorexia is diminished have been identified. Taken together, these observations support the view that SAA is a dedicated

host response that facilitates host survival during an infection, though the mechanism by which SAA imparts a survival advantage remains elusive.

A number of theories have been forwarded to explain the adaptive value of SAA. Since free iron is rapidly reduced during an infection [14, 15], it has been argued that chronic low iron levels might provide a pleiotropic benefit by protecting the host against infections [16]. Consequently, an anorexic response may deny pathogens critical resources such as iron [15]. However, recycling of endogenous reserves (e.g., haemoglobin) represents the primary mechanism by which iron levels are maintained, with dietary reabsorption being highly ineffective. In fact, less than 1% of dietary iron is absorbed, with iron status responding only slowly to a changing nutritional status [17]. Aligning behavioural aspects with immunological prerogatives has also been proposed as a physiological strategy pursued by sickness-associated anorexia. In conjunction with a general sense of fatigue, anorexia may decrease an animal's motivation for engaging in foraging behaviour [12]. Also, foraging animals expose a larger surface from which they may radiate heat, thus limiting the efficacy of a febrile response [12]. In addition, infected animals may be less attentive to their surroundings [12] or more conspicuous [18] and accordingly under greater risk of predation. Thus, decreasing foraging activities would possibly reduce exposure to predators. Furthermore, since many pathogens gain entry via the oral route, anorexia may prevent exposure to a fatal pathogen load [12]. Yet, although LPS administration dramatically decreases food intake in rats, hoarding behaviour seems only modestly reduced if under restricted food access (30 min/day) [19]. In fact, Siberian hamsters (which are dedicated hoarders, unlike house mice and rats) injected with LPS demonstrate a dramatic decline in feeding, while hoarding behaviour remains mostly intact [20]. Consequently, it remains to be explained why an animal would demonstrate hypophagia as an energy-conserving or predation-avoiding strategy but remains actively foraging.

We recently argued [21] that a reduced appetite during an infection may represent an evolutionary conserved strategy for systemically upregulating another evolutionary conserved process, autophagy. A brief overview of the ambiguous results on studies investigating the benefit of nutritional support in critically ill patients is provided, followed by an overview of the nutritional context of immune cells during an infection. Next, a summary of the various mechanisms by which an increase in autophagic activity may influence clinical outcome is provided. This is followed by a discussion addressing some of the key limitations of SAA as an inducer of autophagy, with reference to the clinical implication for nutritional support. Finally, we address outstanding questions regarding the role of autophagy, and how insight into these considerations may lead to more refined nutrition support protocols.

2. Is Nutritional Support Beneficial?

A major goal of nutritional support in critically ill patients is to avoid the loss of muscle mass, a clinical marker for mortality and morbidity. Yet, various lines of evidence suggest that nutritional support may not provide any benefit, and indeed, may potentially be harmful in certain contexts. In a rat model of septic shock, "immunonutrition" with polyunsaturated fatty acids or arginine aggravated disease progression [22]. Similarly, nutritional intervention has not consistently been demonstrated to be effective in critically ill patients. Indeed, it was found in one study that patients receiving early parenteral nutrition (on day 3 in the intensive care unit) had a small but significantly higher incidence of infection [23] while another study showed a decrease in mortality associated with permissive underfeeding (60–70% of caloric goal) [24]. A recent Cochrane meta-analysis reported that the only benefit associated with nutritional therapy is a decrease in nonelective readmission [25]. Indeed, a consortium of experts have advocated that permissive underfeeding rather than full caloric feeding should be applied to critically ill patients suffering sepsis or septic shock [26]. Yet, the mechanism by which a decrease in nutritional intake would be beneficial remains to be fully elucidated.

A similarly puzzling phenomenon is the paradoxical benefit observed during short-term fasting: starvation, up to three days prior to *Listeria* challenge, reduced the mortality rate to only 5% (compared to a fed group with a mortality rate of 95%) [27]. In addition, starvation promoted macrophage activity against bacteria such as *Listeria monocytogenes* (both in vivo and in vitro), which could be further enhanced by LPS administration [28]. M. J. Murray and A. B. Murray [29] also recount an interesting anecdote provided by Edward [30] who noticed that starved hedge-hogs seemed immune to foot and mouth disease. Correspondingly, force-feeding during an infection resulted in a *higher* mortality rate among mice [29]. Thus, there is both clinical and preclinical evidence indicating that nutritional support does not benefit all patients.

3. Starvation: A Calculated Response

It is widely accepted that starvation potently inhibits immune function [31], suggesting that SAA may impede the mobilisation of an effective immune response. Yet, animals have evolved a range of adaptations to cope with nutrition stress [32]. Immune cells in particular occupy a privileged position with regard to the provision of energy-dense substrates. Indeed, during an infection, the expansion of immune effectors is fuelled by peripheral catabolism. In this regard, a number of physiological adaptations ensure that, despite a decrease in feeding, the immune system does not become nutrient deprived.

3.1. Energy-Rich Metabolites and Paracrine Signalling. Activated immune cells are highly dependent on glucose. Indeed, hypoxia-inducible factor (HIF), a major inducer of glycolysis, is necessary for macrophage maturation [33]. Conversely, a switch towards oxidative metabolism is accompanied by an activation of an anti-inflammatory programme [34]. It must be noted that although glycolysis is usually active during hypoxia, activated immune cells, similar to other rapidly dividing cells such as cancer cells and proliferating fibroblasts, engage in a form of oxidative glycolysis: these cells produce ATP via glycolysis irrespective of oxygen tension. Such

aerobic glycolysis (Warburg effect), which is less efficient than oxidative phosphorylation, is likely explained by two possible factors [35]. First, the inefficiency of glycolysis is compensated for by the rapid speed by which a cell can generate ATP via glycolysis. Second, metabolic intermediates of glycolysis are easily fluxed into biosynthetic pathways that are also upregulated in rapidly dividing cells. As an example, the acetyl-CoA which is required for the synthesis of fatty acids is derived from glycolytic pathway. In this regard, the synthesis of fatty acids is critical for immune cell function. In fact, compromising the ability of monocytes to synthesise fatty acids prevents differentiation into mature macrophages [36]. The dependency of fatty acid synthesis is in turn explained by the demand for phospholipid synthesis: an expansion of cellular components such as endoplasmic reticulum (ER), mitochondrial network, lysosomes, and the development of filopodia all make it necessary for lipids to be incorporated into membrane structures [36]. Thus, activated immune cells require glucose for energy production as well as for the biosynthesis of cellular components. It is thus likely that the Warburg effect can be explained by the fact that glycolysis intersects both energy production and biosynthesis.

A number of key regulatory factors ensure that immune cells are also well supplied with glucose. Proinflammatory cytokines TNF [37] and IL-1β [38] induce insulin resistance and thus elevate blood glucose levels. In turn, high glucose levels facilitate the influx of glucose into immune cells via GLUT-1 transporters. Since a GLUT-1 transporter is a facilitative transporter, intracellular glucose is dependent on the extracellular glucose concentration. Thus, elevated glucose levels during an infection are likely to be an adaptive strategy to ensure adequate glucose content in activated immune cells. Similarly, a severe infection is often associated with hypertriglyceridemia. In rats, even low doses of LPS rapidly induce hypertriglyceridemia [39]. High triglyceride levels are maintained through de novo synthesis in the liver [39] and release from adipocytes [40]. Similarly, the development of insulin resistance by LPS binding to TLR4 [41] leads to the secretion of inflammatory cytokines that induce a state of insulin resistance [42]. Thus, infection results in the liberation of energy-rich molecules in circulation which drive immune cell metabolism.

Adipose tissue also seems to be functionally integrated with the immune system, particularly adipocytes which are anatomically associated with lymphoid tissue [43–46]. Since mobilisation of an immune response is costly, special mechanisms exist to suppress immune function during nutrient stress, for example, leptin acting as an "immune-trophic" factor by signalling energy status and thus allowing the optimisation of an immune investment within context of metabolic reserves [47]. In fact, even short-term fasting is able to decrease leptin levels [48]. In this regard, the finely branched lymph vessels increase surface area [44, 45], which facilitates local supply of energy-rich molecules as well as adipocyte-derived paracrine factors [43–45, 49]. Such local paracrine signalling may play an important role during fasting. The finely branched lymph vessels permeating through local adipose deposits represent an anatomical adaptation that may also facilitate paracrine signalling factors between adipocytes and immune cells [43–45, 49]. Supporting this view, adipocytes associated with lymphoid tissue do not respond to normal fasting cues but instead are more sensitive to immune signals, suggesting that these cells are dedicated to assisting immune cells [43–46, 49]. Similarly, bone marrow fat (BMF), whose function remains largely unknown, may function as a "paracrine factory," sustaining local immune cells even in the face of low nutrient availability. Indeed, BMF is highly unresponsive to fasting and exhibits a paradoxical *increase* in anorexic patients [50]. It is tempting to speculate that an increase of BMF during anorexia (and aged individuals [51]) may be an attempt to counteract immune-antagonising signalling context. The fact that BMF cells are, unlike normal adipocytes, not conglomerated but in fact interspersed, indicating a potential paracrine rather than storage function, supports this view [52]. Finally, ovarian cancer cells metastasising to the omentum (a "sail" of adipose tissue, permeated with lymph vessels) were found to be "fuelled" by adipocytes that directly transfer lipids to cancerous cells [53]. Though these authors identified beta-oxidation as a likely endpoint of the fatty acids, it is also likely that the fatty acids derived from these cells may be involved in the synthesis of cellular components. It is likely that these lymphoid-associated adipocytes may respond to signalling queues usually presented by activated immune cells, which are coopted by cancer cells.

These aforementioned observations suggest that adipocytes may play a critical immune-supporting role by providing energy-rich substrate for both biosynthesis and energy production, as well as paracrine factors for sustaining an immune response within a fasted state. Furthermore, recent findings indicate that the close cooperation between the immune system and adipocytes might extend beyond the simple task of supplying energy-rich molecules and paracrine factors. As an example, adipocytes are known to generate antimicrobial peptides [54] and exhibit a phagocytic capacity [55] and since adipocytes can express MHC II [56], they possibly also play a role in epitope expression. It is thus evident that adipocytes are functionally integrated with the immune system. Indeed, it has previously been argued that the advent of adipocytes, a tissue unique to vertebrates [57], may have allowed the evolution of an adaptive immune system [58]. These adaptations ensure that fasting during an infection would, in an otherwise healthy and well-nourished individual, not impede immune function.

3.2. An Altered Amino Acid Profile Leads to an Upregulation of Autophagy. In contrast to glucose and lipids, it has long been recognised that the plasma levels of most amino acids (AAs) undergo a marked decline during a range of different infections or sterile tissue damage [59–64]. Of note, AA levels are in a dynamic state, often exhibiting a rebound effect and are most likely influenced by the severity of an infection or simulation thereof, as well as the time point at which serum AA levels are sampled. Such a decrease in AA occurs in the context of rapid muscle catabolism, suggesting the prime recipients of liberated amino acids may be the liver, where amino acids are used in gluconeogenesis, and immune

cells for rapid cell division or anabolism. The observation that serum levels of branched chain AA (BCAA) often decrease during an infection [60, 65–67], in conjunction with ex vivo studies demonstrating the ability of leucine to inhibit muscle degradation [68], initially generated much excitement as a potential supplement in septic patients. Since critically ill patients exhibit an increase in protein turnover [69], these observations suggest that AA supplementation would be beneficial to critically ill patients. However, results from studies applying supplementation with these or other amino acids have been disappointing and there is currently no definitive study that demonstrates the optimum protein provision in critically ill patients [70].

Low AA profiles during an infection thus raise two questions. Firstly, it remains to be explained why AA supplementation, despite the decline in plasma levels of various AA levels at some point during an infection, failed to consistently demonstrate clinical benefit. Secondly, the reason why various AAs decline at some point during an infection is peculiar, given the fact that a range of other sophisticated adaptations exist to supply immune cells with energy-rich substrates for energy production and biosynthesis.

