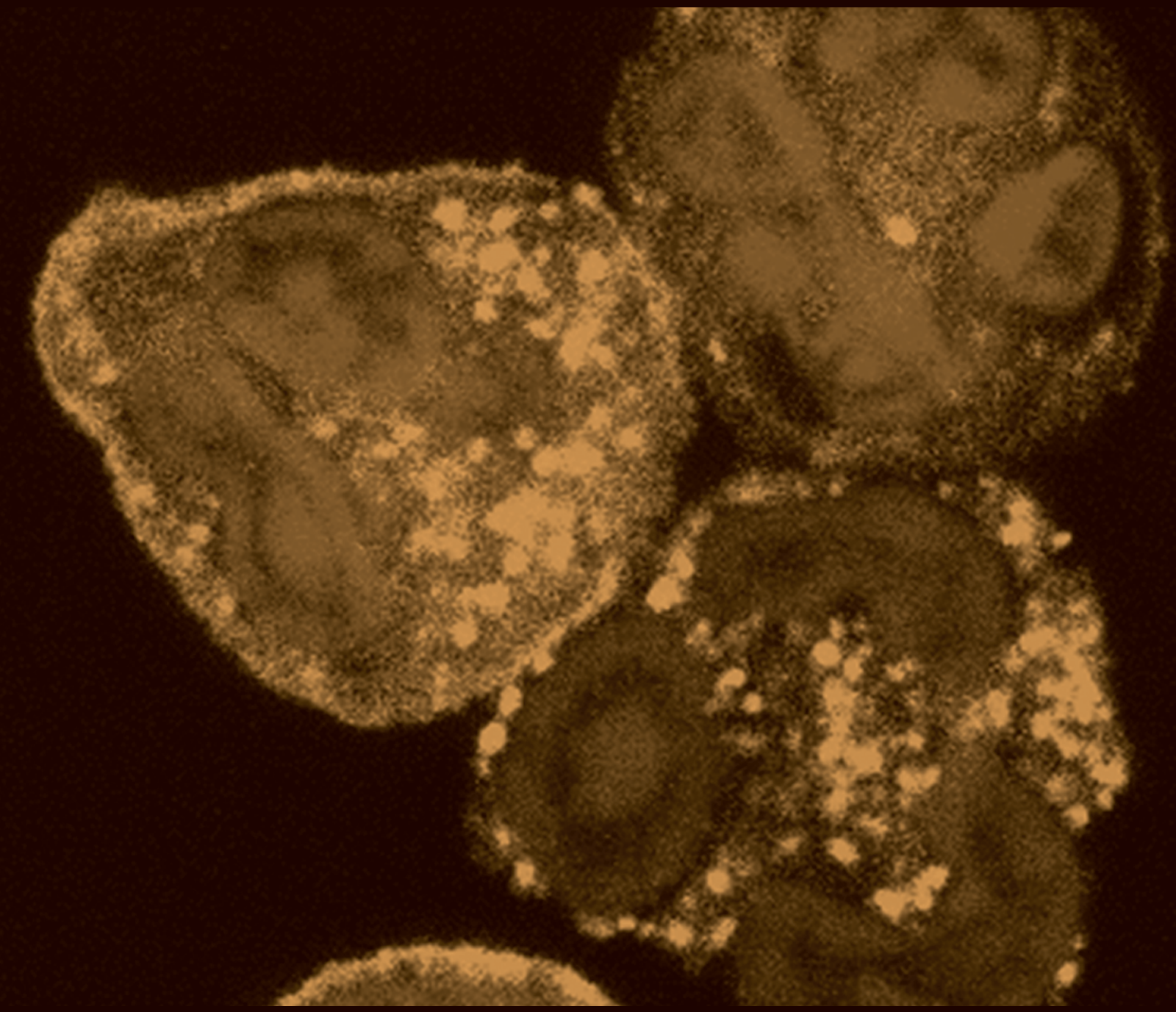
Mediators of Inflammation

Inflammation in Disease: Mechanism and Therapies

Guest Editors: Gustavo Duarte Pimentel, Marília Seelaender, and Fábio Santos Lira



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Editorial Board

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Editorial

Inflammation in Disease: Mechanism and Therapies

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Inflammation in the face of harming stimuli protects the organism; as a result, it is an essential process for survival, in which both innate and adaptive immunity are involved. This process must be tightly controlled and terminated in order to warrant the reestablishment of body homeostasis. Therefore, activation of resident inflammatory cells and the recruitment and modulation of migrating inflammatory cells must be ceased. When failure in neutralising acute inflammation occurs, there is augmented risk for the development of chronic inflammation, leading to several metabolic consequences. In the present special issue, original research studies as well as review articles address the inflammatory process as a key contributor to the development of disease. In addition, pharmacological and nonpharmacological therapies are examined and the molecular and physiological mechanisms of the treatments are discussed.

Among the 24 accepted articles, 5 focus on nutritional therapy and inflammation (D. Estadella et al.; L. Ma et al.; M. J. Kim et al.; S. Scolleta et al.; C. Cunha et al.). The selected articles discuss the effects of the consumption of saturated and trans fatty acids upon tissue lipotoxicity, the influence of resveratrol on lung injury, the beneficial effect of genistein on renal damage, the role of vitamin D in the resolution of inflammation, and finally the capacity of green tea extract to counteract the consequences of obesity.

The ability of exercise to modulate chronic inflammation is the focus of two articles, the first of which discusses the adaptive response of skeletal muscle to inflammation as induced by eccentric overload resistance training (B. N. Ide et al.), while the other considers exercise effects upon spinal cord injury-associated low grade inflammation (E. S. Alves et al.).

Pulmonary diseases and inflammation are the subject debated in 8 from the total of 24 articles selected for this issue, with special emphasis on the possible triggers of inflammation onset in the scenario (Y. Yang et al.; G. Karakiulakis et al.; M. S. Duan et al.; Geve et al.; I. T. Lee et al.; P. Seidel et al.; Y. Shimizu et al.; A. D. Yalcin et al.). In fact, asthma, emphysema, and COPD are extensively discussed in this issue, and studies concerning the effects of treatment with dimethyl fumarate, anti-IgE; activation of muscarinic receptors of immune cells; and proinflammatory signalling are reviewed.

Sleep disorders are also contemplated in articles (V. A. Lemos et al.; D. Rosa et al.; Y. Jin et al.) that demonstrate that sleep apnea contributes to the increased inflammatory cytokine content, a response to hypoxia induced by high altitude.

Finally, 3 studies investigate the role of PPAR, 41BB/4 1 BBL, and mitochondrial dysfunction in metabolic disease (T. H. Tu et al.; F. Monsalve et al.; A. Hernández-Aguilera et al.). In addition, inflammation is discussed in the presence of periodontal disease and endotoxaemic shock (J. D. Corrêa et al.; A. Sampaio et al.).

Collectively, this issue aims at providing insight on the role of acute and chronic inflammation in different diseases, as well as at presenting recently proposed treatment strategies.

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Research Article

Biphasic Modulation of NOS Expression, Protein and Nitrite Products by Hydroxocobalamin Underlies Its Protective Effect in Endotoxemic Shock: Downstream Regulation of COX-2, IL-1β, TNF-α, IL-6, and HMGB1 Expression

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Background. NOS/$^{\bullet}$NO inhibitors are potential therapeutics for sepsis, yet they increase clinical mortality. However, there has been no *in vivo* investigation of the (*in vitro*) $^{\bullet}$NO scavenger, cobalamin's (Cbl) endogenous effects on NOS/$^{\bullet}$NO/inflammatory mediators during the immune response to sepsis. *Methods*. We used quantitative polymerase chain reaction (qPCR), ELISA, Western blot, and NOS Griess assays, in a C57BL/6 mouse, acute endotoxaemia model. *Results*. During the immune response, pro-inflammatory phase, parenteral hydroxocobalamin (HOCbl) treatment partially inhibits hepatic, but not lung, iNOS mRNA and promotes lung eNOS mRNA, but attenuates the LPS hepatic rise in eNOS mRNA, whilst paradoxically promoting high iNOS/eNOS protein translation, but relatively moderate $^{\bullet}$NO production. HOCbl/NOS/$^{\bullet}$NO regulation is reciprocally associated with lower 4 h expression of TNF-α, IL-1β, COX-2, and lower circulating TNF-α, but not IL-6. In resolution, 24 h after LPS, HOCbl completely abrogates a major late mediator of sepsis mortality, high mobility group box 1 (HMGB1) mRNA, inhibits iNOS mRNA, and attenuates LPS-induced hepatic inhibition of eNOS mRNA, whilst showing increased, but still moderate, NOS activity, relative to LPS only. experiments (LPS+D-Galactosamine) HOCbl afforded significant, dose-dependent protection in mice *Conclusions*. HOCbl produces a complex, time- and organ-dependent, *selective* regulation of NOS/$^{\bullet}$NO during endotoxaemia, corollary regulation of downstream inflammatory mediators, and increased survival. This merits clinical evaluation.

1. Introduction

Cobalamin, $C_{63}H_{88}O_{14}N_{14}PCo$ (Figure 1), participates in only two known mammalian enzymatic reactions. Yet, these two Cbl-dependent enzymes, cytosolic methionine synthase (MS) [EC 2.1.1.13], requiring methylcobalamin (MeCbl), and mitochondrial methylmalonyl-CoA mutase (MU) [EC 5.4.99.2], requiring adenosylcobalamin (AdoCbl) [1, 2], are critically involved in key metabolic pathways essential for gene expression and regulation, *via* formation of S-adenosylmethionine (SAM) and methylation, and in protein synthesis and catabolism, cellular respiration, and energy. Activation of methionine synthase also ensures key antioxidant defense status, as it triggers concurrent activation of cystathionine β-synthase (CβS), the pivotal enzyme at the homocysteine junction in the trans-sulfuration pathway to glutathione (GSH) [3].

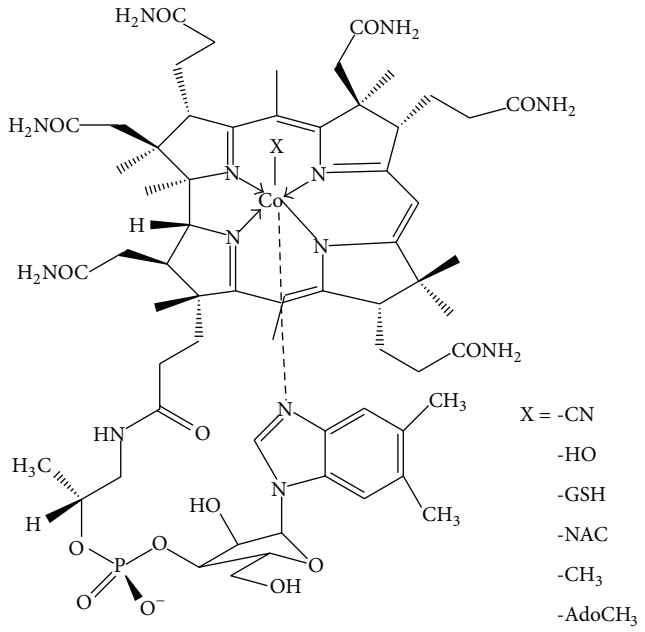

Figure 1: The structure of cobalamin. X = the principal ligands for the cobalt atom, in the upper, β axial position.

Cobalamin is the standard treatment for autoimmune "pernicious" anaemia, and macrocytic or megaloblastic anaemia, as well as for subacute combined degeneration of the spinal cord. However, an increasing body of work suggests that Cbl may also play a central role in the regulation of immunity and inflammation (reviewed in [4]). Cbl confers significant protection in various animal models of shock, from anaphylaxis to trauma and sepsis [5–7], and has remarkable organ/tissue protective effects when used clinically for the treatment of analogous inflammation in CN poisoning (reviewed in [8]). Amongst Cbl's known immunological effects are an augmentation of the CD8+/CD4+ T-lymphocyte ratio and natural killer cell activity [9, 10], both significantly reduced in inflammatory pathology, with negative consequences in septic patients [11].

Interesting homeostatic links between Cbl and pivotal cytokines are also emerging, indicative of complex but still incompletely defined regulatory circuits: MeCbl lowers interleukin-6 (IL-6) expression in peripheral blood monocytes [12], whilst Cbl deficiency raises circulating IL-6 in humans [13] and Cbl physiological status regulates IL-6 levels in rat cerebrospinal fluid [14]. Moreover, in both rodents and humans there appears to be an inverse relation between Cbl physiological levels and tumour necrosis factor alpha (TNF-α) serum levels [15]. *In vitro*, neuronal Cbl deficiency is also associated with increased expression of two TNF-α-converting enzyme secretases [16]. A reasonable hypothesis is that such Cbl/TNF-α/IL-6 regulation may be partly effected *via* Cbl indirect regulation of the central immune regulatory transcription factor, nuclear factor kappa B (NF-κB) [8]. Normal physiological levels of Cbl in spinal fluid appear to correlate with NF-κB quiescence, at least, in a non-inflammatory/non-immune challenge model [17]. Recently, a kinetic study reported that Cob(II)alamin reacts with superoxide at rates approaching superoxide dismutase [18]. CNCbl protects human aortic endothelial cells, and neuronal cells, *in vitro,* against superoxide induced injury [19, 20]. Given that oxidative stress is a major trigger of NF-κB activation, this potential antioxidant effect of Cbl could theoretically lead to NF-κB inhibition. It may also be of critical local importance *in vivo*, as the phagocytic burst includes release of the Cbl carrier, haptocorrin (HC/TC 3), in the immediate vicinity of NADPH oxidase [8, 21] one of the major biochemical sources of superoxide in immune challenge and inflammation [22]. HC/TC3, moreover, is upregulated by IL-1β, itself expressed within fifteen minutes of inflammatory challenge [23]. Nevertheless, though antioxidant effects of Cbls have been observed *in vitro* [24] and may, indeed, be important *in vivo* [20], no systematic analysis of the *in vivo* mechanisms of Cbl conferred protection against inflammation during *acute* immune challenge has hitherto been done.

We wondered if a more comprehensive explanation for Cbl effects on inflammation and immunity, and thence beneficial outcomes in sepsis and other forms of shock, may lie in a potential direct/indirect regulation by Cbl of one or more of the several actions of nitric oxide ($^{\bullet}$NO) as a ubiquitous, cell-signal transduction molecule and second messenger for post-translational modification, whose targets include soluble guanylate cyclase [25]. $^{\bullet}$NO is the product of three nitric oxide synthases (NOS): two constitutive, nNOS (neuronal NOS; NOS I) and eNOS (endothelial NOS; NOS III), and one inducible, iNOS (NOS II), at much higher levels of expression, with the potential to produce 1000-fold higher than normal amounts of $^{\bullet}$NO, during gestation, growth, and the immune response [26]. Cobalamins are known to have effects on $^{\bullet}$NO [27–29], but these have hitherto been thought to be a consequence of Cbl/$^{\bullet}$NO scavenging effects [7, 30–35] demonstrable chemically and *in vitro* [36, 37], but biologically unproven *in vivo* and still controversial [38–40].

Nitrosylcobalamin has not been detected, to date, *in vivo* or *in vitro,* amongst naturally occurring intracellular Cbls [41]. Nevertheless, if the hypothesis that Cbl is involved in NOS catalysis has any substance [42, 43], then it is conceivable that, in analogy to previously observed ferric-heme-NO complex formation at the conclusion of NOS catalysis [44], NOCbl might be transiently formed, just prior to release of free $^{\bullet}$NO by the NOS [43]. Such a theoretical transience and discrete localisation might account for the failure to detect NOCbl *in vivo* to date. Ubiquitous and continuous Cbl scavenging of $^{\bullet}$NO, on the other hand, may pose biochemical hazards. For $^{\bullet}$NO has important antioxidant and cell-signalling actions [25] which might be obstructed by HOCbl's previously proposed, indiscriminate $^{\bullet}$NO scavenging, or even just by a recently proposed, Cbl structural-based, direct inhibition of the NOS *tout court* and nothing else [45]. There is some evidence that HOCbl can discriminate between exogenous $^{\bullet}$NO donors and the natural endogenous donor, *S*-nitrosoglutathione, GSNO, actually prolonging only GSNO-induced, gastric fundus relaxations [46]. This hints at a more complex Cbl/$^{\bullet}$NO regulatory relationship.

Moreover, there are also diverse indications that positive Cbl status is allied to beneficial •NO activity: in diabetic rats, high cobalamin levels correlate with high NOS protein levels, •NO activity, and increased erectile function [47]; Cbl supplementation of vegetarians with low Cbl status significantly increases eNOS •NO release in the brachial artery [48]; in the digestive tract of endotoxemic rats, the highest expression of iNOS is in the ileum, precisely where Cbl is internalized [49], and both Cbl and •NO are known to mediate cell protective effects via ERK1/2 and Akt [50–54]. These protective effects of •NO and Cbl include induction and regulation of heme oxygenase-1 (HO-1) [52, 55–58], which converts biliverdin to the powerful antioxidant bilirubin, and carbon monoxide. CO can then in turn decrease •NO [59]. (For a more comprehensive list of coincidences of Cbl's/ •NO's positive actions, see Table 1 and its related discussion in [43]).

Thus, in these studies we explored an alternative hypothesis to that of Cbl as *just* an •NO, or, indeed, superoxide, mop. We posited that the principal mechanism behind Cbl's beneficial, pleiotropic effects in inflammation may involve a biphasic regulation of NOS expression and protein translation and the ensuing •NO synthesis, during the two distinct pro- and anti-inflammatory phases of the immune response.

2. Materials and Methods

2.1. Animals. Male C57BL/6 mice, weighing 20 to 25 g, were purchased from Harlan, UK, and maintained on a standard chow pellet diet, containing standard amounts of Cbl (50 μg/kg vitamin B12/CNCbl), with tap water supplied *ad libitum*. Animals were kept in a 12:00 h light/dark cycle, and all were housed for 7 days prior to experimentation. All experiments were performed in accordance with UK Home Office regulations (Guidance On the Operation of Animals: Scientific Procedures Act, 1986).

2.2. Cobalamins. The coenzymes, 5′-deoxyadenosylcobalamin, and methylcobalamin; Vitamin B_{12}a, cyanocobalamin, and hydroxocobalamin (CAS 78091-12-0) were purchased from Sigma-Aldrich (UK). Glutathionylcobalamin and N-acetyl-cysteinyl-cobalamin were synthesized and supplied by Professor Nicola Brasch (Kent State University, Ohio, USA). All Cbls (and Cbl-treated animal samples) were protected from light during storage and handling, and were 98% to 99.5% pure.

2.3. Drug Treatment and Experimental Design. 5′-deoxyadenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin (HOCbl), glutathionylcobalamin (GSCbl), and N-acetyl-cysteinyl-cobalamin (NAC-Cbl) were all stored at −20°C, and fresh solutions of them were made using sterile, pyrogen-free, phosphate-buffered saline (PBS; Gibco), prior to the experiments. For the *in vivo* experiments, cobalamins were diluted at 10 mL/kg prior to treatments (with PBS used as vehicle). Cobalamins were administered according to the protocol summarized in Table 1.

2.4. Effects of Endogenous Cobalamins on NF-κB Promoter Activity. RAW 246.7 macrophage cells, stably transfected with NF-κB luciferase reporter construct (Stratagene), were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 1 μg/mL Geneticin, and 50 μg/mL G418. Cells (2×10^4 cells) were seeded in 96-well plates and then preincubated for 1 h with increasing concentrations (1–10–100 μM) of the five principally occurring, intracellular Cbls. Thereafter, at time 0 h, cells were stimulated with *E. coli* LPS (0111 : B4; 1 μg) for 4 h and then processed for measurement of luciferase activity in a luminometer (Luminometer TD-20/20; Turner Designs Instruments).

2.5. Non-Lethal and Lethal Endotoxaemia. Endotoxaemia was induced by the intraperitoneal injection of LPS (0.1 mg/kg), alone (non-lethal) or, in the lethal endotoxaemia protocol, in combination with 1 g/kg D-Galactosamine (Table 1). Sample collection in non-lethal endotoxaemia was carried out at both 4 and 24 h after LPS challenge. Animal survival, in all lethal endotoxaemia experiments, was monitored for a total of 5 days, and all data were analysed using Chi-squared or Kaplan-Meier tests.

Times shown are in relation to time 0 h, when either LPS alone or LPS+D-Gal was administered by intraperitoneal injection. Individual cobalamins were injected into the peritoneum at the doses and times reported in Table 1.

2.6. Sample Preparation for Real-Time Reverse Transcriptase-PCR. Blood (500 μL) was centrifuged (for 5 min, at 2500 rpm), and the plasma then collected for ELISA analysis. 500 μL of TRIZOL reagent (Invitrogen) was added to the remaining fraction. RNA purification was performed as recommended by the manufacturer. Following extraction, RNA (20 μL) was treated with 2 U (1 μL) of TURBO DNase 1 (Ambion, Austin, TX), as described by the manufacturer, to remove any contaminating genomic DNA. An aliquot of the DNA-free RNA (7.6 μL) was then transferred to a new RNase-free tube and reverse-transcribed into complementary DNA (cDNA), using Superscript III Reverse Transcriptase (Invitrogen), as described by manufacturer. The following reagents were used: Oligo dT primers (Invitrogen); 1 μL, 10 mM dNTP (Bioline); 4 μL of 5X first-strand buffer; 1 μL, 0.1 M DTT; 1 μL (40 U) RNaseOUT; and 1 μL (200 U) of Superscript III Reverse Transcriptase (Invitrogen). After synthesis, cDNA was quantified using a Nanodrop ND-1000 and diluted (80 ng/μL) in molecular biology grade water and then loaded into 384-well plates for real-time PCR.

2.7. Real-Time Reverse Transcriptase PCR. Real-time PCR assays were performed on the various samples in order to evaluate the expression of the following genes: GAPDH, RPL32, IL-1β, COX-2, iNOS, eNOS, TNF-α, and HMGB1 (Table 2). For each gene analyzed, reactions were performed using 1 μL of the Qiagen QuantiTect Primer Assay, added to 5 μL Power SyBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and then diluted with 2 μL molecular grade water. A final volume of 8 μL was dispensed into each

Table 1: Protocol for cobalamin treatment of LPS-induced endotoxaemia.

Experimental protocols	Inflammatory stimulus	Treatment	
		Cbl dose	Time of Cbl treatment
4 h non-lethal	LPS (0.1 mg/kg)	0.2 mg/kg	−1 h, +1 h, +2 h
24 h non-lethal	LPS (0.1 mg/kg)	0.2 mg/kg	−1, +1, +2, +6, +22 h
Lethal series I	LPS (0.1 mg/kg)	0.2 mg/kg	−1, +1, +2, +6, +22 h
	+ D-Gal (1 g/kg)	40 mg/kg	+2, +22 h
Lethal series II	LPS (0.1 mg/kg)	40 mg/kg HOCbl	+2, +4 h
	+ D-Gal (1 g/kg)	80 mg/kg HOCbl	+2 h

Table 2

Assay code	Gene and accession number of detected transcripts
QT00100275	NOS2; NM_010927
QT00152754	NOS3; NM_008713
QT00247786	HMGB1; NM_010439
QT00104006	TNF-α; NM_013693
QT01048355	IL-1β; NM_008361
QT01658692	GADPH; NM_008084
QT01752387	RPL32; NM_172086

well and 2 μL of diluted cDNA (160 ng/reaction) was added. Each sample was tested in triplicate for each gene, and PCR reactions were performed using ABI Prism 7900 real-time PCR equipment. The thermal profile consisted of 95°C for 15 min, then 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. This was plotted as a melting curve. The comparison between samples was performed using GAPDH and RPL32 as internal standards. REST MCS software was utilized for the calculation of the relative difference between the test groups.

2.8. iNOS and eNOS Western Blotting. Liver tissues were harvested from ($n = 5$) animals, after LPS endotoxaemia, with or without HOCbl treatment and then homogenized in lysis buffer, which contained a cocktail of protease inhibitors. Protein concentrations prior to loading were determined using the Bradford assay (Sigma): samples were mixed with 6x Laemmli sample buffer, and equal protein amounts (100 μg) then underwent electrophoresis on a 10% polyacrylamide gel in running buffer (0.3% Tris base, 1.44% glycine, and 0.1% SDS in distilled water). This was followed by transfer of the proteins onto PVDF membranes in transfer buffer (using 0.3% Tris base, 1.44% glycine, and 20% methanol, in distilled water). Membranes were blocked for 1 h with 5% nonfat milk solution in TBS containing 0.1% Tween 20. iNOS expression was assessed using a specific monoclonal antibody (1 : 1000; Santa Cruz, USA). The signal was amplified with HRP-linked anti-mouse secondary antibody (1 : 2000) and visualized by ECL (Western blotting detection reagent; Amersham Biosciences, USA). Densitometric analysis was performed using NIH ImageJ software and normalised to tubulin loading controls in the same sample.

2.9. NOS Activity: Nitrate/Nitrite Production Assays. Animals ($n = 5$) were challenged with LPS and treated with Cbls as described above. At 4 h and 24 h after LPS challenge, lung and liver tissue samples were harvested, homogenized, and processed for determination of NOS activity, as measured by nitrate/nitrite end-products of NO. The ultrasensitive, NOS assay used (Oxford Biomedical Research, Oxford, MI, USA: ultrasensitive colorimetric NOS assay: cat no. NB78) employs an NADPH recycling system—$NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and the substrate, L-arginine, but not the cofactor, BH_4,—to ensure that NOS operate linearly for up to 6 hours, as NO-derived nitrate and nitrite accumulate. The assay kit can accurately measure as little as 1 pmol/milliL (~1 milliM) $^{\bullet}$NO produced in aqueous solution. In these studies, the assay was run for 5 h at 37°C. The enzyme nitrate reductase was used to convert all nitrate to nitrite, then Griess reagent employed to quantify nitrite levels, with the generation of a nitrite standard, as recommended by the supplier. The completed reaction was read at 540 nm in a Microtiter plate reader. Data are expressed as mmol nitrite/μg protein.

2.10. Determination of Tumour Necrosis Factor Alpha (TNF-α) and Interleukin (IL-6) Levels. After collection, blood was centrifuged and the plasma separated, under low lighting conditions, then stored at −80°C until performance of the analyses. For determination of circulating TNF-α and IL-6 levels, using ELISA assays, samples were diluted 1 : 10 in the assay diluent, as specified by the manufacturer (R&D, UK). Absorbance was plotted in a standard curve, and data expressed as the content of TNF-α (ng) or IL-6 (pg) per mL of plasma.

2.11. Reagents. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Poole, UK.

2.12. Statistics. Data are shown as a mean ± S.E. of 5 animals per group for the analyses in non-lethal endotoxaemia and, initially, 7–9 per group, then 12 per group, for lethal endotoxaemia survival, series I and II, respectively. Statistical differences were determined by ANOVA, following the Student Newman Keuls test. Chi-square and Kaplan-Meier tests were used for the lethality studies. In all cases, a $P < 0.05$ was taken as significant.

3. Results

3.1. Cobalamins Do Not Inhibit LPS-Induced NF-κB Activation In Vitro. Since Cbl has been shown to prevent NF-κB activation in a non-immune challenge model [17], and activation of NF-κB leads to iNOS induction, we first looked at the effects of the five principally occurring, intracellular Cbls, (CNCbl, HOCbl, GSCbl, and the two mammalian enzyme cofactors for MU and MS, respectively, AdoCbl and MeCbl), on LPS-induced NF-κB activation *in vitro,* using a canonical reporter assay. Although the various incoming forms of Cbl are all reduced or dealkylated soon after cell entry, prior to MS/MU cofactor formation [60], the different incoming forms affect both the rate and ratio of formation of the two known active cofactors, AdoCbl/MeCbl [60–62]. Theoretically, this variability in Cbl cofactor formation may impact on the effects of Cbls in immune challenge with respect to NF-κB activation. Thus, it was important to make this comparison. RAW 264.7 macrophage cells, stably transfected with NF-κB luciferase reporter construct, were preincubated for 1 h with increasing concentrations (1–10–100 μM) of Cbls. Upon LPS stimulation, none of the five Cbls significantly affected or inhibited LPS-driven NF-κB activation at 1 h, with no significant inhibitory effect on NF-κB at a later time point (24 h). CNCbl alone slightly stimulated NF-κB activity, but only at the 1 h time point and when tested at the concentration of 1 μM (Table 3).