4. SAA as an Inducer of Autophagy

4.1. SAA Promotes Upregulation of Autophagy. All eukaryotic cells, when starved of nutrients, activate an ancient catabolic process known as autophagy. Autophagy (derived from the Greek words *auto* meaning "self" and *phagy* meaning "eat") is an evolutionary conserved process by which eukaryotic cells degrade large cellular components into substrates that subsequently can be either used as fuel source, or utilised for the synthesis of critical cellular components. Whereas the proteasome degrade proteins, autophagy is used in bulk degradation of cytoplasmic components, including organelles such as mitochondria or long-lived proteins (process formally known as macroautophagy). Autophagy is rapidly upregulated in starved cells derived from a range of eukaryotes, including plants [71], yeasts [72], invertebrates [73], and mammals [74]. The autophagic process is critical for cell survival during nutrient stress. Transgenic mice with defective autophagic process develop normally but die shortly after birth [75]. Remarkably, it was established that defects in autophagy resulted in these mice being unable to maintain nutrient homeostasis during the transition from placental nutrition to feed-fasting cycles associated with suckling, thus clearly demonstrating the pivotal role of autophagy supplying nutrients during fasting. The evolutionary conserved process of autophagy thus plays a critical role in regulating cellular nutrient status in a fasted state, where it is consistently and robustly upregulated during periods of fasting.

The mechanism by which nutrient stress can induce autophagy has recently been reviewed [76, 77]. Briefly, autophagy is inhibited by mTOR and upregulated by AMPK. A low ATP : ADP ratio results in the activation of AMPK. In turn, activated AMPK inhibits mTOR, a major inhibitor of autophagy. Low energy status is thus one mechanism by which autophagy is upregulated. In this regard, cytokine-mediated insulin resistance may represent a strategy to induce low levels of energy stress in cells, prompting upregulation of autophagy; alternatively, an increase in the cellular AA pool results in the activation of mTOR and a subsequent inhibition of autophagy. As an example, supplementation with BCAA such as leucine protects against muscle wasting induced by a protein-deficient diet [78]. Of note, these authors implicate a decrease in protein degradation, rather than synthesis as the mechanism by which leucine protects muscle mass during AA starvation. This study again emphasises the ability of nutrients such as AA to potently inhibit autophagy. Additionally, depletion of AAs results in the accumulation of unchanged tRNA which in turn also induces autophagy [79]. Finally, under conditions of nutrient deficiency, cells utilise amino acids as a source of energy and metabolic waste products, such as ammonia, accumulate. In this regard, ammonia has also been shown to induce autophagy, but by an mTOR-independent mechanism [80].

Starvation is not the only trigger for autophagy (Figure 1). As an example, ER stress also induces the activation of autophagy [81], where autophagy provides a mechanism for degrading misfolded protein aggregates that cannot be accommodated by the proteasome. In fact, a range of cellular insults including free radicals [82], heavy metals [83], or cytotoxic chemotherapeutics [84] induce autophagy as a general stress response. Also, autophagy is regulated by growth factors [85]. Singling pathways activated by growth factors can directly inhibit autophagy (e.g., Ras activation [86]). Conversely, growth factor withdrawal causes a decreased expression of major nutrient transporters on cell surface, resulting in a decrease in intracellular nutrients, thus leading to the activation of autophagy by nutrient stress. Finally, autophagy is a selective process, being able to target substrates for degradation. As an example, autophagy has been shown to selectively target damaged mitochondria (i.e., mitochondria with a low membrane potential) for degradation, thus playing an important role in mitochondrial "quality control" [87] (a process referred to as mitophagy). The fact that the catabolic process of autophagy can selectively target substrates for degradation also positions autophagy for another key cellular process: host defence against intracellular pathogens. In fact, autophagy is also involved in the degradation of pathogens (formally known as xenophagy) [88].

Therefore, a plausible relationship exists between SAA as a mechanism for upregulating autophagy and the antimicrobial function of autophagy. In fact, the altered AA profile seen in patients may represent a mechanism by which AA withdrawal inhibits mTOR, with a subsequent increase in autophagy. Interestingly, depriving macrophages of growth factors and AAs for four hours upregulated autophagy and provided added protection against *Mycobacterium tuberculosis* (TB) [89]. This suggest that upregulating macroautophagy through fasting can augment immune function.

4.2. The Role of Autophagy during an Infection and Tissue Trauma. In previous work, we argued that autophagy has a number of critical functions during an infection [21] (Figure 2). As an example, a higher rate of autophagic flux would confront intracellular pathogens with a shorter time frame for manipulating the host's intracellular defences. Similarly,

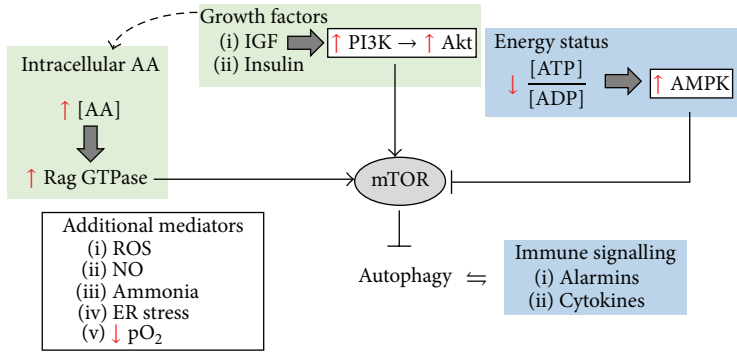

Figure 1: Autophagy plays a key role in energy homeostasis, immune regulation, and a generic stress response to various insults. Growth factor signalling is known to inhibit autophagy directly but may also influence autophagy indirectly, by controlling the cellular import system for AAs. High levels of AAs (in particular, essential AAs such as the BCAA, leucine) also inhibit autophagy through activation of mTOR. In contrast, low energy status upregulates AMPK, which in turn inhibits mTOR, resulting in an increase in autophagy. Autophagy can also be activated by immune effectors including TLR-4 activation or alarmins or via cytokine such as Il1-b and IFN-γ. Also, autophagy modulates these inflammatory mediators, for example, by degrading the inflammasome. Finally, autophagy as a generic stress response is also upregulated under hypoxic conditions, or in response to oxidative stress.

pathogens have evolved mechanisms for inhibiting apoptosis in response to viral subversion of genomic replication machinery [90]. In this regard, autophagy-mediated cell death (autosis) [91] may provide a "backup" cell death programme. In addition, a range of prominent autophagic mediators (e.g., BECN1, ATG5, ATG7, and ATG12) are also involved in apoptotic pathways [92], suggesting that upregulation of autophagy may "prime" cells for cell death. This would also suggest that SAA may be a protocol not only for enhancing the antipathogen activity of immune cells, but also for cell-autonomous defences, thus obstructing the spread of infectious agents. Finally, autophagy is involved in the expression of epitopes in nonprofessional antigen-presenting cells such as muscle [93], endothelium [94], and adipocytes [54], suggesting that SAA-induced autophagy may augment immune operations by recruiting nonimmune cells in mobilising an adaptive immune response. Supporting this view, inhibition of mTOR, a potent initiator of autophagy, enhances the efficacy of influenza vaccine in mice [95]. Similarly, viral inhibition of the proteasome pathway, a strategy to prevent cells from processing and expressing viral epitopes on MHC I, can be compensated for by autophagic processing of endogenous proteins [96].

Moreover, autophagy may also enhance host survival via the nonimmune mechanism. A severe inflammatory episode challenges cells with a number of insults that must be dealt with. Indeed, the development of clinical hypothermia in severe cases of sepsis may be a strategy to avoid overwhelming of cellular survival systems. Supporting this view, in a rat model of severe endotoxemia, rats that were allowed to develop hypothermia (an observation often seen in cases of severe systemic inflammation) demonstrated a significant increase in survivability [97]. Autophagy as a generic cell survival response may play a critical role in addressing the stressors imposed by a systemic inflammatory context. Indeed, insufficient autophagy in rabbits suffering severe burns has been implicated in mitochondrial dysfunction and organ failure [98]. In addition, autophagy may play a role in clearing phagocytosed apoptotic bodies [99], thus avoiding secondary necrosis and subsequent inflammation. Furthermore, autophagy is also implicated in the degradation of the inflammasome complex [100], thus controlling inflammatory tone and rendering cells more responsive to the disease trajectory. Finally, an increase in body temperature within a fever range induces the expression of heat shock proteins [101], suggesting that newly synthesised proteins may occasionally be misfolded. In this regard, autophagy may play an important role in degrading aggregates of misfolded proteins [102]. In summary, it is evident that autophagy is involved in both pathogen clearance and host survival during an infection.

5. Fasting-Induced Autophagy: A Double-Edged Sword?

Host-pathogen coevolution results in the constant development of novel immunological strategies by host, which in turn place evolutionary pressure on pathogens to develop effective counter-measures [103]. In this regard, the fact that autophagy is also upregulated by immunological signals such as LPS and TNF [104] not only emphasises the role of autophagy within an immunological context, but also suggests that pathogens coevolved with the autophagic process as a host defence mechanism. Indeed, pathogens have evolved a number of strategies to subvert the autophagy process [105]. This suggests that fasting may be a strategy to upregulate autophagy as a result of an escalating "arms race" between host and pathogens: by invoking fasting conditions, autophagy is synergistically activated by two independent pathways (immunological activation as well as fasting-induced autophagy). Such a dual activation (by fasting cues and immunological signalling cascades) may render the autophagic process more resistant to "singling attacks" by pathogens attempting to subvert or inhibit autophagy.

However, some pathogens have not only evolved successful measures for subverting host autophagic machinery.

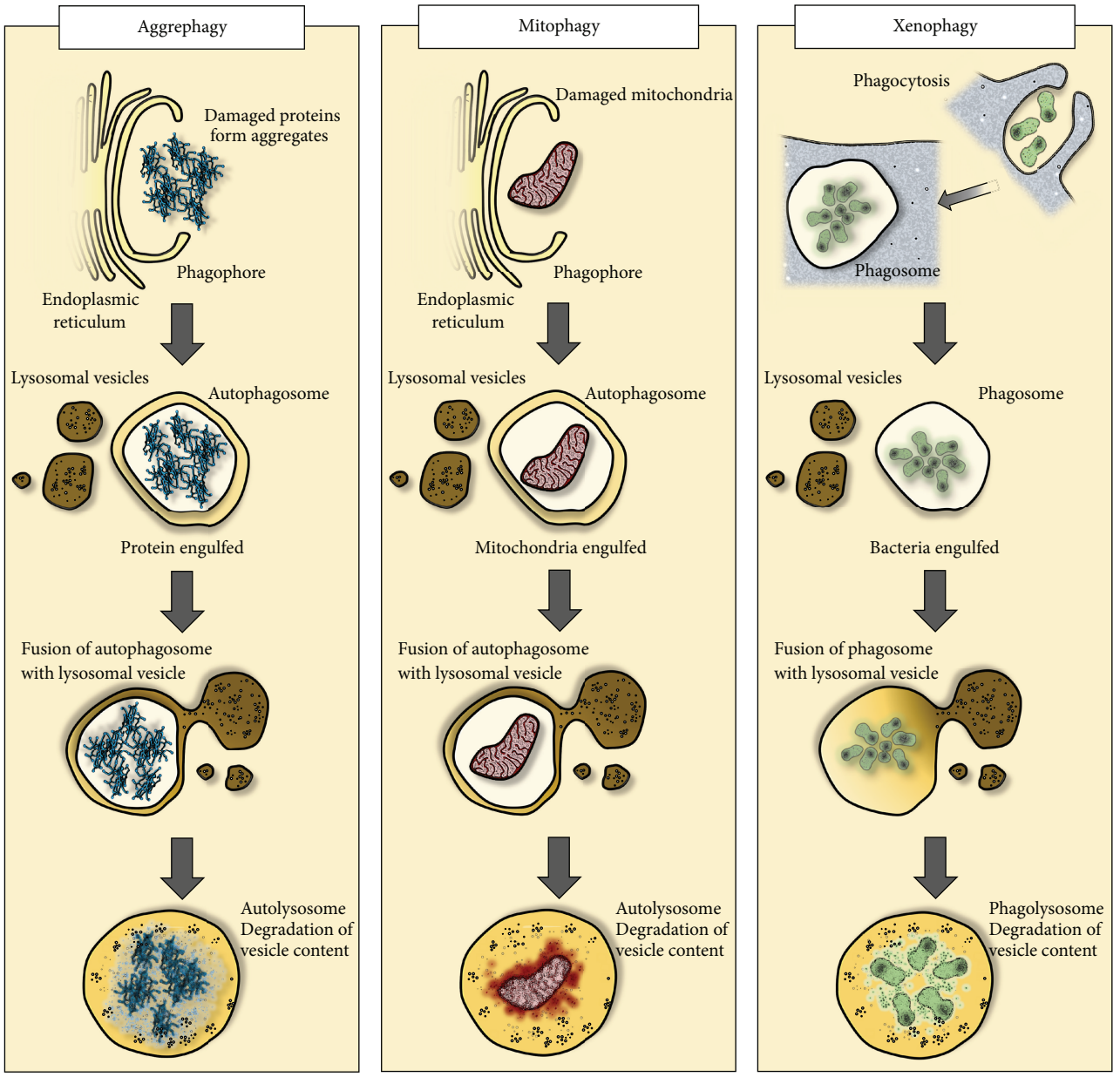

Figure 2: Autophagy plays a critical role in pathogen clearance and host survival. The fibril response as well as oxidative stress resulting from tissue ischemia or immune activation may damage proteins which in turn form toxic aggregates which are cleared by autophagy ("aggrephagy"). Similarly, damaged and dysfunctional mitochondria are targeted for cellular digestion (mitophagy), thus optimising energy generation while diminishing ROS production. Autophagy is also involved in the clearance of intracellular pathogens (xenophagy) in both immune and nonimmune cells. Not demonstrated, autophagic processes are also involved in epitope expression and may provide an alternative form of cell death in viral-infected cells. Also, autophagy may modulate the inflammatory tone by possessing membrane receptors and singling platforms such as the inflammasome.