In two separate experiments, each performed in triplicate, RAW246.7 cells, stably transfected with NF-κB luciferase reporter construct, were seeded in 96-well plates and preincubated for 1 h with increasing concentrations (1–10–100 μM) of individual Cbls, followed by stimulation with *E. coli* LPS (1 μg). At 1 h, and 24 h, following LPS in the respective experiments, cells were processed for measurement of luciferase activity. Basal values of fluorescence were 2.60 ± 0.38 and 3.77 ± 0.31 for 1 h and 24 h incubation, respectively. Data are expressed as a mean ± SEM of triplicate observations. $^{*}P < 0.05$ versus LPS alone.

Table 3: Effects of the naturally occurring endogenous cobalamins in the NF-κB gene reporter assay.

Treatments	NF-κB reporter assay (fold increase over basal)	
	1 h	24 h
LPS	5.63 ± 0.37	4.60 ± 0.21
CNCbl (μM)		
1	*7.10 ± 0.30	5.70 ± 0.90
10	5.53 ± 0.29	6.60 ± 1.70
100	5.23 ± 0.62	5.53 ± 0.74
GSCbl (μM)		
1	6.33 ± 0.37	5.66 ± 0.67
10	6.25 ± 0.32	5.63 ± 0.63
100	5.98 ± 0.26	5.17 ± 0.84
MeCbl (μM)		
1	6.10 ± 0.10	6.53 ± 1.74
10	6.10 ± 0.15	5.11 ± 1.06
100	6.00 ± 0.58	5.26 ± 0.62
HOCbl (μM)		
1	5.67 ± 0.20	5.73 ± 0.91
10	5.98 ± 0.26	5.57 ± 0.78
100	7.00 ± 1.10	6.10 ± 0.66
AdoCbl (μM)		
1	6.23 ± 0.39	5.36 ± 0.77
10	6.26 ± 0.90	5.40 ± 0.70
100	6.20 ± 0.61	4.53 ± 0.32

3.2. Endogenous Cobalamins Enhance Survival in Acute Endotoxaemia. As there was no observable difference between the effects of alkyl and non-alkyl Cbls on NF-κB *in vitro*, we chose to focus these first investigations *in vivo* principally on HOCbl, as a clinically licensed Cbl form, known to be partially converted on cell entry to the two Cbl cofactors, MeCbl and AdoCbl, for MS and MCM, respectively [63]. Furthermore, at supraphysiological doses of 5 g i.v., HOCbl, as a clinical cyanide antidote, has shown remarkable protection against corollary inflammation (analogous to the inflammation seen in SIRS, sepsis, and septic shock), that goes beyond merely acting as a magnet for CN [8].

We therefore next decided to see if the lethality survival protection also conferred by HOCbl in a sepsis/endotoxaemia mouse model [7] was reproducible in a different strain and in a more acute endotoxaemia model. Some groups of animals were alternatively treated with the relatively novel, intracellular Cbl, glutathionylcobalamin (GSCbl) [61, 64] whose clinical effects are untested in sepsis, or with N-acetyl-cysteinyl-cobalamin (NAC-Cbl), a synthetic cobalamin, used as a non-endogenously occurring, thiol Cbl comparison. To gain some information on potential clinical dosage, all Cbls were tested in two distinct, high dosing regimes, with or without prophylactic pretreatment.

In a severe sepsis protocol (LPS+D-Gal), using C57BL/6 mice, we administered a relatively low dose of Cbls (0.2 mg/kg i.p.), equivalent to a maximal concentration of approximately 1 μM (considering a total blood volume of 2.5 mL in the mouse, this concentration being well within the range tested *in vitro*). Individual Cbls were administered i.p. −1 h prior to LPS+D-Gal and then given in repeated doses at +1, +2, +6, and +22 h after LPS+D-Gal. Alternatively, a high dose Cbl protocol (40 mg/kg i.p.) was administered only twice, at +2 and +22 h after LPS+D-Gal, to assess its potential as a rescue regimen. The urine of all Cbl-treated animals was red, within 1 h of administration, an indicator of rapid, high, systemic Cbl saturation (data not shown).

LPS+D-Gal mice rapidly reached 88.9% mortality by 8 h. This did not change further up to 24 h. Animals treated with the relatively low-dose regimen of GSCbl or NAC-Cbl, were protected in the early, 4–8 h time frame (Figure 2(a)). During this period all Cbl-treated animals also exhibited less huddling and pilo-erection (data not shown).

At 8 h after LPS+D-Gal, all relatively low-dose Cbl treatments afforded 25% survival, $^{+}P < 0.05$ versus LPS+D-Gal alone (Figure 2(a)). However, only low-dose HOCbl treatment maintained this level of protection up to 24 h

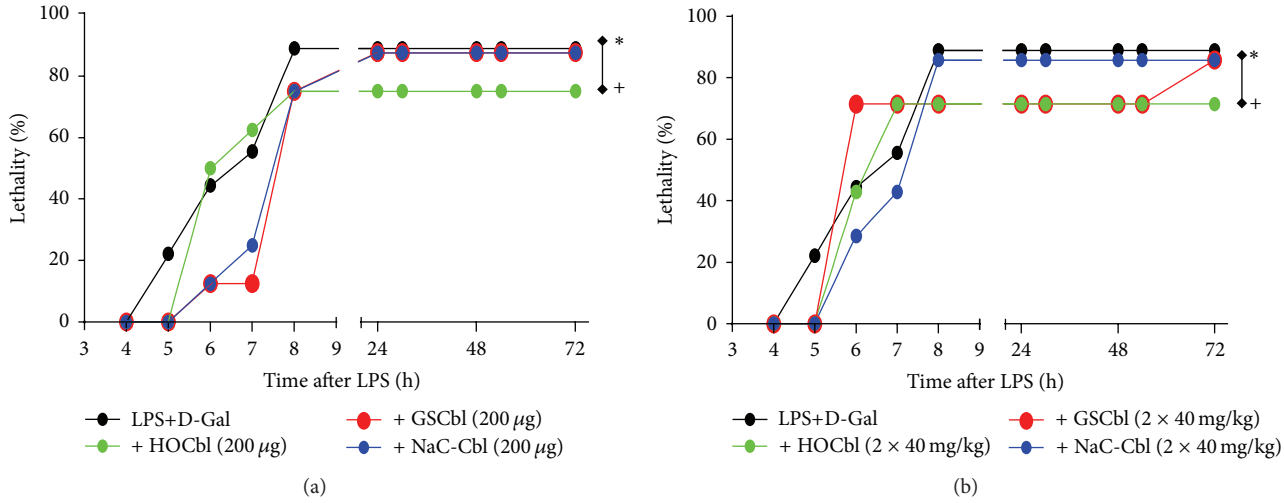

Figure 2: HOCbl protects mice against experimental endotoxaemia. In (a) and (b) mice (n = 8 and 7, resp.) were treated with HOCbl or GSCbl or NAC-Cbl following two distinct protocols, in addition to being injected with LPS (0.1 mg/kg i.p.) + D-Galactosamine (1 g/kg i.p.). Impact on lethality was followed for a total of 5 days. (a) Cobalamins were administered at a dose of 0.2 mg/kg i.p. −1 h, +1 h, +2 h, +6 h, and +22 h and compared to controls given LPS + D-Galactosamine injection alone (Time 0 h). $^{*}P < 0.05$ for GSCbl/NAC-Cbl and + for HOCbl when tested against LPS+D = Gal (n = 9), using the Chi-square or Kaplan-Meier tests. (b) Cobalamins were administered at a dose of 40 mg/kg i.p. +2 h and +6 h and compared to controls given LPS + D-Galactosamine injection alone (Time 0 h). $^{*}P < 0.01$ for NAC-Cbl and + for GSCbl/HOCbl, when tested against LPS+D-Gal (n = 9), using the Chi-square or Kaplan-Meier tests.

(Figure 2(a)). (Indeed, as regards the long-term outcomes, 8 h seemed to be a watershed time point at which the outcome was determined for all groups.) Paradoxically, in view of its early protective effects at the lower dose, the *high-dose* GSCbl regimen was less protective within the first 8 h. High-dose NAC-Cbl, which again provided some degree of protection in the first 6 h, was not significantly different from controls at 24 h. In contrast, high-dose GSCbl and HOCbl, despite the lesser protection of the former in the first hours, offered a consistent 28.60% survival up to 24 h, $^{+}P < 0.01$ versus LPS+D-Gal alone (Figure 2(b)).

Later, at 72 h following LPS+D-Gal, in the GSCbl high-dose group, mortality was equal to that observed in the NAC-Cbl high dose group, 85.72%, close to that of LPS+D-Gal control animals, though this increase in mortality was a late event: with 28.60% survival to 54 h in this group, perhaps indicative of the general Cbl protective trend. Nonetheless, the 25% and 28.60%, respectively, of mice that were alive at 24 h, in each of the two distinct, low- or high-dose, HOCbl-treated groups, exhibited continued survival up to 72 h, $/^{+}P < 0.05$ versus LPS+D-Gal alone/$^{+}P < 0.01$ versus LPS+D-Gal alone (Figures 2(a) and 2(b)) and beyond (data not shown).

II. Since these initial endotoxaemia studies might be considered underpowered, we repeated the lethality survival experiments using larger groups of mice (n = 12) and focussing on HOCbl alone, as having previously shown the most consistent protective effects. This time, given the trend towards improved survival seen at the higher HOCbl dose, two distinct ultra-high doses of HOCbl (40 mg/kg and 80 mg/kg) were tested, with a more concise dose/time frame, +2 h and +4 h only for the 40 mg/kg, and, in the case of the 80 mg/kg dose, a single bolus administration at +2 h. The significant survival advantage of HOCbl treatment results demonstrated over 5 days, in Figures 3(a) and 3(b), not only shows that our HOCbl data is consistently reproducible, but also that increasing the dose of HOCbl significantly increases survival, from 25% up to 33.333%: $^{+}P < 0.01$ for HOCbl (80 mg/kg) by using the Kaplan-Meyer test. By comparison, at 24 h in the LPS-only group, there was 90% mortality.

To gain information about the mechanisms behind the consistent protection afforded by HOCbl, and to observe any potential impact on the NOS, a non-lethal protocol was next deployed. Then the expression of inflammatory mediator genes in liver and lung was analysed, in both the pro- and anti-inflammatory phases of the immune response, at the 4 h and 24 h time points.

3.3. HOCbl Selective Promotion/Modulation of eNOS/iNOS mRNA, Inhibition of IL-1β, and Cox-2 Expression: 4 h Time Point. The early effects of HOCbl treatment on eNOS mRNA appeared organ dependent, with significant promotion of eNOS mRNA in the lung and attenuation in the liver (Figures 4(a) and 4(b)). For eNOS, in LPS-only animals we observed a decrease in the lung of −2.9 ± 0.1, whereas there was an increase of 2.1 ± 0.1-fold change in LPS+HOCbl-treated animals (Figure 4(a)). Paradoxically, in the liver of LPS-only treated animals, there was an increase of eNOS expression of up to ~15-fold compared to up to ~4-fold change only in LPS+HOCbl—treated animals (Figure 4(b)).

Liver and lung iNOS and COX-2 gene expression levels were increased in LPS-only treated animals when compared to that of PBS-only injected mice, whose value was set as 1. However, as for eNOS, the effects of HOCbl on iNOS expression were once more organ selective, failing to inhibit the rise in iNOS mRNA in the lung, but attenuating

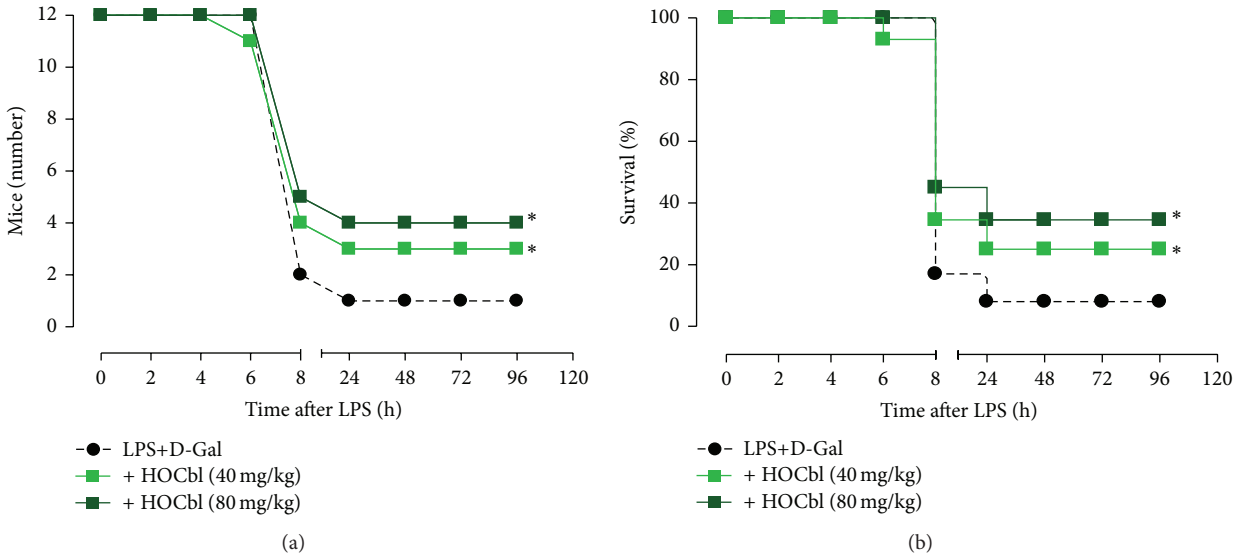

FIGURE 3: Ultra-high dose HOCbl consistently improves survival in experimental endotoxaemia. Mice ($n = 12$ per group: 2 Cbl active treatment groups plus 1 LPS-only group) were treated with HOCbl, following two distinct protocols, +40 mg/kg at 2 h and +4 h post LPS + D-Gal, and +80 mg/kg at only +2 h following LPS (0.1 mg/kg i.p.) + D-Galactosamine (1 g/kg i.p.). Impact on lethality was followed for a total of 5 days. $^*P < 0.05$ for HOCbl at both doses, when tested against LPS+D-Gal ($n = 12$), using the Chi-square test. $^*P < 0.01$ for HOCbl (80 mg/kg) by using the Kaplan-Meier test.

it in the liver (Figures 4(c) and 4(d)). Strikingly, in spite of HOCbl's failure to completely inhibit iNOS expression, HOCbl was a consistent inhibitor of COX-2 mRNA in both liver and lung, bringing its degree of expression back to and below that of PBS-injected mice (Figures 4(e) and 4(f)). HOCbl treatment also had a consistent regulatory effect on IL-1β expression, which was moderately and significantly decreased in lung and completely inhibited in liver (Figures 4(g) and 4(h)).

3.4. HOCbl Has Early Promotional Effects on Translation of eNOS/iNOS Protein. To determine efficiency of translation of the post-LPS increased NOS mRNA, we assessed eNOS and iNOS protein expression by Western blot in 4 h liver samples. As predicted by our hypothesis that sepsis may involve a failure in translation of the NOS, this revealed that whilst in the LPS-only challenged group there was a significant depression of eNOS protein translation, that was at odds with its high mRNA expression, HOCbl significantly promoted eNOS protein translation, above the levels of both PBS control and LPS-only treatment groups (Figure 5(a)). A similar paradoxical pattern was observed in hepatic iNOS protein translation, with significant depression of iNOS protein translation in the LPS-only challenged group, and promotion of iNOS protein in the HOCbl+LPS treated group (Figure 5(b)). We confirmed that these effects of HOCbl on NOS protein promotion were not random or artifactual, but were specific to Cbl, by repeating the LPS non-lethal endotoxaemia experiment using either GSCbl or NAC-Cbl treatment and performing Western blots for eNOS/iNOS protein. Once again, we observed a significant early promotion of eNOS/iNOS protein by these other Cbls, when compared to LPS only (Figures 5(a) and 5(b)).

3.5. HOCbl Moderates High $^\bullet$NO Synthesis at 4 h and 24 h after LPS. However, when NOS activity was measured (using a nitrite production assay) both in the early post-LPS challenge, pro-inflammatory phase and in the late anti-inflammatory, resolution phase, a further paradoxical result emerged, suggesting that HOCbl may exert some post-translational modification of NOS activity. Levels of nitrite at 4 h showed an inverse relation to levels of NOS protein, with significantly higher levels of nitrite in the LPS-only eNOS/iNOS-depressed group and significantly lower levels of nitrite being generated in the HOCbl/eNOS/iNOS-promoted group (Figure 6(a)). (That this was a general, reproducible Cbl effect was confirmed, as stated previously, by also doing the Western blot with 4 h GSCbl/NAC-Cbl-treated liver samples and also running the NOS activity assay with both thiol-Cbl treated samples.) Here we again observed a correlation between GSCbl/NAC-Cbl promoted high NOS protein in the Western blots and decreased nitrite in the NOS activity assay (Figure 7).

At 24 h following LPS, levels of NOS-derived nitrite, as measured in tissue samples, were even higher than at 4 h in both the LPS-only and HOCbl-treated groups. Nevertheless, HOCbl consistently showed *relatively* less nitrite production than LPS only, in both lung and liver tissue samples (Figures 6(b) and 6(c)).

3.6. HOCbl Regulates TNF-α Expression and Protein but Leaves Circulating IL-6 Protein Levels Unchanged. Since high

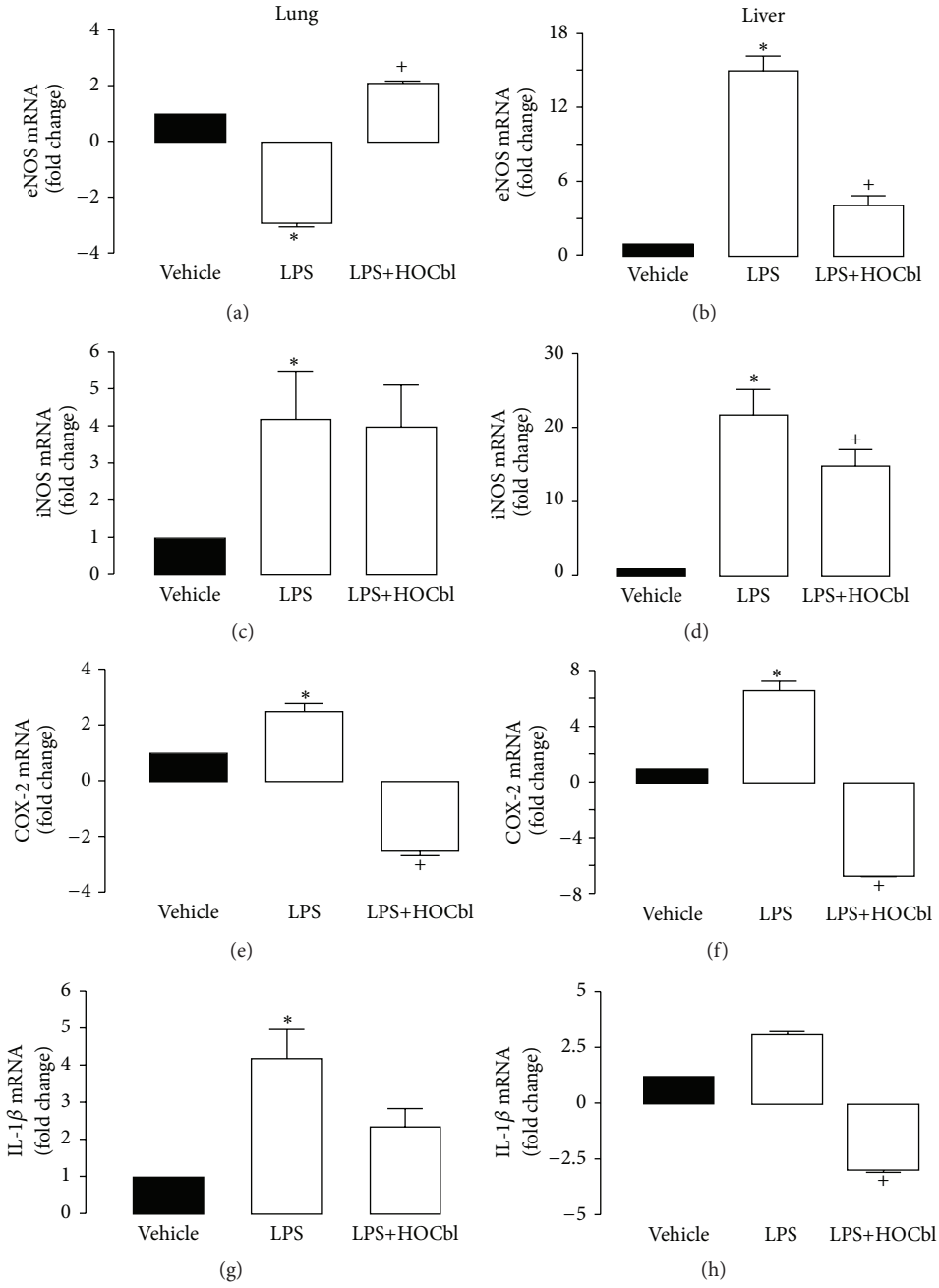

Figure 4: HOCbl selectively modulates NOS enzymes and inhibits COX-2, IL-1β mRNA, in lung and liver, at 4 h following LPS-induced endotoxaemia. Mice ($n = 5$) were treated with HOCbl, at the low dose protocol (0.2 mg/kg i.p.) and compared to LPS only (0.1 mg/kg i.p. at time 0 h). Organs (lung and liver) were harvested at 4 h after LPS and gene expression was quantified in tissue extracts by real-time PCR, using GAPDH and RPL32 as internal standards. ((a), (b)) eNOS mRNA data; ((c), (d)) iNOS mRNA data; ((e), (f)) COX-2 mRNA data; ((g), (h)) IL-1β mRNA data. Values are a mean ± SEM of triplicate observations. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS-only group.

iNOS expression/protein and $^{\bullet}$NO activity in the sepsis literature are associated with high TNF-α, IL-6, and ensuing toxicity, we evaluated how HOCbl might impact upon systemic levels of TNF-α and IL-6 triggered by LPS. Plasma levels of these cytokines were quantified using ELISA. Levels of IL-6 protein were not significantly lower in the HOCbl-treated group (Figure 8(a)) However, HOCbl treatment significantly ($P < 0.05$) attenuated the post-LPS-induced increase in circulating plasma TNF-α, as measured at the 4 h time point, (~50% reduction: Figure 8(b)). Consistent with its effects on plasma TNF-α, HOCbl also showed some protection from the inhibitory effects of LPS on TNF-α mRNA in the lung and significant attenuation of TNF-α mRNA in liver (Figures 8(c) and 8(d)).

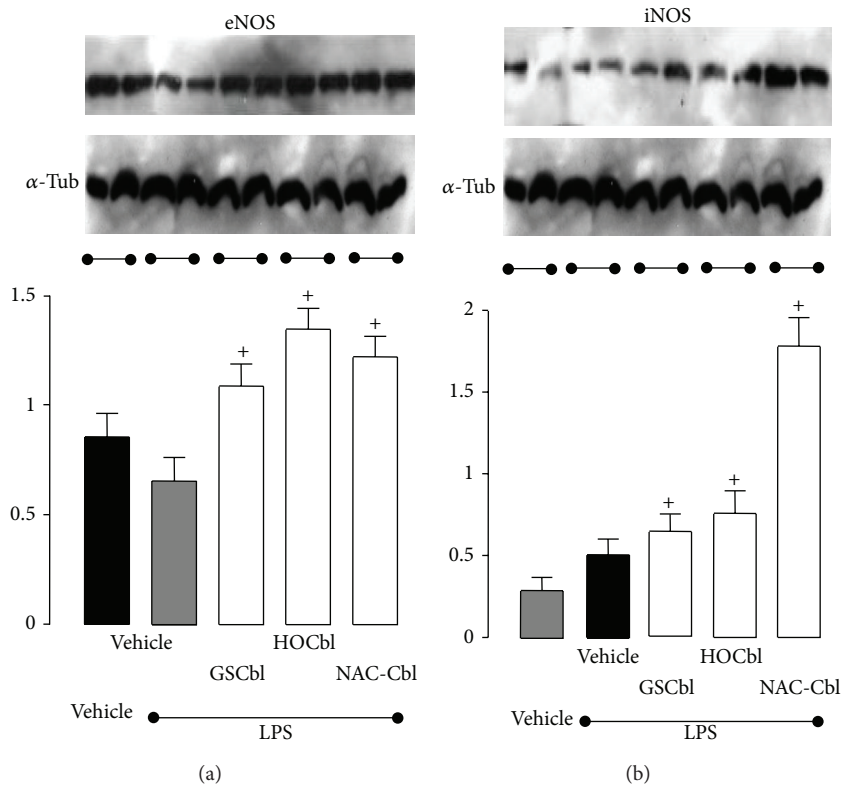

Figure 5: Early (4 h) promotional effect of HOCbl on iNOS and eNOS protein expression in liver samples from endotoxemic mice: mice ($n = 5$) were treated with HOCbl at the low dose protocol (0.2 mg/kg i.p.) at −1 h, +1 h, and +2 h and challenged with LPS (0.1 mg/kg i.p.) at time 0. Liver samples were collected and processed as described in Materials and Methods, for the Western blot analysis of iNOS and eNOS protein expression. Upper panels ((a), (b)) show membranes probed for iNOS, eNOS, with tubulin as the loading control; lower panels show densitometric analysis of the blots. Values are a mean ± SEM of triplicate experiments. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS group.

3.7. HOCbl Late Effects on NOS and Cox-2 mRNA: 24 h Time Point. In the resolution phase of the immune response to LPS, the effects of HOCbl treatment on NOS expression displayed a degree of organ selectivity, though most notable with respect to iNOS expression. Whilst HOCbl did not change LPS-induced eNOS mRNA inhibition in the lung, it significantly attenuated its inhibition in the liver, from −75- to −58 fold-change, respectively, for LPS only and LPS+HOCbl (Figures 9(a) and 9(b)).

HOCbl effects on iNOS mRNA in the lung were more distinctive, with ~80% inhibition compared to LPS-only (vehicle group). In the liver, HOCbl treatment attenuated the LPS-induced inhibition of iNOS mRNA by ~40% (Figures 9(c) and 9(d)). The consistent HOCbl tissue inhibition of Cox-2 mRNA, seen at the early pro-inflammatory phase time point of 4 h, persisted at 24 h, showing a significantly greater degree of inhibition than LPS-only: 7- versus 2.5-fold for LPS-only in the lung; 115- versus 50-fold for LPS-only in liver (Figures 9(e) and 9(f)).

3.8. HOCbl Inhibition of HMGB1 mRNA at 24 h following LPS. Given the early regulatory impact of HOCbl on NOS/$^{\bullet}$NO activity, and COX-2, IL-1β, and TNF-α, we expected to see related downstream beneficial effects on expression of the late (≥18 h) effector of endotoxaemia, high mobility group box 1 (HMGB1). This prediction was confirmed. Where LPS-only presented an inconsistent picture, HOCbl treatment consistently inhibited HMGB1 mRNA: in the lung, from an increase of 2.5 in LPS-only to a near threefold decrease (setting levels of expression even lower than those observed in the control group, taken as a value of 1); and in the liver, to a more significant degree, even beyond the remarkable inhibitory effect of LPS-only (Figures 9(g) and 9(h)).

To conclude the 24 h gene expression analyses, tissue levels of IL-1β and TNF-α mRNA were also quantified using RT-PCR. In resolution, HOCbl treatment significantly increased inhibition of hepatic IL-1β (in line with its inhibitory effect at 4 h) and inhibition of TNF-α in the lung, whilst also, paradoxically, decreasing LPS inhibition of TNF-α in the liver (Table 4). Of note was the fact that the late effects of HOCbl on TNF-α mirrored the degree of late iNOS expression, in both lung and liver, as, indeed, did LPS-only (Figures 9(c) and 9(d) and Table 4).