This is well exemplified by *Trypanosoma cruzi*. The pathogen's internalisation into mouse embryonic fibroblasts is hampered by deletion of key autophagic proteins, Atg5 and Beclin 1, and similarly is promoted by conditions that enhance autophagy (inhibition of mTOR by both rapamycin and AA starvation) [106]. This indicates that upregulation of autophagy may promote the growth of *Trypanosoma cruzi*. In fact, these authors point out that the disease course of *T. cruzi* infections may be adversely affected by the nutritional state of infected individuals: in undernourished individuals, with high rates of hepatic autophagy, the growth of *Trypanosoma cruzi* may be enhanced. Therefore, it is likely that similar clinical contexts exist in which nutritional support may be implemented in mediating therapeutic inhibition of autophagic processes.

Furthermore, aggressive nutritional support may be necessary in patients who are already undernourished. As

mentioned earlier, leptin plays a key role in maintaining immune function. In fact, leptin expression is also markedly increased in response to an immune challenge [107]. Conversely, mice lacking leptin or leptin receptors exhibit defects in their immune function [108]. In this regard, leptin levels parallel body fat mass, indicative of long-term nutritional status [109]. In undernourished individuals, chronically low leptin levels have been implicated in the immune insufficiency observed in these individuals. Thus, a physiological difference between fasting and starvation is a key point that needs to be investigated. Nutritional state prior to an insult may play a critical role in targeting patients that would benefit most from nutritional support, and those who would tolerate permissive underfeeding with a more favourable outcome.

This also points to another key aspect of nutritional support, namely, the timing of nutritional intervention. As argued here, maintaining high levels of autophagy may be advantageous during the initial phases of an infection or injury. However, during the resolution phase, nutritional support may become crucial in sustaining anabolic repair processes. Supporting this view, a recent study in critically ill children [110] has demonstrated superior outcome with late (postponed by 1 week) parenteral nutrition, reaffirming an observation also made for adults [23, 111]. A contributing factor is the possibility that withholding parenteral nutrition during the early phase of an injury/infection may promote higher levels of autophagy when it is most beneficial. In contrast, nutritional supplementation may be more advantageous during the resolution phase, when macronutrients are required for effective tissue repair.

The ambiguous results from clinical trials suggest that some patients may benefit from nutritional support whereas others may in fact be negatively affected by nutritional interventions. Clinical trials are often performed on "critically ill" patients without distinguishing between the nature and the cause of the illness. Reevaluating clinical benefit in the context of different disease settings may point out the context in which preservation of autophagic processes may be more important than supplying metabolic substrates. There is also a need to develop good markers for mapping the disease trajectory, thus allowing for optimised nutritional protocols where nutrients are administered when they are needed most.

6. Future Directions

The role of autophagy during an infection, and possibly also in the context of tissue trauma, highlights a number of issues regarding the role of nutritional therapy in critically ill patients. Nutritional support increases circulating AA levels which inhibit autophagy. Similarly, the secretion of bile salts in response to a meal also inhibits hepatic autophagy. This raises the question whether dietary formulation could be designed to supply patients with nutrients without inhibiting autophagic processes. As mentioned previously, bile acids that are reabsorbed after a meal inhibit hepatic autophagy. This would suggest that enteral nutritional formulations that avoid extensive bile flow may avoid downregulation of autophagy. Bile acid release is initiated by the gastric peptide hormone cholecystokinin, which is secreted in response to fatty acids in the gastric tract. However, the length of the fatty acids plays a critical role, as it was found that fatty acids shorter than C12 [112] do not stimulate the secretion of cholecystokinin [112]. This would suggest that short chain fatty acids may provide a source of energy without inhibiting autophagy. The role of dietary composition that may supply metabolic substrates with minimum impact on autophagic processes should therefore be investigated in future studies.

In addition, intensive insulin therapy may also play a role in derailing autophagy. A high blood glucose level is an independent marker for increased incidence of infection and clinical outcome in intensive care patients [113], supporting the notion that insulin therapy may lower blood glucose and enhance survival. However, it is not clear whether high glucose levels represent a more aggravated condition, or whether high glucose levels lead to the development of various pathologies. It was found in earlier studies that controlling glucose levels via aggressive insulin therapy (IT) resulted in clinical benefits in intensive care patients [24]. Yet, a number of concerns have been raised regarding the validity of this trial [114]. Indeed, a subsequent trial was terminated ahead of schedule due to increased adverse effects and in particular an increase in complications resulting from hypoglycaemia in patients receiving IT [115]. In this regard, permissive underfeeding may also avoid further exacerbate already elevated glucose levels without the need for coadministration of insulin. In addition, we suspect that these occasional adverse effects may result from insulin's ability to inhibit autophagy via the mTOR signalling pathway [116]. Thus, permissive underfeeding may represent a superior form of nutritional support by both upregulating autophagy and avoiding the further increasing glycaemic index via nutritional support.

There is also a need to elucidate the numerous roles that autophagy plays in a variety of cells over the course of the disease. Previous studies [117, 118] have identified an early upregulation of autophagy in proliferating T cells. Indeed, inhibition of mTOR results in an enhanced T cell mediated immune response and enhanced immune cell memory [119], clearly implicating the role of mTOR in T cell function. However, a recent study [120] where a conditional knockout system was deployed, demonstrated, contrary to previous findings, that autophagy is not upregulated in CD8 cells during the initial phase of an infection. From day 8, autophagy is robustly upregulated; yet, at this point (day 8), the virus had already been cleared. Thus, the stimulus for SAA is removed at a time when autophagy is upregulated, indicating that autophagy plays a continuous role in the developing disease state and that SAA is not the only mechanism by which cells induce autophagy. Moreover, the function of autophagy in this context seems to be primarily involved in T cell survival by means of a metabolic shift towards fatty acid metabolism. Thus, although autophagy plays a crucial role in viral control (by promoting cell survival of memory T cells), this process must be independent of SAA and thus does not support an interpretation of SAA as the "autophagic trigger." Furthermore, it has been demonstrated that T cells exhibit pronounced metabolic plasticity [121], which may assist these cells in surviving the range of environments they encounter as migrating cells. Collectively, these observations suggest that

though autophagy has an important function in various immunological contexts, these autophagic processes are active, independent of SAA.

Elucidation of autophagic circuits may also be of interest, both in understanding pathogen subversion and to optimise nutritional support. A low ATP : AMP ratio upregulates AMPK, which upregulates autophagy, whereas low AAs inhibit mTOR, a potent inhibitor of autophagy [122]. Thus, autophagy may be upregulated by inhibition of mTOR, as a result of either AA starvation, or mild energy stress. It is thus possible that autophagy may be preferentially induced by different nutritional triggers, though it should be noted that these processes are not mutually exclusive. It is also well appreciated that animals are capable of "self-medicating" by selecting food sources with specific properties in order to rid themselves of various diseases [123]. Indeed, altered food preference has also been reported in invertebrates. Two groups have independently observed that African armyworms prefer and exhibit increased survival when fed a diet with a higher protein-to-glucose ratio during bacterial [7] or viral [8] infections. This suggests that AMPK-activated autophagy may possibly be more important in invertebrates (whereas AA might be the putative autophagic "trigger" in vertebrates). Alternatively, it is possible that invertebrates, during an infection, metabolise AAs as fuelled source, thus increasing ammonia levels with a subsequent increase in autophagy [80]. In fact, a similar mechanism has been observed in a clinical setting: it was shown that LPS impaired the hepatic removal of ammonia via ureagenesis, which might explain the increase in ammonia observed in patients during sepsis [124]. It is tempting to speculate that such a "metabolic dysfunction" is in fact a strategy to elevate ammonia levels slightly, thus increasing the stimuli for upregulating autophagy systemically. Future studies may elucidate the functional value of substrate utilisation, as well as the accumulation of metabolic waste or intermediates in regulating autophagic processes.

Finally, the fact that pathogens have evolved strategies for subverting autophagic process necessitate careful study designs that measure autophagy flux [125]. However, an increase in autophagic elements such as vesicles may indicate an increase in autophagic activity. It may also signify a defect in the autophagic process, such as an inability of lysosomes to fuse with phagosomes, and it may be a direct result of pathogen subversion of autophagic process. Moreover, various proteins involved in autophagy are also degraded by the autophagic process itself [126]; thus, immunoblotting of key proteins may give rise to misleading results.

7. Conclusion

The prevalence of SAA across various species suggests that avoiding feeding during an infection is an evolutionary conserved response and consequently must represent an adaptive response. Yet, the metabolic demand of mobilising an immune response motivates the implementation of aggressive nutritional support. Clinical trials and results from animal studies have not consistently demonstrated an advantage of nutritional supplementation. Similarly, as outlined in our introduction, a mechanistic justification for SAA as a beneficial process has not been conclusively identified. The role of SAA as a strategy for upregulating autophagy not only provides a rational basis for explaining the clinical value of permissive underfeeding, but also explains the contrasting results between studies: some patients may benefit from autophagy-mediated cell survival and the antipathogenic effects of this process, whereas some would not. Indeed, in the context of some pathogens, upregulating autophagy may in fact be detrimental. Stratifying patient groups according to the trauma or infectious agents involved may provide insight into the context in which aggressive nutritional support may be warranted and where permissive underfeeding may provide a superior strategy. Finally, there is a need to optimise scheduling when nutritional support is introduced. It is likely that patients would benefit most from nutritional support if administered during the resolution phase, where a shift from catabolic to anabolic metabolism is observed.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Inverse Relationship of the *CMKLR1* Relative Expression and Chemerin Serum Levels in Obesity with Dysmetabolic Phenotype and Insulin Resistance

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Background. In obesity there is a subclinical chronic low-grade inflammatory response where insulin resistance (IR) may develop. Chemerin is secreted in white adipose tissue and promotes low-grade inflammatory process, where it expressed *CMKLR1* receptor. The role of chemerin and *CMKLR1* in inflammatory process secondary to obesity is not defined yet. *Methods*. Cross-sectional study with 134 individuals classified as with and without obesity by body mass index (BMI) and IR. Body fat storage measurements and metabolic and inflammatory markers were measured by routine methods. Soluble chemerin and basal levels of insulin by ELISA and relative expression of *CMKLR1* were evaluated with qPCR and $2^{-\Delta\Delta C_T}$ method. *Results*. Differences ($P < 0.05$) were observed between obesity and lean individuals in body fat storage measurements and metabolic-inflammatory markers. Both *CMKLR1* expression and chemerin levels were increased in obesity without IR. Soluble chemerin levels correlate with adiposity and metabolic markers ($r = 8.8\%$ to 38.5%), $P < 0.05$. *Conclusion*. The increment of *CMKLR1* expression was associated with insulin production. Increased serum levels of chemerin in obesity were observed, favoring a dysmetabolic response. The results observed in this study suggest that both chemerin and *CMKLR1* have opposite expression in the context of low-grade inflammatory response manifested in the development of IR.