4. Discussion

Our studies present a picture of complex and far-reaching homeostatic regulation of the activation, expression, and

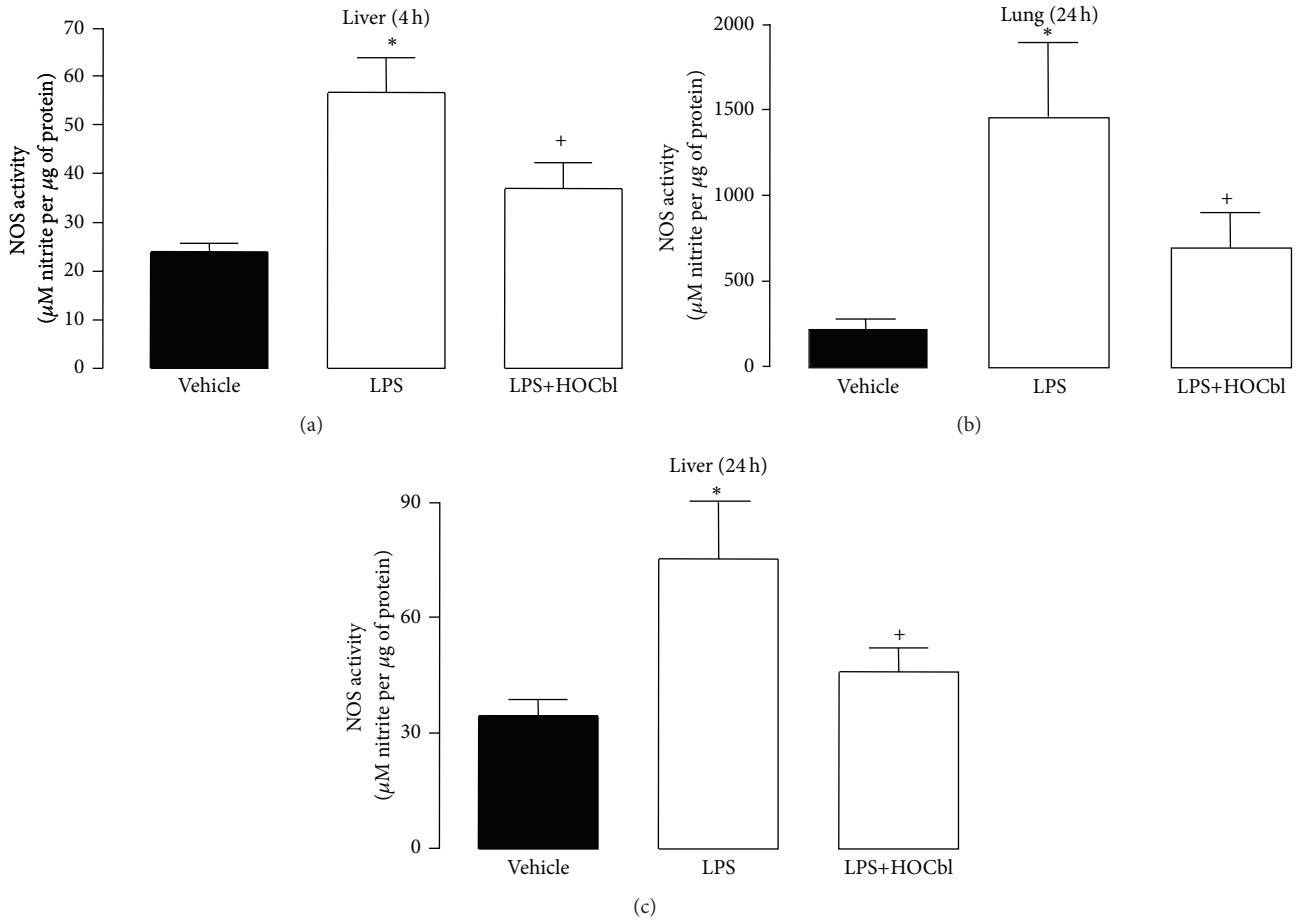

Figure 6: Modulatory effects of HOCbl on NOS activity in tissue homogenates of endotoxemic mice at 4 h and 24 h. Liver ((a), (c)) and lung (b) tissue samples were collected 4 h and 24 h after LPS challenge (0.1 mg/kg; i.p.); two groups of mice ($n = 5$ per group) having previously been treated with HOCbl (resp., 0.2 mg/kg i.p. at −1 h, +1 h, and +2 h and at −1 h, +1 h, +2 h, +6 h, and +22 h) and also challenged with LPS at time 0. NOS activity at 4 h and 24 h was assayed as described in Materials and Methods, and shown as μmol of NO generated per μg protein. Values are a mean ± SEM of triplicate observations. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS group.

Table 4: Effects of HOCbl on pro-inflammatory cytokine gene expression, at 24 h following LPS-induced endotoxaemia.

Gene	Organ	LPS	LPS + HOCbl	P value (versus LPS)
IL-1β	Liver	−8.30 ± 0.30	−10.6 ± 0.3*	0.0056
	Lung	2.70 ± 0.50	2.1 ± 0.1	0.3046
TNF-α	Liver	−86.90 ± 0.10	−68.6 ± 0.2*	<0.0001
	Lung	−2.10 ± 0.20	−11.4 ± 0.1*	<0.0001

Mice were treated with HOCbl at the low dose protocol (0.2 mg/kg i.p. at −1 h, +1 h, +2 h, +6 h, and +22 h) and compared to LPS only (0.1 mg/kg i.p. at time 0 h). Twenty-four hours after LPS stimulation, organs (liver and lung) were harvested and processed for assessment of IL-1β, TNF-α, gene expression by real-time PCR, using GAPDH and RPL32 as internal standards. Values are a mean ± SEM of triplicate observations. * denotes statistical significance for $P < 0.05$.

translation of NOS, $^{\bullet}$NO synthesis, and inflammatory mediators by HOCbl during the immune response. We propose that this regulation accounts for the noted survivals in rodent endotoxaemia, both in our more acute, septic shock models I and II (a modest but significant 25%/28.60%, and 25%/33.333%, survival) and in a previous sub-acute, sepsis model (performed with CNCbl/HOCbl—$n = 10$ animals per group—30%/40% survival [7]). The regulation we show here may also explain the observed organ/tissue-protective effects of HOCbl in the clinical treatment of CN poisoning and the ensuing shock, which appear to go beyond what may be expected from the Cbl binding of CN alone [8]. Furthermore, given the supraphysiological, saturating doses of Cbl used in our studies, if Cbl had been acting just as an $^{\bullet}$NO scavenger, or even as a NOS inhibitor, it seems unlikely that it would have permitted the increasing rise in NOS activity (as indexed by nitrite), both early and late, or that its effects would be so subtle, complicated, and ultimately beneficial.

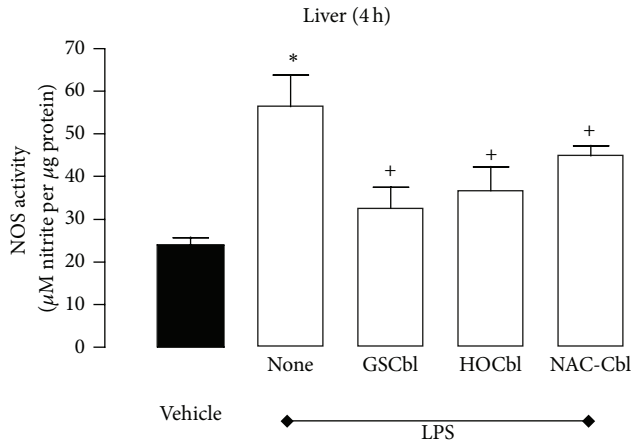

FIGURE 7: The effects of thiol Cbls on activity of NOS. Liver tissue samples were collected 4 h after LPS challenge (0.1 mg/kg; i.p.); 4 groups of mice ($n = 5$ per group) having previously been treated with either LPS-only or HOCbl/or GSCbl or NAC-Cbl plus LPS at time 0 h (0.2 mg/kg i.p. at −1 h, +1 h, and +2 h and at −1 h, +1 h, +2 h, +6 h, and +22 h). NOS activity at 4 h was assayed as described in Materials and Methods, and shown as μmol of NO generated per μg protein. Values are a mean ± SEM of triplicate observations. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS group.

4.1. Paradoxes of Thiol Cobalamin Effects. The thiol Cbls, on the other hand, present some mysteries, about which one can only give speculative answers at this point. The survival advantage exhibited in the early hours by comparatively low dose GSCbl/NAC-Cbl treated animals, but not with HOCbl treatment, may be due to the known higher reduction potential/lability of thiol Cbls [64], resulting in more rapidly available intracellular Cbl. Cbl is likely to be, at least in part, functionally deficient in sepsis, as indexed by the down-regulation of the Cbl receptors, megalin and cubilin, in the kidneys of endotoxaemic mice [65], the kidney being known, as a Cbl homeostatic regulator, to reduce its Cbl uptake in states of Cbl deficiency [66]. Pertinently, megalin is down-regulated via the LPS-induced ERK1/2 signaling pathway [67], through which, as noted earlier, both Cbl and $^{\bullet}$NO achieve their coincidentally regulatory, beneficial effects.

Although the beneficial supply of some extra GSH, as a consequence of thiol Cbl lability, might be proposed as an alternative explanation for early survival protection, it is noteworthy that, at the higher dose, GSCbl showed no early hours protection. Moreover, in a new series of endotoxaemia lethality, *in vivo* survival studies that compared protective effects of *all* endogenous Cbls against those of GSCbl and NAC-Cbl, we observed that though GSCbl/NAC-Cbl still consistently conferred the same early/pre-8-hour protection compared to the 4 other Cbls, paradoxically, both thiol Cbls repeatedly produced poor long-term survival outcomes, equivalent to or worse than LPS-only, whereas HOCbl/CNCbl/MeCbl/AdoCbl all consistently showed significant protection, with CNCbl/MeCbl in particular, producing even better survival results than the current HOCbl-centred study (Brancaleone, Dalli et al. 2011, unpublished data).

GSCbl is certainly known to produce a more rapid early increase in MS activity (together with fourfold greater formation of AdoCbl), when compared to HOCbl [61]. Such an increase in MS activity, normally rapidly deactivated by oxidative stress [68–70], would increase synthesis of the methyl donor, S-adenosylmethionine (SAM), which inhibits LPS-induced gene expression by modulating histone methylation [71]. Whilst this may have initial short-term benefits, as observed, there are also negative long-term consequences from excessive inhibition of the necessary, pro-inflammatory gene expression at this stage. This may be why MS expression and activity are *transiently* decreased by 25% and 30% *early on* in the normal pro-inflammatory phase of the immune response to LPS [72], as well as to allow for GSH synthesis modulation [72].

The consequent, equally paradoxical failure of the other thiol Cbl, NAC-Cbl, to significantly increase survival beyond 8 h, at both high- and low-dose protocols, may also be attributable to the fact that NAC is particularly unstable as a Cbl ligand and may therefore have acted independently of Cbl as NAC alone. Further, though NAC can (1) act as an antioxidant by increasing GSH levels (2) it can equally act as a pro-oxidant [73], increasing disulfides, GSSG, [74] and (3) is counter-indicated in sepsis, since, whilst NAC enhances phagocytosis, it also suppresses the bactericidal respiratory burst in ICU patients, with potential negative outcomes [75].

Indeed, consistent with this independent, paradoxical observation, additional data from our *in vivo* endotoxaemia model shows that in the early 4 h phase of the immune response NAC-Cbl, in contrast to HOCbl/GSCbl, significantly increases circulating PMN, specifically granulocytes, yet decreases the intensity of CD11b ex-pression (see Supplementary data available online at http://dx.doi.org/10.1155/2013/741804 (and Researchgate)). The adhesion molecule/complement receptor, CD11b, is a marker of neutrophil activation, and its high expression normally correlates with a strong respiratory burst [76].

It is conceivable then that such NAC-promoted suppressive effects ultimately outweighed the very early benefits in survival protection conferred by NAC-Cbl.

4.2. Cbl, NF-κB, and iNOS. The initial *in vitro* observation that all major endogenous Cbls do not inhibit NF-κB activation, even at 24 h, may appear surprising, particularly in view of the Cbl beneficial outcomes *in vivo*. However, it has previously been shown *in vivo* that early inhibition of NF-κB in immune challenge increases and prolongs inflammation, and that persisting late NF-κB activation (24/48 h) permits its resolution [77]. Moreover, this failure to inhibit NF-κB by Cbl during inflammation, with positive outcomes, has now also been observed by a group who were aware of our findings [57] and, independently, in a Cbl cancer model by Marguerite et al. (2012, Marc Marten personal communication to Wheatley).

Further, since activation of NF-κB is linked to induction of iNOS, and since inadequately low levels of $^{\bullet}$NO—and, indeed, iNOS gene knockout in mice [78]—and NOS

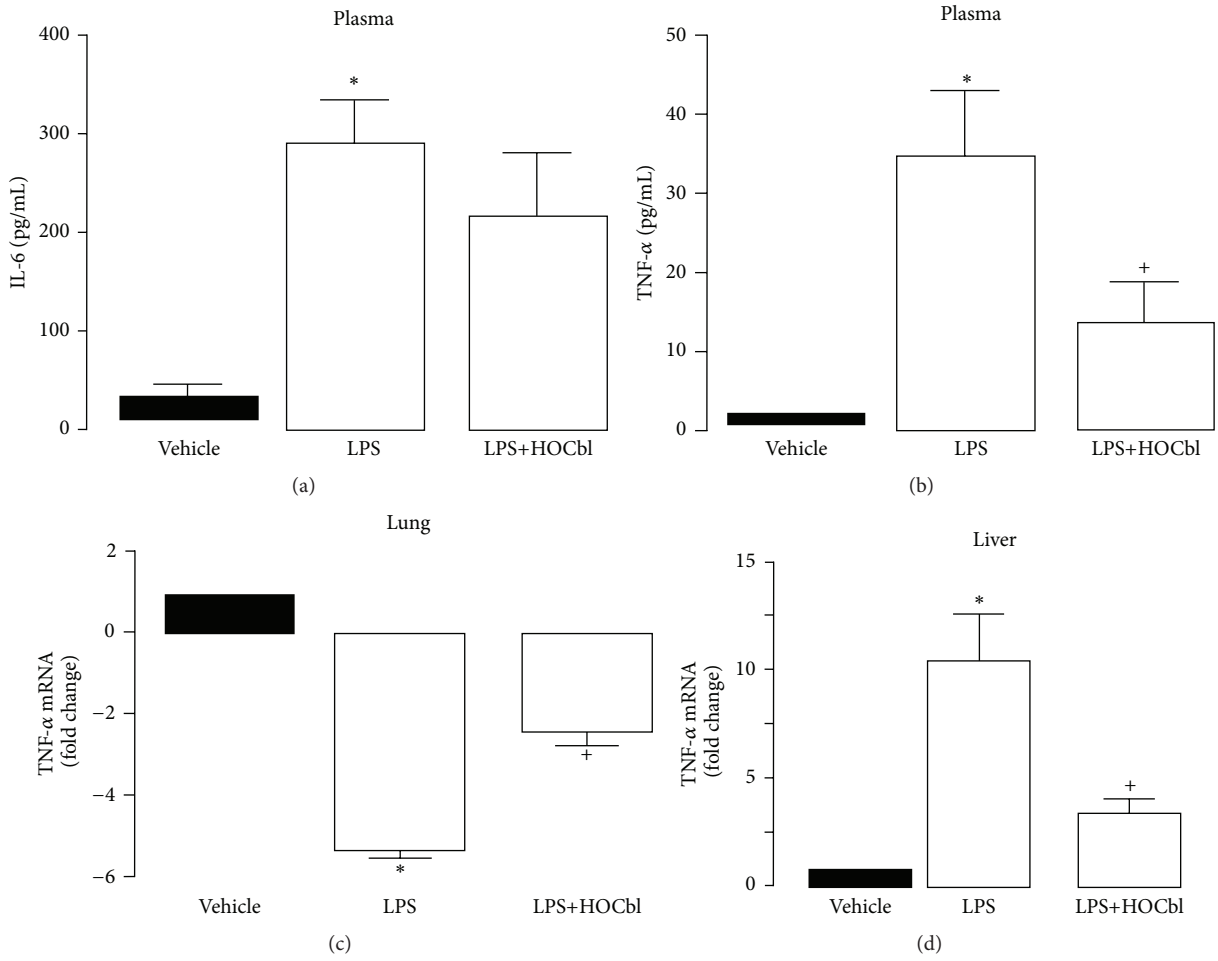

Figure 8: Selective effects of HOCbl on IL-6 and TNF-alpha at 4 h following LPS-induced endotoxaemia. Mice ($n = 5$) were treated with HOCbl, at the low dose protocol (0.2 mg/kg i.p.) at −1 h, +1 h, and +2 h and compared to LPS (0.1 mg/kg i.p. at time 0). Organs (lung and liver) were harvested at 4 h subsequent to LPS, and gene expression quantified in tissue extracts by real-time PCR, using GAPDH and RPL32 as internal standards. ELISA was used to measure plasma levels of IL-6 (a) and TNF-α (b). Tissue expression of TNF-α mRNA in lung (c) and liver (d) extracts. Data are a mean ± SEM of 5 mice per group. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS group.

inhibitors in clinical trials [79, 80] have been implicated in sepsis morbidity and mortality [81], we adopted the hypothesis that NOS translation may, in contradiction to the common view, actually be depressed in sepsis and NOS catalytic activity "uncoupled" or malfunctioning. Previously observed high $^{\bullet}$NO in sepsis is believed to comprise a greater ratio of the more toxic $^{\bullet}$NO species, such as peroxynitrite, ONOO- [82], which inhibits eNOS [83], as opposed to more antioxidant/cytotoxic $^{\bullet}$NO forms, such as *S*-nitrosothiols/GSNO [84, 85] or $^{\bullet}$NO itself; although overhigh levels of GSNO also have negative effects in sepsis-like inflammation [85]. Since Cbl is known to promote GSH [3, 86–88], whose synthesis is induced simultaneously with that of iNOS [89], it should theoretically alter the ratio of GSNO/$^{\bullet}$NO to ONOO- and related species [43], so that the more positive actions of $^{\bullet}$NO predominate [42] (Figure 10). Therefore, we predicted that Cbl would not inhibit iNOS expression and translation early on. This proved correct, with significant HOCbl (and GSCbl/NAC-Cbl) early promotion of both iNOS and eNOS proteins and significantly lower LPS-only iNOS/eNOS protein. Since eNOS is known to be depressed in sepsis, with adverse cardiovascular consequences [90], this early effect of Cbl may have positive clinical implications.

There is an apparent contradiction in the data showing relatively low iNOS/eNOS mRNA leading to strikingly high protein translation in the LPS+HOCbl treated animals, in direct contrast to the LPS-only group, where strikingly high iNOS/eNOS mRNA yielded much lower levels of iNOS/eNOS protein. That this is not an artefact, but a specific Cbl effect, also observed by others [47], is seen by the comparable inverse results achieved with the two, thiol Cbls. (These collective Cbl results were also mirrored by decreasing nitrite/NOS activity, in inverse proportion to the ascending levels of HOCbl/GSCbl/NAC-Cbl iNOS protein.)

We propose that this paradox may actually be an index of Cbl/NOS regulation in endotoxaemia and that the high mRNA levels in LPS-only animals may be due to the observed phenomenon of "relaxed control of RNA synthesis" when

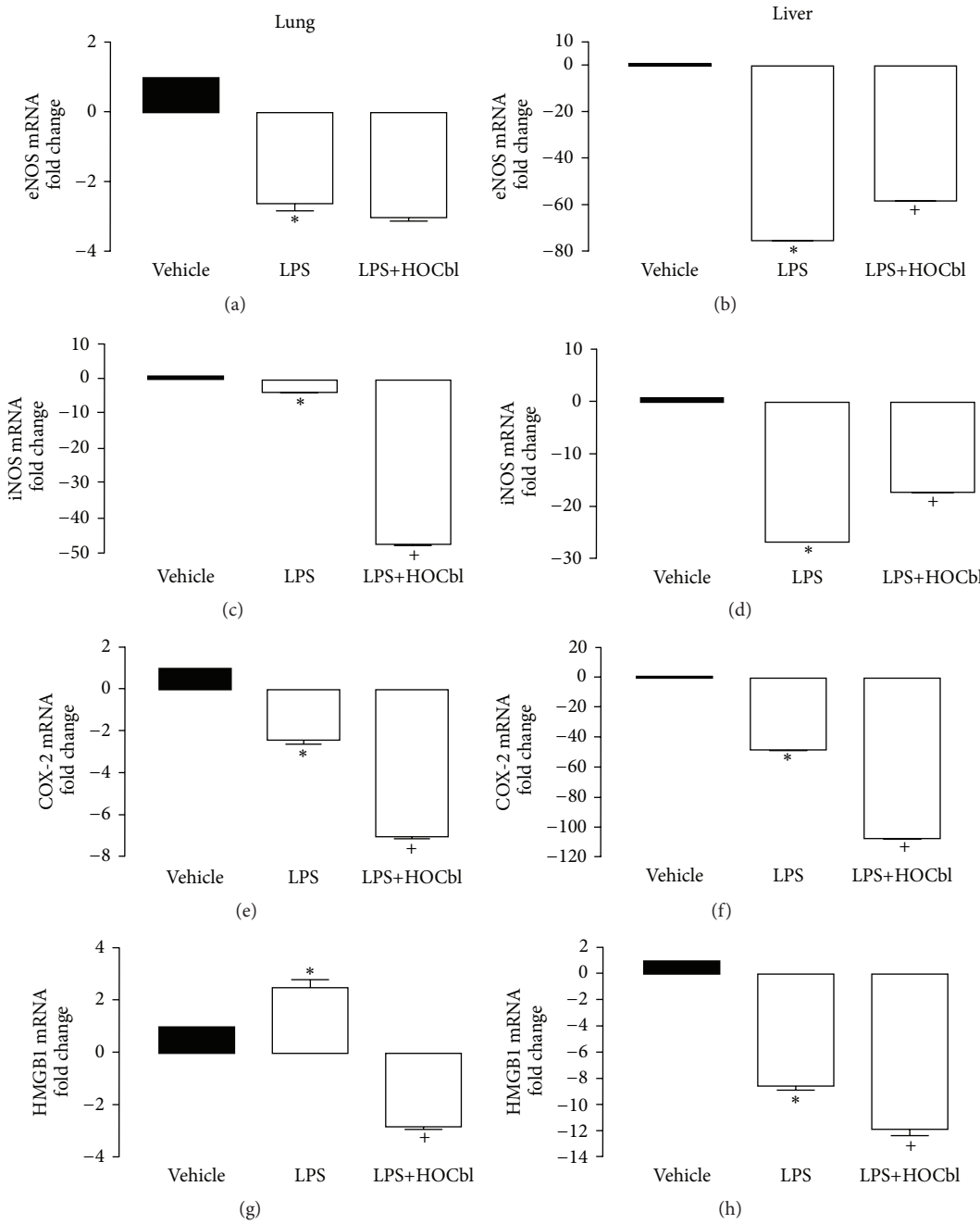

Figure 9: HOCbl modulates eNOS/iNOS, COX-2, HMGB1 gene expression in lung and liver at 24 h subsequent to LPS-induced endotoxaemia. Mice ($n = 5$) were treated with HOCbl (0.2 mg/kg i.p low dose regimen (Table 1)) and compared to LPS 0.1 mg/kg (given i.p. at time 0 h). Organs (lung and liver) were harvested at 24 h after LPS and gene expression was quantified in tissue extracts by real-time PCR, using GAPDH and RPL32 as internal standards, and PBS-injected group set as 1. ((a), (b)) eNOS mRNA data; ((c), (d)) iNOS mRNA data; ((e), (f)) COX-2 mRNA data; ((g), (h)) HMGB1 mRNA data. Values are a mean ± SEM of triplicate observations. $^{*}P < 0.05$ versus vehicle (PBS-treated) control; $^{+}P < 0.05$ versus LPS group.

SAM/methyl groups are deficient [92], as in folate or Cbl deficiency [93], thus consequential on the functional Cbl deficiency of endotoxaemia/sepsis, and more permanent MS inactivation by LPS, discussed earlier. Furthermore, it is theoretically possible that abnormal cell function in sepsis may result in much of the mRNA produced by the LPS-only group being "masked" and unavailable for efficient translation. It is possible also that the translated protein may be unstable and degrade at a faster rate. This is certainly known to be the case with eNOS mRNA in hypoxia [94] and in the presence of high TNF-α [95], both characteristic of sepsis. In contrast, given Cbl's impact on