1. Introduction

Obesity which is the excess storage of white adipose tissue (WAT) and low-grade inflammation are the key factors for development of insulin resistance (IR) [1–4].

In WAT primed immune cells are recruited as adiposity increases, and these cells became resident cells (mainly macrophages) and secrete proinflammatory adipokines that promote further recruitment of circulating monocytes [4–7]. Later, they polarize towards M1 macrophages, favoring an inflammatory subclinical chronic state [6, 8–10].

Chemerin is an adipokine secreted by adipocytes; it is closely associated with amount of fat and distribution. As a chemoattractant protein, chemerin acts as a ligand for the coupled G-receptor protein (ChemR23) and participates in both adaptive and innate immunity [11]. In humans, chemerin gene (*RRARES2*) is highly expressed in WAT and to a lesser extent in liver and lungs. On immune cells, chemerin is known to stimulate chemotaxis of dendritic cells, macrophages, and NK cells. Meanwhile, its receptor, ChemR23 gene (*CMKLR1*), is expressed in dendritic cells, monocyte/macrophages, and endothelial cells [11–14]. ChemR23 is involved in the differentiation of adipocytes and increased intracellular glucose or lipids promote its expression [14].

The interaction of chemerin/ChemR23 has been shown to reduce cytokines, chemokines, and phagocytosis, proving to be important in the inflammatory process associated with obesity [12, 14, 15].

In this context, chemerin/ChemR23 axis has been shown to impact IR development, which influences the clinical course and severity of obesity-related diseases [10]. However, the association with immunometabolic markers and chemerin and its receptor ChemR23 is scarce. Therefore, the aim of this study was to characterize the inflammatory and metabolic phenotype of subjects with obesity-IR state based on the chemerin soluble levels and its receptor *CMKLR1* expression.

2. Material and Methods

2.1. Study Design. In this cross-sectional study a total of 134 adults, aged 20 to 59 years, were recruited from general population in the west of Mexico. We included individuals who at the time of enrollment did not present glucose intolerance, infectious diseases, hypertension, pregnancy, anemia, cardiovascular disease, malignancy, and renal and metabolic diseases such as type 2 diabetes mellitus (T2DM). Subjects with current medication use were excluded.

Subjects were classified in two forms, based on obesity and then by IR. First, they were classified according to the recommendations' of World Health Organization, by body mass index (BMI), waist circumference (WC), waist-hip ratio (WHR), and waist to height ratio (WHtR), in two groups: subjects with obesity, if any of the following conditions were present: BMI ≥ 30.0 kg/m^2, WC ≥ 90.0 cm, WHR ≥ 0.90, and WHtR ≥ 52.5 in Men and WC ≥ 80.0 cm, WHR ≥ 0.80, and WHtR ≥ 53.0 in women, and lean subjects that are lower in these measurements. For detection of IR in subjects with obesity they were secondly classified according to Gayoso criteria in two groups: with and without IR [16].

For ethical purposes, participants were informed about the study and signed a consent form following the Helsinki declaration guidelines [17] and the institutional (Guadalajara University) review boards' committees.

2.2. Subjects' Medical History and Physical Examination. All individuals who satisfied inclusion criteria were clinically evaluated by a physician who performed a complete medical history. Assessment of general health status and vital signs were included: blood pressure (measured 3 times with the subject in the sitting position for 15 minutes before the evaluation), heart and respiratory rate, and body temperature.

2.3. Subjects' Body Fat Storage Measurements. We evaluated height, which was measured to the nearest 1 mm by using a stadiometer (Seca GmbH & Co. KG. Hamburg, Germany); body weight and total and trunk body fat mass (absolute, kg, and relative, %) were determined by bioelectrical impedance analysis (TANITA BC-418 Segmental Body Composition Analyzer, Tokyo, JPN) to the nearest 0.1 kg. WC, hip circumference (HC), and coronal abdominal diameter were measured by using an anthropometric fiberglass tape (GULICK® length 0–180 cm precision ±0.1; USA). At the level of the iliac crest (L4-5) sagittal abdominal diameter was measured using a sliding-beam, abdominal caliper (precision ±0.1 cm, Holtain Ltd. Crosswell, Crymych, Pembs., SA41 3UF, UK) with the patient lying in a supine position on the examination table [18]. Five measures of skinfold thickness (i.e., abdominal, bicipital, tricipital, subscapular, and suprailiac) were obtained on the right side of the body by using a Harpenden skinfold caliper (opened 80 mm with precision of ±0.2 mm, constant pressure: 10 g/mm^2; Holtain Ltd. Crosswell, Crymych, Pembs., SA41 3UF, UK). All these measurements were carried out by the same Physician, in duplicate following the procedures recommended by anthropometric indicators measurement guide [19, 20].

To determine obesity and adiposity indexes the following calculations were used: BMI, kg/m^2 = weight (kg)/height^2 (m); WHtR = WC (cm)/height (cm) [21]; WHR = WC (cm)/HC (cm); conicity index (CI) = WC (cm)/$0.109\sqrt{(\text{weight (kg)/height (cm)})}$; BFR = total body fat mass (kg)/height (cm); total adipose area (TAA, cm^2) = WC$^2/4\pi$; visceral area (VA, cm^2) = π(WC/2π − abdominal skinfold)^2; subcutaneous abdominal area (cm^2) = TAA − VA [18]; visceral adiposity index (VAI): for males, VAI = (WC/36.58 + (1.896BMI))6(TG/0.81)6(1.52/HDLc) and females, VAI = (WC/39.68 + (1.886BMI)) 6(TG/1.03)6(1.31/HDLc) [22]; homeostasis model assessment-insulin resistance (HOMA-IR) = [basal glucose mg/dL × (basal insulin μUI/mL)/405] [23, 24].

2.4. Metabolic, Inflammatory Markers, and Chemerin Levels Measurements. Individuals included in the study were fasting 12 hours before the blood samples were taken, allowing them to clot at room temperature, and then were centrifuged at 1509 RCF (Rotanta 460R, Andreas Hettich GmbH & Co.

Table 1: Status of body fat storage in individuals in the study group.

Study group	Lean	Obesity without IR	Obesity with IR
BMI (kg/m^2)	[21.6 (18.5–24.7)]	[32.0 (30.1–34.7)]	[32.6 (30.2–34.9)]
$n = 134$	49	32	53
Measurement			
Height (cm)	166.0 ± 8.4	162.8 ± 7.8	163.8 ± 9.0
Body weight (kg)	60.0 ± 9.9	90.1 ± 12.8[a]	97.3 ± 18.9[a]
Total body fat mass (%)	20.3 ± 5.9	40.3 ± 6.8[a]	40.6 ± 6.9[a]
Total body fat mass (kg)	12.0 ± 3.6	36.6 ± 8.8[a]	39.8 ± 12.4[a]
Trunk body fat mass (%)	23.8 ± 10.6	38.1 ± 11.4[a]	35.4 ± 10.5[a]
Trunk body fat mass (kg)	5.9 ± 2.4[b,c]	18.4 ± 3.9[a,c]	19.4 ± 6.1[a,b]
Waist circumference (cm)	78.3 ± 6.6	109.6 ± 11.9[a]	113.7 ± 13.2[a]
Hip circumference (cm)	93.3 ± 4.8	115.7 ± 12.4[a]	118.8 ± 10.8[a]
Coronal abdominal diameter (cm)	30.2 ± 6.4[b,c]	38.0 ± 8.7[a,c]	44.9 ± 12.0[a,b]
Sagittal abdominal diameter (cm)	16.7 ± 2.1	24.6 ± 3.4[a]	25.9 ± 4.4[a]
Total adipose area (cm^2)	491.2 ± 82.3	967.9 ± 216.6[a]	1042.5 ± 249.6[a]
Visceral area (cm^2)	227.4 ± 373.0	292.7 ± 516.4	599.7 ± 836.1[a]
Subcutaneous abdominal area (cm^2)	314.2 ± 324.0	696.8 ± 560.8[a]	562.6 ± 908.7

BMI: body mass index [$\overline{x}$ (min–max)]. Data are shown in $\overline{x}$ ± SD. [a]Difference *versus* lean. [b]Difference *versus* obesity without IR. [c]Difference *versus* obesity with IR ($P < 0.05$, ANOVA, Tukey *post hoc*). IR: insulin resistance.

KG.) for 10 minutes at 20°C. Serum was collected and stored at −86°C until further analysis.

We quantified serum concentration of glucose and nonesterified fatty acids (NEFA) with routine enzymatic methods; triglycerides (TG) and total cholesterol (TC) with routine colorimetric methods, high and low density lipoprotein cholesterol (HDLc and LDLc, resp.), apolipoproteins A1 (Apo-A1) and B (Apo-B), and high sensitivity C reactive protein (CRP) with immunoturbidimetry methods (Randox Laboratories 55 Diamond Road, Crumlin Co. Antrim, Northern Ireland, UK); and erythrocyte sedimentation rate (ESR) with Wintrobe method [25]. And the low density lipoprotein cholesterol (VLDLc) was obtained with the Friedewald formula [26].

Through using commercial enzyme-linked immunoabsorbent assays (ELISA) soluble levels of insulin were determined (sensitivity of 0.399 μUI/mL) (ALPCO 26-G Keewaydin Drive, Salem, NH 03079), and chemerin was determined with a limit of detection of 1.08–7.8 ng/mL (R&D Systems, Minneapolis, USA).

2.5. CMKLR1 Relative Expression Analysis. Mononuclear cells from the subjects studied were isolated by density gradient media with separating solution Lymphoprep™ (AXIS-SHIELD PO Box 6863 Rodelokka, 0504 Oslo, Norway). Total RNA was isolated from purified mononuclear cells, using TRIzol® LS Reagent (Ambion RNA Life Technologies, 5791 Van Allen Way, Carlsbad, CA 92008) based on the single-step RNA isolation modified method reported by Chomczynski [27]. Complementary DNA synthesis (cDNA) was performed with 2 μg of each total RNA sample using a reaction size of 20 μL, with oligo (dT) 18 primer (100 ng/μL), RNase free, DEPC treated water, and SuperScript Reverse Transcriptase III kit (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404) and stored at −20°C until used for expression analyses.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) was conducted using the StepOne™ detection system, EXPRESS SYBR® GreenER™, and ROX™ qPCR SuperMix Universal, and sequence detector software v2.3 (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404) was used for data analysis. A threshold cycle (CT) value was determined from each amplification plot.

In brief, *CMKLR1* mRNA expression was performed in a final reaction volume of 20 μL (10 μM forward and reverse primer, 500 nM ROX, 1X SYBR Green qPCR master mix, and cDNA 1000 ng). The conditions of the reaction were as follows: holding 95°C/10 min, cycling (35 cycles of 95°C/15 s, 60°C/60 s), and melt curves 95°C/15 s, 60°C/60 s, and 95°C/15 s. Expression of target genes was normalized by the endogenous reference gen *RPS28*; sequence specific primers were forward: 5′-GGTCTGTCACAGTCTGCTCC-3′, and reverse 5′-CATCTCAGTTACGTGTGGCG-3′ and for *CMKLR1* target gen forward: 5′-GTGGTGGTCTAC-AGCATCGT-3′ and reverse: 5′-ATGGCGGCATAGGTG-ATATGG-3′.

The relative expression fold change of target gene was calculated using the comparative CT method with $2^{-\Delta\Delta C_T}$ equation [28]. To ensure accuracy of data, experiments were done in duplicate, blank, internal controls and melt curve data were collected with applications of StepOne detector software (Cat. 4376357).

2.6. Statistical Analysis. Data were analyzed with statistics software SPSS v21 (IBM Inc., Chicago, IL, USA) and GraphPad Prism v6.01 (2014 Inc. 2236 Beach Avenue Jolla, CA 92037). Results are given as mean ± standard deviation (SD). The data distribution of clinical and laboratory variables

was evaluated with Z Kolmogorov-Smirnov test. The clinical and laboratory characteristics of study group were compared with one way ANOVA with Tukey *post hoc*. Data from serum concentrations of chemerin and insulin, laboratorial assessment, and adiposity variables were subjected to Pearson correlation tests. A two-tailed P value < 0.05 was considered statistically significant.

3. Results

3.1. Status Assessment of Body Fat Showed High Adiposity and IR. In this study, the observed frequency of obesity and IR was 63% and 39%, respectively, while IR was not present in lean individuals (with average BMI of 21.6 kg/m^2, 18.0 to 24.7). For individuals classified with obesity, IR prevalence was 62%. The adiposity measurements are shown in Table 1.

The fat distribution assessed by the skinfold thicknesses showed magnitudes as follows: the group with obesity and IR > obesity without IR group > lean group (Figure 1(a)). Adiposity indexes were increased in both groups with obesity *versus* lean subjects, except VAI whose increase is observed in individuals with IR *versus* lean subjects (Figure 1(b)).

3.2. Individuals with Obesity and IR Presented a Subclinical Inflammatory State and Dyslipidemia. Lean individuals presented lower levels of glucose, insulin, and HOMA-IR when compared with individuals with and without IR, whereas NEFA presented no changes in different groups (Figure 1(c)); lean individuals showed lower levels of triglycerides and LDLc compared to subjects with and without IR, also lean individuals presented lower levels of total cholesterol and VLDLc when compared to obesity group with IR. Lean subjects also presented lower levels of Apo-B compared with individuals without IR whereas HDLc and Apo-A1 presented no statistical changes (Figure 1(d)). Levels of CRP and ESR were higher in obesity with IR *versus* lean (6.83 ± 5.65, 17.46 ± 11.67, $P < 0.001$; resp.).

3.3. Obesity without IR Showed a Contrasting Context on Chemerin Levels. Increased levels of soluble chemerin were observed in obese individuals without IR compared to obese individuals with IR and lean subjects groups (Figure 2(a)).

3.4. Chemerin and Insulin Levels Were Associated with Dysmetabolic Phenotype and Body Fat Adiposity Markers. Positive correlations of chemerin and insulin were observed with the increase in the status of body fat and subcutaneous fat accumulation with lipid profile (Table 2).

3.5. Higher CMKLR1 Relative Expression Was Associated with Obesity without IR. Increased expression levels of *CMKLR1* receptor were observed in individuals with obesity without IR *versus* lean and obesity with IR individuals (Figure 2(b)).

A detailed test by tertiles was performed (describing the first tertile as lower expression, second tertile as intermediate expression, and third tertile as higher expression); we found increased accumulation of abdominal fat mass and metabolic markers between individuals with high expression *versus* individuals with low expression of *CMKLR1*, independent of the presence of IR and/or obesity. Other relevant information provided in this analysis is that there is an inverse correspondence in insulin levels, HOMA-IR, and NEFA regarding the expression of *CMKLR1* (Table 3).

Table 2: Correlation of chemerin and insulin serum levels with the dysmetabolic phenotype and body fat adiposity markers status in the study groups.

Measurements	Chemerin (ng/mL)	Insulin (μUI/mL)
	Correlation (%)	
Body weight (kg)	−2.8	**34.7****
BMI (kg/m^2)	15.3	**46.2****
Body fat mass (%)	**38.5****	**41.5****
Total body fat mass (kg)	**26.1****	**46.7****
Waist circumference (cm)	**18.8***	**36.4****
Hip circumference (cm)	**21.6****	**41.5****
Waist-hip ratio	5.8	**16.9****
Body fat ratio	**31.5****	**47.1****
Waist to height ratio	**26.6****	**38.8****
Conicity index	**30.7****	**18.3****
Coronal abdominal diameter (cm)	8.4	**13.0***
Sagittal abdominal diameter (cm)	15.7	**46.4****
Total adipose area (cm^2)	**18.8***	**36.4****
Visceral area (cm^2)	**19.2***	**37.0****
Subcutaneous abdominal area (cm^2)	17.6	**19.4****
Glucose (mg/dL)	−6.3	**22.5****
Skinfold thickness (mm):		
Abdominal	−10.3	**17.3****
Bicipital	**32.7****	**45.8****
Tricipital	**30.4****	**39.0****
Subscapular	11.1	**38.8****
Suprailiac	**16.1***	**36.5****
Triglycerides (mg/dL)	**20.4***	**26.1****
LDLc (mg/dL)	13.0	**12.5***
VLDLc (mg/dL)	**20.4***	**20.9****
HDLc (mg/dL)	**17.4***	10.1
NEFA (mmol/L)	**30.2****	9.0
CRP (mg/L)	14.1	**31.5****
ESR (mm/h)	**30.2****	1.8

IR: insulin resistance. HDLc, LDLc, and VLDLc (lipoproteins of high, low, and very low density cholesterol, resp.); NEFA: nonesterified fatty acids; CRP: C reactive protein; ESR: erythrocyte sedimentation rate. $^{**}P < 0.001$, $^{*}P < 0.05$, Pearson correlation test.

4. Discussion

Two main findings emerge from this study: first, the *CMKLR1* receptor expression was associated with obesity and its features, and second, its ligand chemerin was associated not only with obesity, but also with metabolic dysfunction such as dyslipidemia and IR.

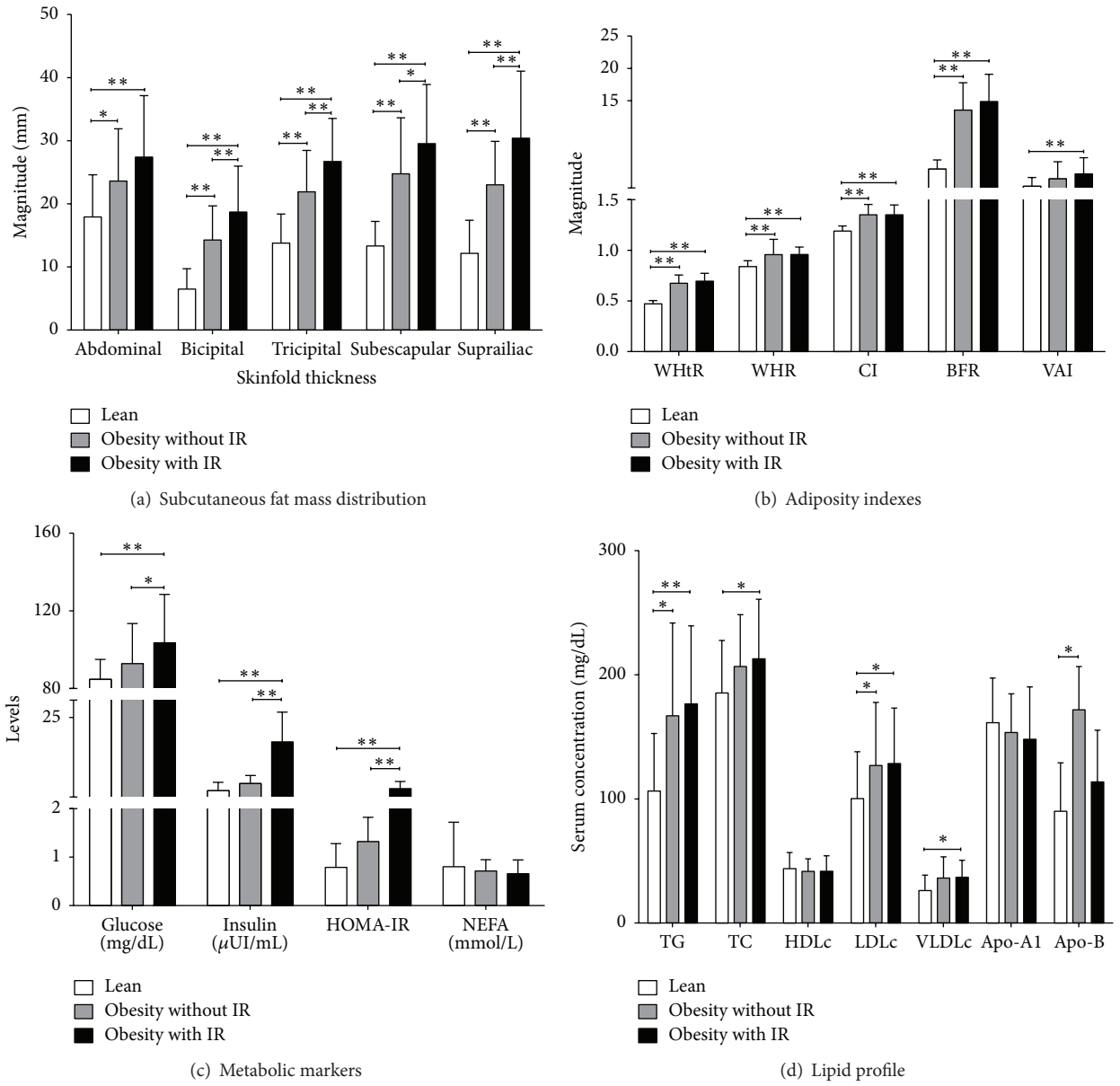

Figure 1: Body fat mass distribution, adiposity indexes, and metabolic markers in the study groups. Lean: [BMI: 21.4 (18.5–24.7) kg/m^2] $n = 49$; obesity without IR: [BMI: 32.0 (30.1–34.7) kg/m^2] $n = 32$; obesity with IR: [BMI: 32.6 (30.2–34.9) kg/m^2] $n = 53$. IR: insulin resistance. WHtR: waist to height ratio; WHR waist-hip ratio; CI: conicity index; VAI: visceral adiposity index; BFR: body fat ratio; HOMA-IR: homeostatic model assessment of insulin resistance; NEFA: none esterified fatty acids; TG: triglycerides; TC: total cholesterol; HDLc, LDLc, and VLDLc (lipoproteins of high, low, and very low density cholesterol, resp.). Data are shown in $\overline{x} \pm$ SD. $^{**}P < 0.001$, $^{*}P < 0.05$ (ANOVA, Tukey *post hoc*).

Adipose tissue has been suggested to be an important source of low-grade inflammation based on three biological aspects: quantity (total mass and relative proportion), anatomical distribution, and phenotype of resident cells [10, 29].

Regarding the quantity and site, our study supported that dysfunctional WAT may play an important role in low-grade inflammation, because the individuals with obesity shown increased accumulation of fat manly in the abdominal region along with low-grade inflammation and dysmetabolic phenotype (represented by dyslipidemic state and increased adiposity indexes), although not all individuals with obesity were IR.

Another important point from this study is that it supports the fact that the IR is a component for developing a chronic-degenerative disease. The IR first promotes dyslipidemia (as an intermediate event) leading to metabolic syndrome and subsequently development of T2DM [1, 3, 30].

The dysmetabolic phenotype observed in individuals with obesity in our study can be explained in the context

Table 3: Adiposity and metabolic markers in the relative expression levels of *CMKLR1*.

Tertile	First	Second	Third
CMKLR1 relative expression	[0.546 (0.034–1.060)]	[4.148 (1.148–8.934)]	[69.787 (9.009–473.260)]
% obesity	48.7	44.1	51.7
% IR	59.0	52.9	48.3
Measurement			
Trunk body fat mass (%)	26.02 ± 12.0	32.32 ± 9.0[a]	33.37 ± 6.4[a]
Coronal abdominal diameter	35.15 ± 12.0	42.18 ± 10.5[a]	40.97 ± 8.9
VAI	2.44 ± 2.6	3.33 ± 2.3	4.54 ± 2.41[a]
Insulin (μUI/mL)	15.22 ± 11.4	13.08 ± 8.4	8.74 ± 5.1[a]
HOMA-IR	3.49 ± 2.5	3.05 ± 1.9	2.13 ± 1.4[a]
NEFA	0.725 ± 0.2	0.618 ± 0.26	0.500 ± 0.25[a]

The expression levels of *CMKLR1* are in relative units [$\overline{x}$ (min–max)]. Data are shown in $\overline{x}$ ± SD and were classified by tertiles. [a]Difference *versus* first tertile ($P < 0.05$, ANOVA, Tukey *post hoc*). IR: insulin resistance. VAI: visceral adiposity index; HOMA-IR: homeostatic model assessment of insulin resistance; NEFA: nonesterified fatty acids.