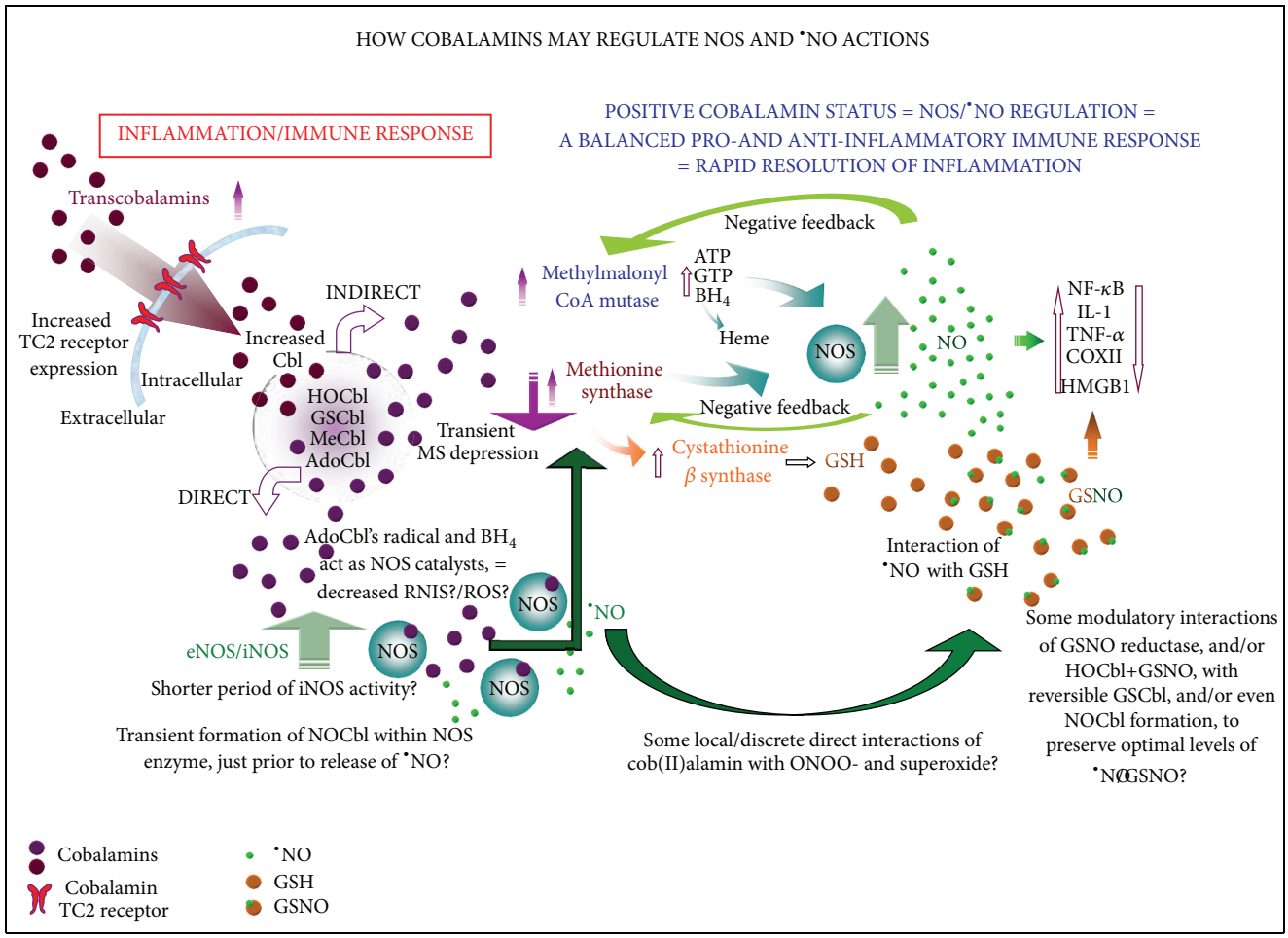

Figure 10: Hypothetical scheme showing how cobalamins may modulate the NOS/•NO species, both indirectly and directly, with downstream effects, during the course of the immune response. Immune challenge upregulates—via the AP-1/Ras signalling pathway—the circulating Cbl carrier proteins, haptocorrins, /HC-TCI & III (which, when desialylated, are taken up by the hepatic Ashwell receptor, thus transporting more MeCbl to the liver for increased synthesis of acute phase proteins). At the same time, the Cbl tissue carrier proteins, the transcobalamins, /TCII are also upregulated, as a result of crosstalk between the transcription factors, NF-κB and Sp-1, the TCII promoter. The increased supply of intracellular Cbl is partly converted to the active cofactors, AdoCbl and MeCbl, in a high AdoCbl to low MeCbl ratio. When methionine synthase/MS, along with SAM synthesis, is initially decreased by LPS challenge in activated monocytes/macrophages, methylmalonyl CoA mutase/MU activity takes precedence [91], triggering increased expression of TCII cell receptors. Part of the consequent extra AdoCbl synthesised may then be directed towards NOS catalysis. A combined proportional increase in activity of MU and, later, MS ensures that all the necessary components for NOS synthesis/assembly, substrates and cofactors, are in plentiful supply. This prevents excess generation of ROS and RNIS during NOS catalysis. These are further decreased because part of the •NO produced may combine with GSH, or other thiols, to form the more beneficial, antioxidant *S*-nitrosothiols/*S*-NOS. •NO is then released, as needed, in targeted amounts, by the action of GSH-dependent GSNO reductase and/or possibly by interaction with HOCbl, yielding GSCbl and •NO. Increasing levels of GSNO/•NO rapidly downregulate IL-1β, COX-2, TNF-α, HMGB1 expression, and, at the conclusion of the second, anti-inflammatory phase of the immune response also inhibit iNOS, NF-κB, and down-regulate MU, MS, and CBS. The lower right part of the scheme shows a possible direct catalytic regulation of iNOS by AdoCbl's lower axial ligand base, the dimethylbenzimidazole (DMBI), and the adenosyl radical, generated after NOS enzyme-induced homolysis of the Co-C bond, which would reduce formation of RNIS/ROS, increase the ratio of •NO/GSNO to ONOO- and related species, and make NOS catalysis more productive, thereby lessening the duration of iNOS activity and ensuring the beneficial effects of •NO predominate (see [43]).

the two coenzymes, MS and MU, upon Cbl treatment, the septic cell should afford a degree of metabolic normality and thus economic efficiency in transcription/translation.

A remarkable study, over half a century ago, demonstrated that Cbl is capable of reactivating a diversity of key enzymes after acute oxidant stress, most of them also negatively affected in sepsis, including glucose-6-phosphate-dehydrogenase, lactate dehydrogenase, lysine, ornithine, and glutamic decarboxylase [96]. This last is critical for the supply of alpha-ketoglutarate in the Krebs cycle, which is depressed in sepsis, with consequent lower ATP production that is associated clinically with increased mortality [97]. In turn,

supply of alpha-ketoglutarate determines the availability of L-arginine, glutamate, and glutamine, all of which also decrease in sepsis, with adverse consequences, in the case of L-arginine especially for the NOS [98]. Observed low levels of L-arginine in sepsis [99] are associated with increased reactive nitrogen species, including high ONOO-, and reactive oxygen species production during NOS catalysis [98], interfering with the normal, more beneficial $^{\bullet}$NO cell signalling, necessary for the efficient resolution of the pro-inflammatory phase of the immune response.

4.3. HOCbl Reciprocal Regulation of iNOS/NO and TNF-α. iNOS-derived $^{\bullet}$NO is known to have a direct regulatory correlation to levels of TNF-α [100, 101]. This iNOS/$^{\bullet}$NO/TNF-α regulation seems operative here with HOCbl treatment, not LPS-only. Since HOCbl permitted a moderate rise in $^{\bullet}$NO (at least, as measured by NOS nitrite end-products), in tandem with moderate levels of TNF-α mRNA and protein, and since Cbl status has an inverse relation to TNF-α levels [15], it seems reasonable to conclude that such Cbl/TNF-α regulation occurs downstream of HOCbl/NOS/$^{\bullet}$NO regulation. Additional evidence for this in our studies may be seen even in the resolution phase of the immune response, where HOCbl-related levels of iNOS expression showed a direct correlation to those of TNF-α, even to the degree of inhibition, in both lung and liver tissues (Figures 9(c) and 9(d)/Table 3). Importantly, however, in the early pro-inflammatory phase HOCbl iNOS/$^{\bullet}$NO regulation does not completely inhibit TNF-α: 50% reduction being observed in our experiments. This is a critical point as anti-TNF-α mAb treatment increases sepsis mortality in the clinic, since some degree of TNF-α production and consequent early pro-inflammatory signalling is essential for an effective immune response [102].

4.4. HOCbl Regulation of IL-6, IL-1β, and Cox-2. Also noteworthy, and crucially consistent with such HOCbl/iNOS/TNF-α regulation, is HOCbl's apparently selective failure to inhibit IL-6 early on. IL-6 is essential for induction of acute phase proteins, whilst simultaneously also decreasing pro-inflammatory cytokines and increasing anti-inflammatory factors [103]. IL-6 regulation of pro-inflammatory factors includes regulation of TNF-α and IL-1β [104], expression of the latter also determining that of Cox-2, involved in arachidonic acid-derived prostaglandin and leukotriene synthesis [105]. IL-1β is rapidly expressed (~15 min post LPS) [106], whereas iNOS, which may be induced by IL-1β, is not fully expressed until 6 h after LPS [107].

We have observed that treatment with high doses of endogenous Cbls (HOCbl/GSCbl) promotes iNOS mRNA expression as early as 2 h following LPS (unpublished data), possibly fast forwarding the immune response. This, together with the HOCbl-promoted high NOS protein, controlled rise in $^{\bullet}$NO synthesis, and thence a moderate production of TNF-α/IL-6 may form a feedback loop accounting for the tight HOCbl regulation of IL-1β, and consequently also Cox-2, as seen at 4 h after LPS (Figure 10 scheme).

4.5. HOCbl Inhibition of Late HMGB1 Gene Expression. Cobalamin-promoted NOS/$^{\bullet}$NO early regulation of TNF-α/IL-6/IL-1β/Cox-2 seems also to be consistent with, and accounts for, the later inhibition of HMGB1 mRNA. If expressed at >18 h and then released extracellularly, HMGB1 can trigger further late release of TNF-α, IL-1β, and inflammatory products from COX-2, iNOS, and excessive ROS and RNI species, leading to pathology [108]. It is known that the nervous system can modulate circulating TNF-α levels *via* release of acetylcholine by the vagus nerve [109]. But our studies show that Cbl—essential for acetylcholine synthesis [110]—is the first known endogenous inhibitor of late HMGB1 mRNA expression. Both nicotine and ethyl-pyruvate have been used to block extracellular release of HMGB1 [109] but, in addition to the fact that HOCbl appears to impact on HMGB1 much further upstream, at least in tissues, neither drug is endowed with the safety profile of Cbl [111] and, more pertinently, neither is known to exert such a central, endogenous regulation of the immune response. Further Cbl/sepsis studies should include measurement of plasma HMGB1 levels. But, on the evidence of the general anti-inflammatory regulation observed in our studies, we predict that extracellular release of HMGB1, from macrophages and PMN, should be negligible with HOCbl treatment.

4.6. A New Paradigm for the Cbl/NOS/$^{\bullet}$NO Relationship? The theory that Cbl may impact on the NOS indirectly, through the contribution of its two known mammalian coenzymatic functions to NOS substrate and cofactor assembly and, indeed, to assembly of the NOS protein itself [39], may further explain our findings, including the Cbl-promoted high NOS protein (Figure 10 scheme). Furthermore, a deficiency of any of the NOS substrates and cofactors (the likely result of Cbl functional deficiency in endotoxaemia) is known to result in less tightly "coupled" NOS activity and increased free radical generation [112, 113], with a corollary increase in inflammatory mediators and prolonged period of NOS activity, indexed by our observed higher LPS-only NOS nitrite levels.

(In a forthcoming study we will also analyse more exactly how Cbl may shift the ratio of $^{\bullet}$NO/GSNO/ONOO and related species).

It may also be that Cbl, as AdoCbl and its radical, takes a *direct*, active part in NOS catalysis, as a third mammalian Cbl cofactor [43]. From this perspective, the high NOS protein seen with high Cbl administration may be a classical instance of the cofactor promoting coenzyme assembly. Such a central, direct Cbl/NOS, catalytic interaction would further reduce excess production of toxic forms of $^{\bullet}$NO, as well as superoxide and other related ROS and RNI species. The consequent, more precise, pro- and anti-inflammatory signalling should again result in a shorter, more effective period of NOS activity, thus lower detectable nitrite levels (as seen) with the beneficial signalling and antioxidant effects of $^{\bullet}$NO predominant. However, direct Cbl scavenger interactions, in discrete intracellular compartments, with primarily toxic RNIS, such as ONOO-/ONOOH/NO_2, with which Cbl interacts *ex vivo*, [114], may also play a part and cannot be

ruled out as contributing to a more complex picture behind our observed results (Figure 10).

5. Conclusions

These novel observations on the mechanism behind cobalamin protection in endotoxaemia suggest that we may be looking at the ideal natural, selective and collective regulator of the NOS, and thence of cytokines and other pivotal factors, in immune challenge and sepsis. In fact, it is now accepted that anti-inflammatory therapies (based on blocking a specific mediator) fail *toutcourt* in sepsis and that a more *modulatory* approach, which regulates the homeostatic inflammatory response, (in itself beneficial), could be successful. Thus, our findings may have significant clinical implications, not only for the treatment of sepsis, but also for other analogous inflammation-driven conditions, such as cancer and malaria, where NOS/$^{\bullet}$NO deregulation, and consequent loss of control over key inflammatory mediators, are equally pathogenic [115, 116].

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

The authors Mauro Perretti and Carmen Wheatley share senior authorship.

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Review Article

Peroxisome Proliferator-Activated Receptor Targets for the Treatment of Metabolic Diseases

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Metabolic syndrome is estimated to affect more than one in five adults, and its prevalence is growing in the adult and pediatric populations. The most widely recognized metabolic risk factors are atherogenic dyslipidemia, elevated blood pressure, and elevated plasma glucose. Individuals with these characteristics commonly manifest a prothrombotic state and a proinflammatory state as well. Peroxisome proliferator-activated receptors (PPARs) may serve as potential therapeutic targets for treating the metabolic syndrome and its related risk factors. The PPARs are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily. So far, three isoforms of PPARs have been identified, namely, PPAR-α, PPAR-β/δ, and PPAR-γ. Various endogenous and exogenous ligands of PPARs have been identified. PPAR-α and PPAR-γ are mainly involved in regulating lipid metabolism, insulin sensitivity, and glucose homeostasis, and their agonists are used in the treatment of hyperlipidemia and T2DM. Whereas PPAR-β/δ function is to regulate lipid metabolism, glucose homeostasis, anti-inflammation, and fatty acid oxidation and its agonists are used in the treatment of metabolic syndrome and cardiovascular diseases. This review mainly focuses on the biological role of PPARs in gene regulation and metabolic diseases, with particular focus on the therapeutic potential of PPAR modulators in the treatment of thrombosis.

1. Introduction

According to World Health Organization global status reports, 80% of the 347 million people with diabetes globally will die of cardiovascular disease [1], and it will be the 7th leading cause of death in 2030 [2]. Moreover, International Diabetes Federation (IDF) predicts that people with metabolic syndrome have a fivefold greater risk of developing type 2 diabetes mellitus (T2DM). Until now a quarter of the world's adults has metabolic syndrome, and it is becoming more common due to a rise in obesity. In the future, it may overtake smoking as the leading risk factor for heart disease. To define, metabolic syndrome is a cluster of metabolic abnormalities which includes hyperlipidemia (elevated triglycerides (TG), low serum high-density lipoprotein (HDL) cholesterol), hypertension, central obesity, and elevated blood glucose [3]. However, the risk of developing metabolic syndrome is closely linked to overweight, obesity, and lack of physical activity. Furthermore, insulin resistance also may raise the risk for metabolic syndrome. Accumulating data reveals that the prevalence of this syndrome within individual cohorts varies with age, gender, and ethnicity. The pathological factors responsible for this syndrome can be high waist measurement of 35 inches or more for women or 40 inches or more for men, a high triglyceride level of 150 mg/dL or higher (The mg/dL is milligrams per deciliter—the units used to measure triglycerides, cholesterol, and blood sugar.), and a low HDL cholesterol level sometimes called as "good" cholesterol because it helps to remove cholesterol from arteries. An HDL cholesterol level of less than 50 mg/dL for women and less than 40 mg/dL for men is a risk factor, and also high blood pressure of 130/85 mmHg or (The mmHg

is millimeters of mercury—the units used to measure blood pressure) high fasting blood sugar level between 100 and 125 mg/dL is considered prediabetes. A fasting blood sugar level of 126 mg/dL or higher is considered diabetes. A fasting blood sugar level of 100 mg/dL or higher (or being on medicine to treat high blood sugar) is a metabolic risk factor.