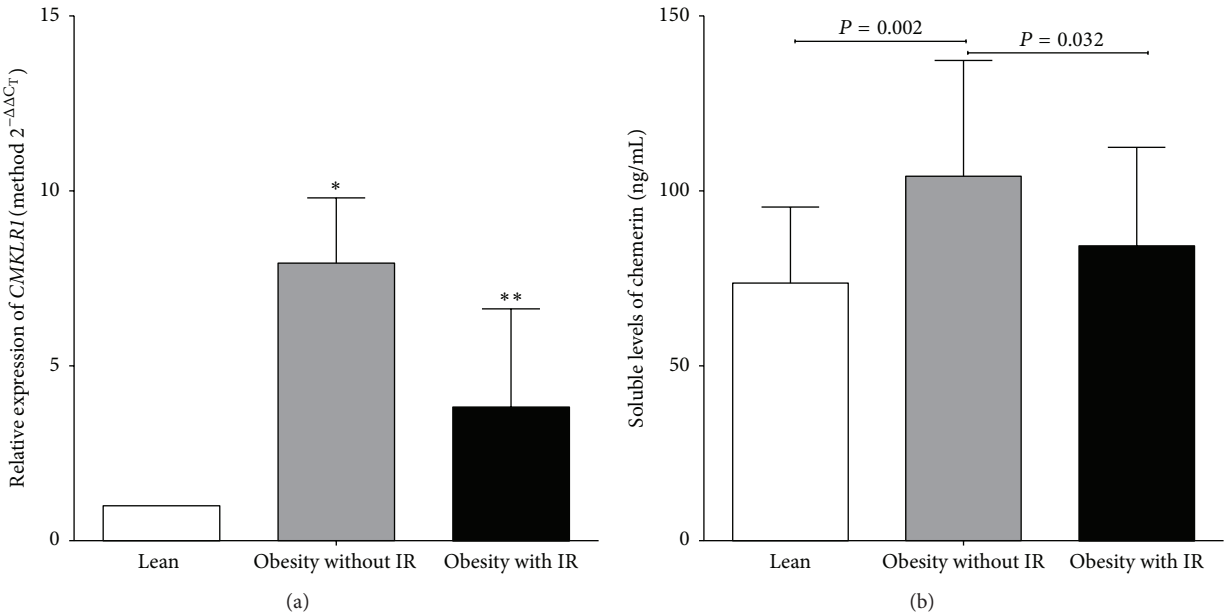

Figure 2: Levels of soluble chemerin and relative expression of *CMKLR1* in the study groups. (a) Relative expression of *CMKLR1*. Method $2^{-\Delta\Delta C_T}$: *difference between obesity without IR and lean (7.9-fold; $P = 0.003$), and **obesity with IR *versus* lean (3.8-fold; $P < 0.001$). (b) Serum levels of chemerin (ANOVA, Tukey *post hoc*). IR: insulin resistance.

of immune system dysregulation that exists in IR by the development of two alternative mechanisms not exclusive: in one the WAT of individuals with obesity have increased resident M1 macrophages able to produce chemokines and in additional one, it has been found that high concentrations of fatty acids induce expression of TNFα and Toll-like receptor-4 signaling [4, 30]. This pathway converges with insulin signaling in adipocytes affecting positively glucose transport, glycogen synthesis, and cell differentiation while negatively affecting the lipolysis and gluconeogenesis [8, 31, 32].

The polarization of monocytes/macrophages to M1 in WAT is favored by coupling of chemerin through ChemR23 that in turn displayed a proinflammatory profile, where chemotactic ligand-receptor interaction regulates continued migration of monocytes to WAT [13, 14]. Soluble chemerin produced by adipocytes and resident macrophages M1 in WAT binds to ChemR23 with high affinity, and its levels decrease as recruited circulating monocytes differentiate into macrophages [12–14, 33, 34].

This study evaluated the gene expression of *CMKLR1* receptor in circulating monocytes. It presented 7.9- or 3.8-fold higher expression in individuals with obesity without IR or obesity with IR, respectively, than lean individuals. Interestingly the increase in expression levels was directly associated with the proportion of abdominal fat mass and body dimensions, whereas an inverse association was observed with the production of insulin, nonesterified free fatty acids, and HOMA-IR.

In this regard, previous study showed that the chemerin/ChemR23 signaling does not affect the inflammatory

response in *ex vivo* human macrophages [35]. The expression levels of *CMKLR1* found in our study suggest that the level of expression is higher in early activation of primed circulating monocytes but decreases at later stages, which can be explained based on the reports in other studies in animal models [31, 36], although changes of *CMKLR1* expression in human macrophage differentiation/polarization still remain to be established.

The association of *CMKLR1* expression levels, observed in our study, can be explained due to IR being closely associated with excess in abdominal accumulation of WAT [30] and based on other reports in which an adipocyte cell line expression of *CMKLR1* was analyzed during differentiation process where upregulation was in the early stage whereas a downregulation was observed in late stages [37, 38]. Proinflammatory stimulus such as TNFα or adiponectin has been shown to upregulate gene expression of *CMKLR1* in differentiated adipocytes [39]. This shows the complexity of the *CMKLR1* expression and functionality that is not dependent on the expression of its ligand but also dependent on the cell type presented on the disease.

Nevertheless, the limitations of this study were that protein levels of the ChemR23 in monocyte were not assessed, limiting complementary studies about the functional receptor. Another limitation that should be taken in consideration is that this same receptor is known to bind with the same affinity to the lipid mediator resolving E1, which has anti-inflammatory properties. The quantification of such ligand was not done.

One of the main findings was the increased serum levels of chemerin in individuals with obesity without IR *versus* individuals with IR and lean. In parallel correlation with indicators of adiposity and metabolic markers was observed.

In this regard, previous studies report conflicting results on chemerin levels in different diseases with an inflammatory component such as chronic pancreatitis (94.0 ng/mL) [40], T2DM individuals (179.0 ng/mL) [41], lipodystrophy (234.3 ng/mL) [42], and obesity without diabetes (590.08 ng/mL) [43]. Chemerin levels were increased, except for the levels reported in rheumatoid arthritis (35.0 ng/mL) [44] (an inflammatory disease *per se*), although this can be explained based on treatment; however, other studies have suggested that chemerin may be the functional link between chronic inflammation and obesity-related T2DM and cardiovascular disease [45].

Our results can be explained because monocytes/macrophages are decisive in the pathogenic process of IR, based on the fact that they are an important source of proinflammatory markers (TNFα, IL-6, chemerin, and C reactive protein), along with increased levels of expression of adipokines, chemokines, and proinflammatory cytokines associated with an equivalent increase in hyperplastic and hypertrophic adipocytes [13, 30, 46].

One important observation from this study is that, in the context of obesity, soluble chemerin levels enhanced both the inflammation and dysmetabolic phenotype, showing how an opposite change in the expression of *CMKLR1* takes place once IR is established.

In previous reports it is postulated that chemerin production has a dual profile pro/anti-inflammatory [9, 15]. In this context we suggest that, in the initial stage of IR, the increase in chemerin levels promotes dysmetabolic profile arising from the dysfunctional adipose tissue [36]. Although in the setting of obesity during the process of establishment of IR the levels of chemerin can be decreased, the dysmetabolic profile is maintained. Its scenery might be explained due to that action of chemerin where it regulates the insulin metabolism [6].

5. Conclusions

This is the first study that links the increment of *CMKLR1* expression with insulin production, showing an association with fat mass and corporal dimensions, while the increased serum levels of chemerin in obesity were observed, favoring a dysmetabolic response.

Taking together, the results observed in this study suggest that both chemerin and *CMKLR1* have opposite expression in the context of low-grade inflammatory response, manifested in the development of IR.

Functional studies are necessary to clarify the biological functions of chemerin signaling in the pathogenesis of IR.

Competing Interests

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

Authors' Contributions

Fernanda-Isadora Corona-Meraz and Rosa-Elena Navarro-Hernández equally contributed to this work.

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Research Article

Correlation of *A2bAR* and *KLF4/KLF15* with Obesity-Dyslipidemia Induced Inflammation in Uygur Population

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In this paper, the researchers collected visceral adipose tissue from the Uygur population, which were divided into two groups: the normal control group (NC, $n = 50$, $18.0\,kg/m^2 \leq BMI \leq 23.9\,kg/m^2$) and the obese group (OB, $n = 45$, $BMI \geq 28\,kg/m^2$), and then use real-time PCR to detect the mRNA expression level of key genes involved in inflammation signaling pathway. The findings suggest that, in obese status, the lower expression level of *A2bAR*, *KLF4*, and *KLF15* of visceral adipose tissue may correlate with obese-dyslipidemia induced inflammation in Uygur population.

1. Introduction

Obesity, particularly abdominal obesity, has become worldwide health problem, which is closely related to the increased lipolysis of adipocyte and blood lipid level [1]. Importantly, lipid metabolism disorder is closely related to chronic inflammation induced by obesity [2]. The Uygur and Kazak are two of the main minorities of Xinjiang Region, the former of which is more likely to suffer obesity and type 2 diabetes mellitus (T2DM) [3]. Our group has previously reported that at the same body mass index (BMI) level both male and female Uygur subjects had significantly greater value of WHR and visceral fat content, compared with Kazak subjects [3]. It remains unclear why Uygur population is more susceptible to obesity and T2DM.

Kruppel-like factors (KLFs), as a transcription factor family, are composed of 17 members with zinc finger structure, widely involved in cell proliferation, differentiation, and embryonic developmental regulation [4]. KLF4 has anti-inflammation effects and can promote fatty acid β-oxidation [5, 6]. KLF15, like KLF4, is a newly discovered transcription factor that plays an important role in glucose homeostasis and lipid accumulation in cells [7]. The A2b adenosine receptor (A2bAR) is expressed on the cell membrane and involved in lipid metabolism and inflammation [8, 9]. Visceral adipose tissue, as one of the principal locations of the systemic inflammation response, plays an important role in the regulation of body energy metabolism [10]. In the process of inflammation induced by abdominal obesity, whether KLF4 and KLF15 play an important role and whether the A2bAR correlates with KLF4 and KLF15 are not found.

Thus, our study intends to evaluate the mRNA expression level of *A2bAR*, *KLF4/KLF15*, and key inflammation signaling pathway genes in visceral adipose tissue from Uygur population to investigate the correlation of *A2bAR* and *KLF4/KLF15* with obesity-dyslipidemia induced inflammation in Uygur population.

2. Materials and Methods

2.1. Subjects. We enrolled 172 Uygur subjects between the ages of 20 and 90 years between January and December

2014 from People's Hospital of Xinjiang Uygur Autonomous Region for physical examination and evaluation of dyslipidemia. We collected visceral adipose tissue from 95 Uygur subjects and analyzed mRNA expression level of key genes related to inflammation. Those subjects were divided into two groups: the normal control group (NC, $n = 50$, $18.0\,kg/m^2 \leq BMI \leq 23.9\,kg/m^2$) and the obese group (OB, $n = 45$, $BMI \geq 28\,kg/m^2$). Exclusion criteria included type 1 diabetes (T1DM): various pathogenic factors that resulted in the lacking source of insulin and the fasting insulin that is lower than $5\,\mu$IU/mL; tumors: the patients who were diagnosed with all kinds of tumors by the doctor; acute inflammation: the patients with the symptoms including sudden onset, short duration, and the granulocyte infiltration that were diagnosed with acute inflammation; kidney disease: the patients with massive proteinuria, hypoproteinemia, and hyperproteinemia; and, what is more, the patients who recently use drugs to interfere with glucose and lipid metabolism.

2.2. Anthropometric and Clinical Parameters. We measured the following clinical parameters using standard procedures: height, weight, body mass index (BMI), waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), systolic blood pressure (SBP), and diastolic blood pressure (DBP). BMI was calculated by dividing weight (in kilograms) by height (in meters) squared. WC and HC were measured using a flexible tape with tension calipers at the extremity (Gulick-Creative Health Product, Inc., Plymouth, MI), midway between the xiphoid and umbilicus during the midexpiratory phase and at the maximum circumference in the hip area, respectively. WHR was calculated by dividing WC by HC.

2.3. Measurement of Biochemical Indexes. The fasting plasma glucose was detected using the glucose oxidase-peroxidase method [11]. Total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were detected using a standardized automatic biochemistry analyzer (Japan, Olympus AU2700). Low HDL-C, high TC, high LDL-C, and high TG were defined as HDL-C < 1.03 mmol/L, TC > 5.17 mmol/L, LDL-C > 2.59 mmol/L, and TG > 1.70 mmol/L, respectively. The subjects who have any of the above indexes of abnormal blood lipid level were defined as dyslipidemia [12].

2.4. Tissue Samples. On the day of abdominal surgery, we take the visceral adipose tissue whose size is about 3 cm × 3 cm and avoid the burning of sampling process. After the finish of the sampling process, we repeatedly wash the sample three times with PBS buffer solution and then take the visceral adipose tissue into the cryopreserved tubes, tagging name, gender, medical record number, and group. Snap-freezing in liquid nitrogen until RNA extraction was performed. Total RNA was extracted from the tissue within a week and then stored at −80°C.

2.5. RNA Isolation and Real-Time PCR. Total RNA was isolated from visceral adipose tissue using TRIZOL reagent (cat. # 15596-026, Life Technologies, Carlsbad, CA, USA) and purified using an RNeasy Mini Kit (cat. # 74106, QIAGEN, GmbH, Germany). RNA purity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was performed as follows: 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min. Real-time PCR was performed using SYBR Premix Ex Taq (Takara) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sequences were listed in Table 1, using GAPDH as an internal control. One microliter ($1\,\mu$L) of each RT reaction product was amplified in a $20\,\mu$L PCR reaction using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The PCR protocol was performed as follows: 95°C for 30 s and 40 cycles consisting of 5 s at 95°C and 34 s at 60°C. Dissociation curves were analyzed using the Dissociation Curve 1.0 Software (Applied Biosystems) to detect and eliminate possible primer-dimer artifacts. All reactions were performed in triplicate. The relative amounts of target gene transcripts were calculated using the comparative cycle-time method.

2.6. Subject's Consent and Ethics Statement. All subjects provided informed and voluntary consent prior to enrollment in this study. This consent included understanding that clinical information and biological samples would be used for research. The consent form and ethical approval were provided by the Medical Ethics Committee at First Affiliated Hospital, Shihezi University School of Medicine (reference number 2014LL22).

2.7. Statistical Analysis. SPSS statistical package (version 13.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Clinical characteristics and biochemical data were expressed as mean ± SD; mRNA expression levels were expressed as mean ± SEM. t-test was used for the comparison between different groups. The correlation analysis was tested by Pearson method, and P value < 0.05 was defined as statistical significance.

2.8. Quality Control of Laboratory Testing. This study implemented strict quality control methods for the collection of subjects' general information and visceral adipose tissue. A team of researchers designed questionnaires and two independent operators performed molecular biology techniques. Attempts were made to choose subjects from different group who were well matched in terms of age and gender.

3. Results

3.1. The Detection Rate of Dyslipidemia in Uygur Population. The detection rate of total dyslipidemia was 61.05%, with increasing of age; the detection rate of high blood TC was 14.53% and similarly demonstrated an increasing trend with age; the detection rate of high blood TG was 22.67%, of which detection rate decreased with the age in men but increased in women; the detection rate of low HDL-C was 42.44%; the detection rate of high LDL-C was 3.49%. The above indexes were higher than those observed in Han and

Table 1: Primers used in real-time PCR of target gene.

Gene	Sequence ID	Primer name	Primer (5′-3′)	Fragment (bp)
A2bAR	*XM_011523660.1*	*A2bAR*-F	GGTCATTGCTGTCCTCTG	121
		A2bAR-R	TTCATTCGTGGTTCCATCC	
MYD88	*XM_006713170.1*	*MYD88*-F	CCGCCTGTCTCTGTTCTTG	115
		MYD88-R	GTCCGCTTGTGTCTCCAGT	
SRC	*XM_011529014.1*	*MCP-1*-F	CGAGAAAGTGAGACCACGAA	131
		MCP-1-R	GTGCGGGAGGTGATGTAGA	
NF-κB	*XM_011532009.1*	*NF-κB*-F	CTGAGTCCTGCTCCTTCCA	103
		NF-κB-R	CTTCGGTGTAGCCCATTTGT	
KLF4	*NM_001314052.1*	*KLF4*-F	GGCACTACCGTAAACACACG	140
		KLF4-R	CTGGCAGTGTGGGTCATATC	
TNF-α	*NM_000594.3*	*TNF-α*-F	GTGACAAGCCTGTAGCCCAT	111
		TNF-α-R	TATCTCTCAGCTCCACGCCA	
APN	*NM_004797.3*	*APN*-F	ATGGCCCCTGCACTACTCTA	104
		APN-R	CAGGGATGAGTTCGGCACTT	
MCP-1	*NM_002982.3*	*MCP-1*-F	GATCTCAGTGCAGAGGCTCG	155
		MCP-1-R	TTTGCTTGTCCAGGTGGTCC	
GAPDH	*NM_001256799.1*	*GAPDH*-F	GGTGGTCTCCTCTGACTTCAA	211
		GAPDH-R	TCTTCCTCTTGTGCTCTTGCT	

Kazak population except the detection rate of high LDL-C (Tables S1–S6 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7015620).

3.2. Clinical Characteristics of Uygur Subjects in the NC and OB Group. We selected 95 Uygur subjects and divided them into two groups: NC group ($n = 50$) and OB group ($n = 45$). The clinical characteristics were shown in Table 2. The SBP, DBP, and TC in the OB group were higher than in the NC group but were not statistically significant. The weight, WC, HC, WHR, BMI, TG, and TC of individuals in the OB group were significantly higher than those in the NC group ($P < 0.05$).

3.3. mRNA Expression of Key Genes in Inflammation Signaling Pathway of Visceral Adipose Tissue. The chip analysis revealed significantly differential higher expression of *KLF4* and *KLF15* in normal weight subjects of Uygur population compared to obese subjects, while there is no difference in the Kazak or Han population, respectively (Figure S1, Table S7). Then, we evaluated the key genes mRNA expression level of visceral adipose tissue from NC and OB group in Uygur population. The results were shown in Figure 1. Compared with the NC group, the mRNA expression level of *MCP-1* was slightly higher in the OB group; however this difference was not statistically significant. The levels of *TLR4*, *NF-κB,* and *TNF-α* were significantly higher in the OB group as compared to the NC group ($P < 0.05$), while the expression levels of *A2bAR* and *APN* were lower in the OB group, and the levels of *KLF4* and *KLF15* were significantly lower in the OB group ($P < 0.05$).

Table 2: Comparison of subject metrics and biochemical parameters between NC and OB group.

Testing index	NC	OB
Case number	50	45
Age	47.42 ± 17.39	45.94 ± 10.01
Weight (kg)	63.08 ± 7.74	**79.96 ± 11.10****
WC (cm)	89.62 ± 15.29	**112.42 ± 8.56****
HC (cm)	93.40 ± 8.64	**106.23 ± 18.23****
WHR	0.96 ± 0.16	**1.07 ± 0.08****
BMI	22.92 ± 2.54	**31.56 ± 3.13****
SBP (mmHg)	120.52 ± 22.61	129.74 ± 20.95
DBP (mmHg)	80.28 ± 15.2	82.82 ± 14.17
FPG (mmol/L)	5.0 ± 0.85	5.0 ± 0.72
TG (mmol/L)	2.60 ± 1.43	**3.76 ± 1.89****
TC (mmol/L)	4.87 ± 1.20	5.08 ± 1.03
LDL (mmol/L)	2.64 ± 0.80	**3.02 ± 0.77***
HDL (mmol/L)	1.22 ± 0.47	1.45 ± 0.80

WC: waist circumference; HC: hip circumference; WHR: waist-to-hip ratio; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; TG: triglycerides; TC: cholesterol; HDL: high density lipoproteins; LDL: low density lipoproteins. *t*-test: values are given as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with NC group.

3.4. The Correlation of KLF4/KLF15 and Dyslipidemia Indexes in OB Group. The results were shown in Figure 2. In OB group, the mRNA expression level of *KLF4* was significantly negatively correlated with BMI, TG ($P < 0.05$), and

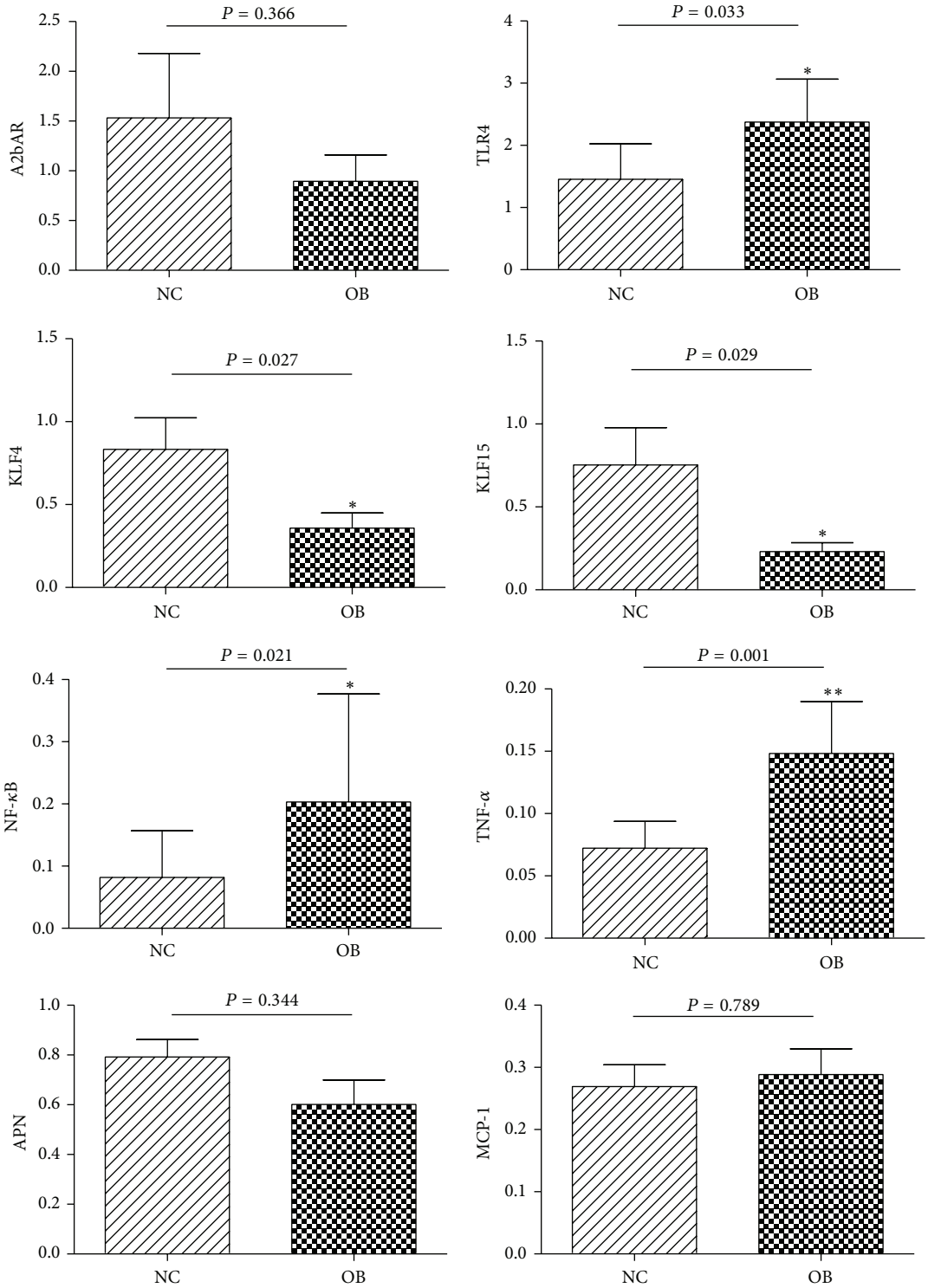

Figure 1: The mRNA expression level of critical gene in inflammation signaling pathways. t-test, $^{*}P < 0.05$, $^{**}P < 0.01$. The difference between the two groups has statistical significance.

negatively correlated with LDL while *KLF4* was positively correlated with HDL. The mRNA expression level of *KLF15* was significantly negatively correlated with TG and LDL ($P < 0.05$) and negatively correlated with BMI, while *KLF15* was positively correlated with HDL.

3.5. The Correlation of KLF4/KLF15 and Key Genes of Inflammation Signal Pathway in OB Group. The results were shown in Figure 3. In OB group, the mRNA expression level of *KLF4* was significantly positively correlated with *A2bAR* and *NF-κB* while negatively correlated with *TNF-α* ($P < 0.05$). The

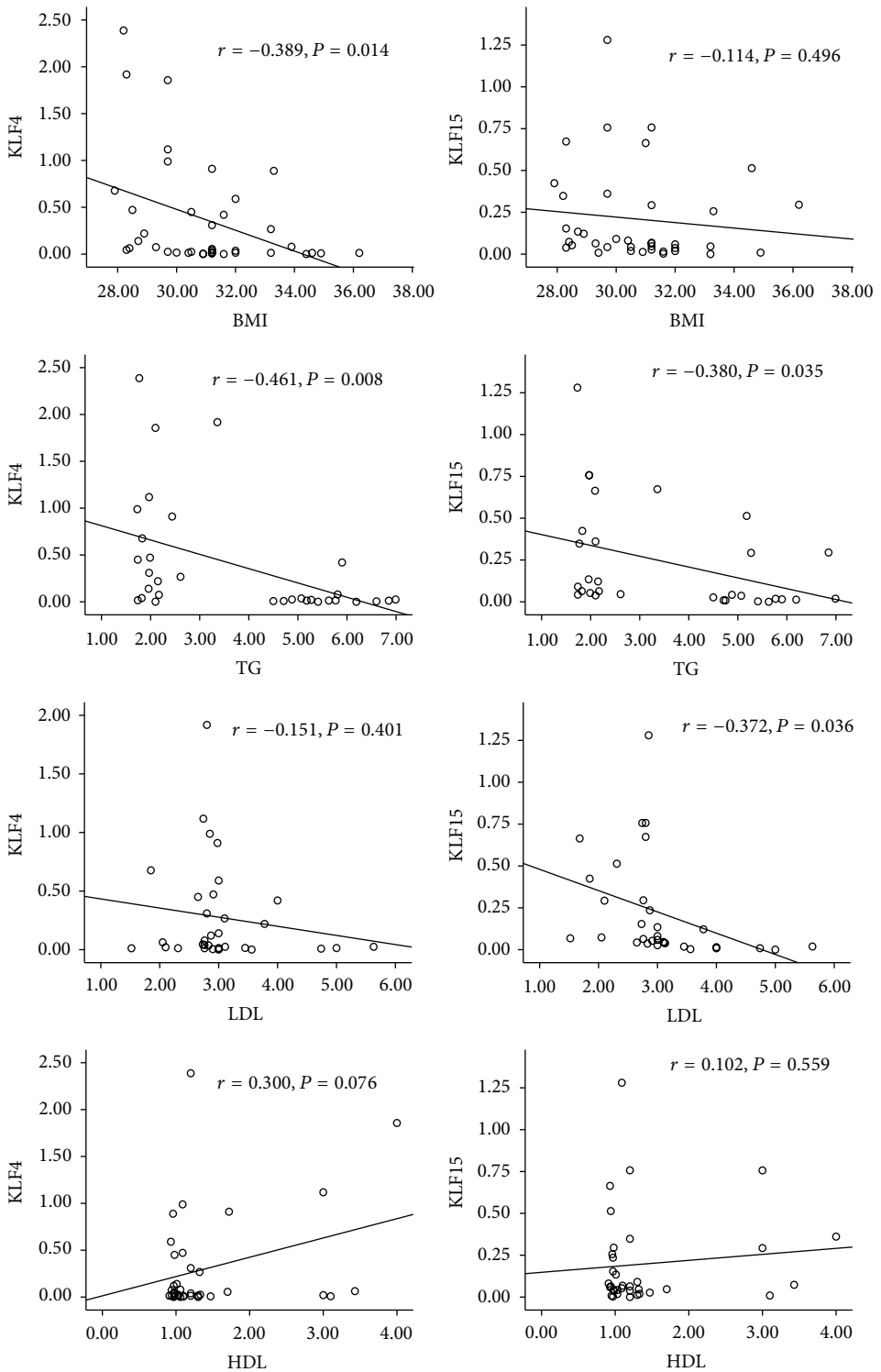

Figure 2: The correlation of the *KLF4*, *KLF15,* and dyslipidemia indexes. Pearson analysis, $P < 0.05$. The correlation between the two groups has statistical significance.

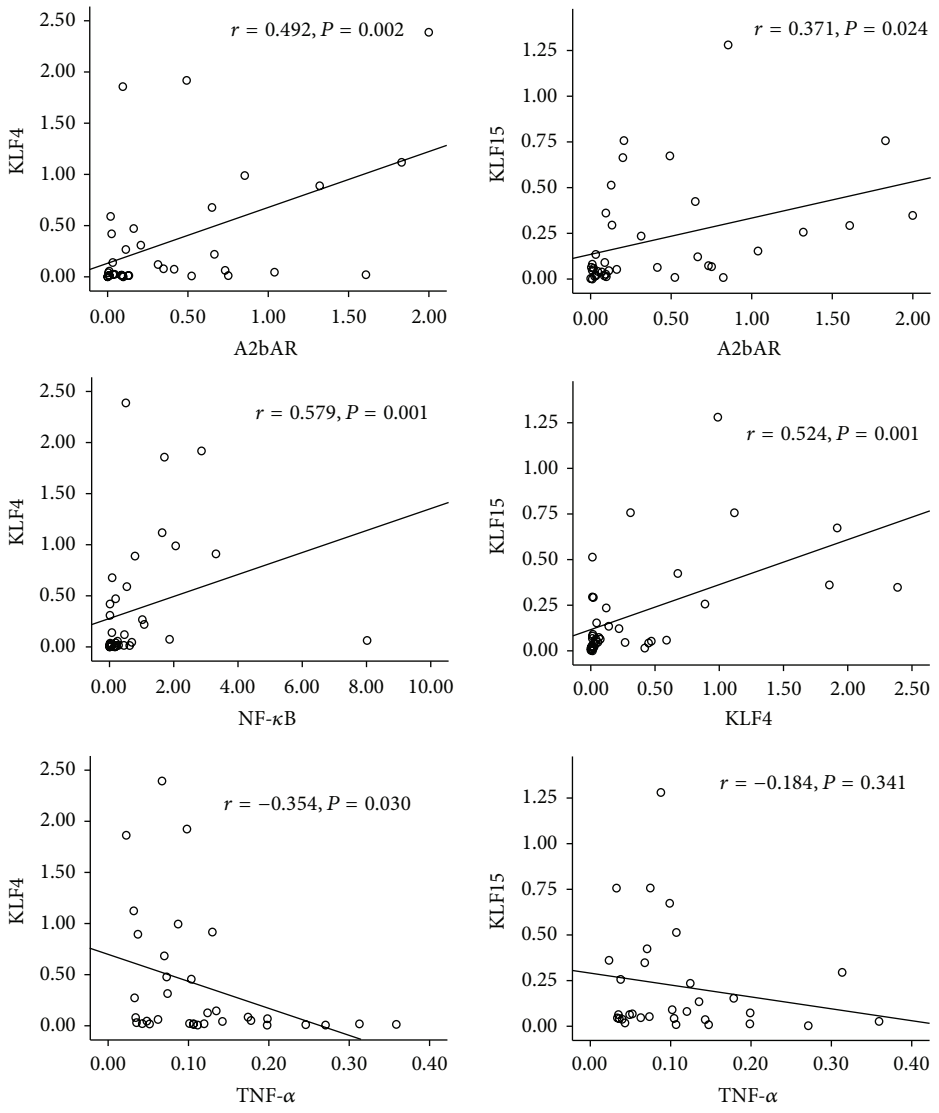

Figure 3: The correlation of *KLF4*, *KLF15,* and the key genes of inflammation signal pathway. Pearson analysis, $P < 0.05$. The correlation between the two groups has statistical significance.

mRNA expression level of *KLF15* was significantly positively correlated with *A2bAR* and *KLF4* ($P < 0.05$) while negatively correlated with *TNF-α*.

4. Discussion

Abnormal lipid metabolism induced by obesity is considered one of the core indicators of metabolic syndrome and may be associated with hypertension, dyslipidemia, and T2DM [13]. In the current study, we evaluated the detection rate of dyslipidemia. Our results noted that, in Uygur population, the detection rate of total dyslipidemia was 61.05%, high TC was 14.53%, high TG was 22.67%, low HDL-C was 42.44%, and high LDL-C was 3.49%. More importantly, the detection rates of total dyslipidemia, high TC, high TG, and low HDL-C were higher in Uygur than in Han and Kazak population, which indicates that the Uygur population has more lipid metabolism disorders. The mRNA expression profile chip analysis by our previous research has demonstrated that mRNA expression levels of *KLF4* and *KLF15* were significantly decreased in subjects with obesity, suggesting these genes may play an important role in lipid metabolism in Uygur population.

Recently, it was reported that KLF4-deficient macrophages exhibited lower ability to perform fatty acid oxidation [6]. Moreover, overexpression of KLF4 can increase the M2 macrophages (anti-inflammation) marker protein expression while it can decrease M1 macrophages (inflammation) marker protein expression [6]. KLF4 overexpression reduced the expression of MCP-1 in J774a cells [6]. Recent studies have suggested that KLF15 regulated lipid uptake and utilization in skeletal muscle [14]. In cultured 3T3-L1 adipocytes, treatment with TNF-α significantly reduced the mRNA expression of *KLF15* [15]. Moreover, *KLF15* gene ablation attenuated anti-inflammation adipolin expression in adipocytes and KLF15

can significantly attenuate the p300-dependent p65 activation on both the MCP-1 and VCAM-1 promoter [15].

Our findings in Uygur population supported previous chip results and demonstrated that *KLF4* and *KLF15* in the NC group were significantly higher than in the OB group. More importantly, in the OB group, the mRNA expression level of *KLF4* was significantly negatively correlated with BMI, TG, and *TNF-α*. The mRNA expression level of *KLF15* was significantly negatively correlated with TG and LDL and positively correlated with *KLF4*. The above results suggest that the *KLF4* and *KLF15* may collaborate to impact lipid metabolism and inflammation. Interestingly, our results demonstrated that *KLF4* significantly correlated with *NF-κB*. Recent research has found that KLF4 may physically interact with the subunit P65 of NF-κB to limit inflammation in vascular endothelial cells [16]. This research may explain why KLF4 was positively correlated with NF-κB in our study. However, this phenomenon in the adipose tissue has not been reported in the literature to date.

Eisenstein et al. proposed that KLF4 and A2bAR were significantly positively correlated in adipose tissue of American population [17]. A2bAR knockout animals demonstrated elevated liver TG concentrations, which indicated impaired lipid metabolism. Moreover, in A2bAR knockout animals, CCL2, TNF-α, and IL-6 level were elevated, whereas IL-10 and IFN-γ concentrations were decreased in the epididymal tissue [18]. A2bAR activation ameliorates the course of diabetes and inflammation in low-dose streptozotocin-treated and nonobese diabetic mice [18]. We noted that A2bAR expression level was higher in the NC group as compared to the subjects in the OB group in visceral adipose tissue of Uygur population. Moreover, *A2bAR* significantly correlated with *KLF4* and *KLF15*. The above results indicate that, in the context of obesity, the positive correlation between *A2bAR* and *KLF4/KLF15* may play an important role in obesity-dyslipidemia induced inflammation of visceral adipose tissue in Uygur population.

It is found that free fatty acid can serve as an agonist of the toll-like receptor 4 (TLR4) complex. Stimulation of TLR4 activates proinflammation pathway and induces cytokine expression in various cells [19]. Thus, we evaluated the *TLR4* mRNA expression level. The expression of *TLR4* was significantly higher in the OB group in Uygur population, which indicates that the high level of blood lipid may promote the release of inflammation factors by upregulating the expression of *TLR4*.

Above all, our findings suggest that, in obese status, the lower expression level of *A2bAR*, *KLF4,* and *KLF15* of visceral adipose tissue may correlate with obesity-dyslipidemia induced inflammation in Uygur population.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Cuizhe Wang and Xiaodan Ha contributed equally to the paper.

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