From investigations, metabolic syndrome has been found to be associated with a greater risk of coronary heart disease, stroke, diabetes, and cardiovascular mortality than the risk conferred by each one of its individual components [4]. Even though the etiology of metabolic syndrome has not yet established definitely, but growing incidences and pathetic states raise an alert to reduce the risk factors of metabolic syndrome through better lifestyle and the treatment with better therapeutics which are the cornerstones for the management of metabolic syndrome. During the last decade, it has been shown that pharmacological activations of peroxisome proliferator-activated receptors (PPARs) are effective therapeutic approaches to correct some aspects of metabolic syndrome mainly hypertriglyceridemia (fibrates) and type 2 diabetes mellitus (thiazolidinediones) [5].

2. Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptor (PPAR) is a subfamily of nuclear hormone receptors [6–8], that function as ligand-activated transcription factors to regulate various biological processes. Peroxisome proliferation was first reported in rats treated with clofibrate in the 1960s [9]. Later on, a number of compounds were discovered which share the same characteristic of inducing peroxisome proliferation. Thus, they were named as peroxisome proliferators. In 1990, the first receptor for these compounds was cloned from mouse liver and named it as peroxisome proliferator-activated receptor (PPAR) [10]. Shortly, it was realized that PPARs in fact represent a group of three receptors PPAR-α, PPAR-β/δ, and PPAR-γ [11]. All PPAR isoforms function mainly as transcription factors [12]. They control and regulate the expression of large number of genes involved in regulating the intermediary metabolism of glucose and lipids, homeostasis, adipogenesis, insulin sensitivity, immune response, cell growth, and differentiation [6, 12–15]. A variety of endogenous and synthetic ligands for PPARs have been identified [16], activation of PPARs by a suitable ligand will result in the recruitment of coactivators and loss of co-repressors that remodel chromatin and activate gene transcription. Furthermore its diverse distribution in tissue has been shown to have multiple functions upon activation such as adipogenesis, fatty acid oxidation, and anti-inflammation [17].

During the last decade it has been extensively demonstrated that risk factors of metabolic syndrome often associated with obesity, characterized by macrophage infiltration and activation in adipose tissue and liver can be treated by PPARS targets [18]. In fact, inflammation a major determinant of health complications seen in overweight and obesity, which underscores the link between nutrition, metabolic organs, and the immune system, can be regulated by PPARs targets [19–21]. This review focuses on the characterization of PPARs family, mechanism of action, ligand selectivity, and physiologic role of the PPAR family and then discusses the understanding of the pathogenic roles of metabolic syndrome and its treatment with PPARs agonists, with particular focus on the therapeutic potentials of PPAR modulators in the treatment of thrombosis.

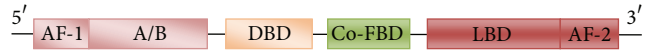

Figure 1: Schematic representation of the functional domains of PPARs. The PPARs are composed of four different domains. The A/B domain is located in the N-terminal region with AF-1 which is responsible for phosphorylation, C domain is involved in DNA binding domain, the D domain is the region of cofactors coupling and E/F domain is the specific domain for the ligands, associated with E/F domain is AF-2, which promotes the recruitment of cofactors necessary for the process of gene transcription.

2.1. Mechanisms of Action of PPARs. All three isoforms of PPAR possess similar structural and functional features. Principally, four functional domains have been identified in PPARs, called A/B, C, D, and E/F (Figure 1). The N-terminal A/B domain contains a ligand-independent activation function 1 (AF-1) [22], responsible for the phosphorylation of PPAR. The DNA binding domain (DBD) or C domain promotes the binding of PPAR to the peroxisome proliferation response element (PPRE) in the promoter region of target genes [23]. The D domain or co-FBD is a site for cofactors coupling. The E/F domain or ligand-binding domain (LBD) is responsible for ligand specificity and activation of PPAR binding to the PPRE, which increases the expression of targeted genes. Recruitment of PPAR cofactors is to assist the gene transcription processes carried out by the ligand-dependent activation function 2 (AF-2), which is located in the E/F domain [24].

Substantial progress has been made to delineate the molecular mechanisms that mediate PPAR-regulated gene expression and associated cellular functions. In the classical model of PPAR activation, PPAR with RXR nuclear receptor is heterodimerized with PPRE termed DR-1, which consists of direct repeats of AGGTCA separated by a single intervening nucleotide [25, 26]. Activation of transcription through this dimer is blocked by associated corepressor proteins, such as nuclear receptor corepressors (NCoR), histone deacetylases (HDAC), and G-protein pathway suppressor 2 (GPS2) [27, 28]. The addition of ligand causes dissociation of the corepressor proteins followed by the recruitment of coactivators such as PPAR coactivator (PGC-1), the histone acetyltransferase p300, CREB binding protein (CBP), and steroid receptor coactivator (SRC)-1. Formation of the PPAR activation complex leads to histone modification (e.g., through acetylation) and altered expression of genes involved in fatty acid metabolism, lipid homeostasis, and adipocyte differentiation [29, 30]. Like PPARs, RXRs exist in three different isoforms, resulting in different combinations of heterodimers. The formation of different heterodimers seems to influence promoter recognition on the target gene sequences resulting in various metabolic processes. Furthermore, activation of

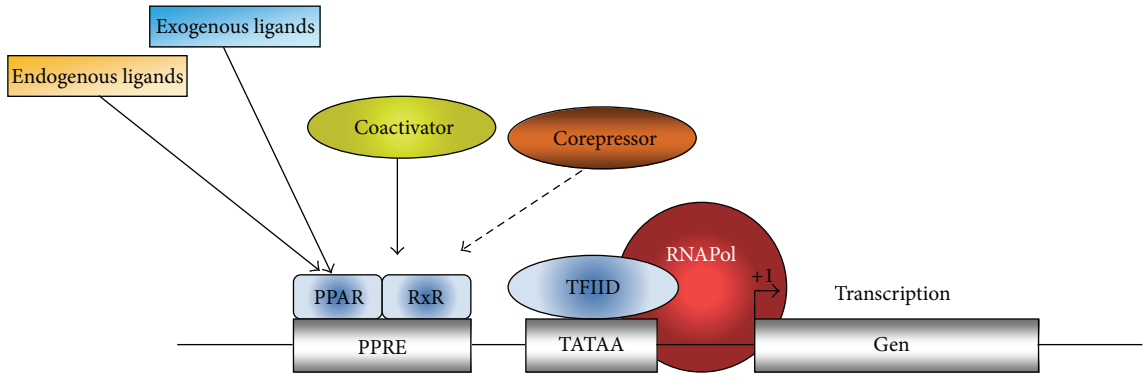

Figure 2: Gene transcription mechanisms of PPAR. In inactivated state, PPAR interacts with the corepressor, and this complex has Histone deacetylase activity, thus inhibiting the transcription process. After the binding of the exogenous ligand (drug) or endogenous ligand (fatty acids, prostaglandins, etc.), with the PPAR it is activated and it heterodimerizes with RXR and recruits coactivators, which have histone acetylase activity facilitating the transcription of several genes.

PPARs by natural and synthetic ligands, other factors such as RXR, PPREs and cofactors also play a fundamental role in the process of desired transcription. The mechanisms by which activated PPARs initiate gene transcription are illustrated in Figure 2.

Recent studies of new millennium reveal new ways to activate PPARs and affect physiology. Although PPAR is commonly parked on DR-1 with RXR waiting for a ligand to activate it, there appear to be other modes of PPAR action. As with other nuclear receptors, heat shock proteins (HSP) may facilitate the folding of newly translated PPARs. The association of PPAR with Hsps, and perhaps other proteins, may keep PPAR in the cytoplasm until the appropriate ligand binds the PPAR in LBD, leading to protein dissociation and nuclear import of PPAR. In an even simpler scenario, PPAR may remain soluble, presumably in the nucleus. Ligand binding then facilitates the heterodimerization of PPAR with RXR. Another pattern for PPAR action involves its heterodimerization with a nuclear receptor other than RXR, such as the thyroid hormone receptor (THR). In these cases, the DNA pattern recognized by the heterodimer may vary from the usual DR-1 pattern. Finally, activation of PPAR by its ligand may allow it to associate with other transcription factors, such as p65 or c-jun. The binding of PPAR with p65 will prevent completion of signaling through the NF-κB pathway, and binding with c-jun will interfere with AP-1 signaling pathway.

3. Ligands for PPAR Isoforms

From elucidated crystal structure studies of PPAR, the divergent amino acid sequence in the LBD of the three PPAR isoforms is thought to provide the molecular basis for ligand selectivity. And a large ligand-binding pocket (1300 Å) is found to exist in all three PPAR isoforms, allowing diverse and structurally distinct compounds access to the LBD [31] and enabling PPAR to sense a broad range of endogenous and exogenous compounds. A variety of endogenous and exogenous compounds, including fatty acids and their metabolites [32], industrial chemicals such as herbicides and plasticizers as well as synthetic pharmaceutical agents have been shown to bind to activate PPAR [33]. These ligand activated PPARs regulate metabolic activities leading to FA catabolism, lipid storage, glucose metabolism, cardiovascular risks and other effects, such as those affecting inflammation [34–36]. A variety of ligands, including n-3 and n-6 fatty acids (FAs), eicosanoids, and a few endocannabinoids and phospholipids, have been identified as PPAR endogenous ligands. Although many fatty acids are capable of activating all three PPAR isoforms [37, 38], some preference for specific fatty acids by each PPAR has been demonstrated. Table 1 shows fatty acids and their derivatives, including 8-hydroxyeicosatetraenoic acid, the arachidonic acid lipoxygenase metabolite LTB4, and arachidonate monooxygenase metabolite epoxyeicosatrienoic acids which have been shown to potently activate PPAR-α [33, 37, 39]. Synthetic lipid-lowering drug fibrates and clofibrates are also potent ligands for activating PPAR-α and are clinically proved to be lipid lowering drugs [40–42]. Endogenous arachidonic acid cyclooxygenase metabolite prostacyclin, the linoleic acid 15-lipoxygenase-1 product 13-S-hydroxyoctadecadienoic acid and synthetic compounds including L-165041 and GW2433 have been found to be selective PPAR-β/-δ ligands [43, 44]. Naturally occurring PPAR-γ ligands including 15-deoxy-(12,14)-prostaglandin J2 [45] and oxidized metabolites of linoleic acid 9-hydroxy- and 13-hydroxy-octadecadienoic acids have been identified [46, 47]. Furthermore, synthetic antidiabetic TZD including rosiglitazone (Avandia) and pioglitazone (ACTOS) are potent PPAR-γ selective agonists and have been very effective in improving glycemic control via insulin sensitization (see Table 1). Interestingly, recent investigations reveal the existence of PPARs isoforms even in human platelets. This on upregulation of these PPARs inhibits platelet activation through nongenomic mechanisms [48]. Furthermore, the most crucial factor noticed is that the activity of these ligands depends on their presence in cells or tissues enriched in PPARs, their binding specificity toward the different PPARs and the availability of coregulators that can act either as coactivators or corepressors of transcription. Given the variety and anatomic distribution of endogenous

Table 1: Potential targets of PPARs for prevention and treatment of metabolic syndrome.

Ligands	Receptor	Selective	Function
Saturated Fatty acid	PPAR-α (liver, adipose tissue, kidney, heart, skeletal, muscle, large intestine)	Endogenous lipid with PPAR-α agonist active	Lipid and glucose metabolism
Unsaturated Fatty acid		Endogenous lipid with PPAR-α agonist active	Lipid and glucose metabolism
CP775146		Selective, high affinity PPAR-α agonist	Lipid and glucose metabolism
Fenofibrate		PPAR-α agonist	Treatment of hypertriglyceridemia and dyslipidemia
GW7647		Highly selective, potent PPAR-α agonist. Orally active	Lipid homeostasis, beta oxidation
Oleylethanolamide		PPAR-α agonist	Lowers body weight and hyperlipidemia
Palmitoylethanolamide		Endogenous lipid with PPAR-α agonist active	Anti-inflammatory, reduces pain
WY14643		Selective PPAR-α agonist	Atherosclerosis and anti-inflammation and prevent hyperinsulinemia
GW6471		Selective PPAR-α antagonist	Antagonist fenofibrates
MK886		Selective PPAR-α antagonist	Inhibit PPAR-α, β, γ activities
Fatty acids (i) Docahexanoic acid (ii) Arachidonic acid (iii) Linolenic acid	PPAR-β (Ubiquitous)	Endogenous lipid with PPAR-β agonist active	Anti-inflammation
GW501516		Highly selective, potent PPAR-β agonist	Increases serum HDL-c in atherogenic dyslipidemia and decreases fasting blood sugar.
GW0742		Potent PPAR-β agonist	Anti-inflammatory
L-165,041		PPAR-β selective agonist	Hyperlipidemia, hyperglicemia, ateroesclerosis, and obesity
GW610742		PPAR-β selective agonist	Treatment diabetic and nephropathy
FH535		PPAR-β selective antagonist	Treatment diabetic and nephropathy
GSK0660		PPAR-β selective antagonist	Inverse agonist PPAR-β/γ
GSK3787		Potent and selective PPAR-β antagonist	Inverse agonist PPAR-β/γ
NSAIDs		PPAR-β selective antagonist	Cancer
Linolenic acid	PPAR-γ (adipose tissue, lymphoid tissue, colon, liver, heart)	Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
Arachidonic acid		Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
15d-PGJ2		Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
9-HODE		Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
13-HODE		Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
15-HETE		Endogenous lipid with PPAR-γ agonist active	Anti-inflammation
Ciglitazone		Selective PPAR-γ agonist	Inhibits cell proliferation
GW1929 hydrochloride		Selective PPAR-γ. Orally active	Decreases glucose, fatty acids, and triglyceride
LG100754		PXR:PPAR agonist	Sensitizes PPAR-γ
nTZDpa		PPAR-γ selective agonist	Anti diabetic, anti carcinogenic
JTT-501 (isoxazolidinedione)		PPAR-γ selective agonist	Anti diabetic
Pioglitazone hydrochloride		Selective PPAR-γ agonist	Anti diabetic
S26948		Selective PPAR-γ agonist	Anti diabetic
Troglitazone		Selective PPAR-γ agonist	Anti diabetic
FH535		PPAR-γ antagonist	Inhibits Wnt/β-catenin signaling
GSK0660		PPAR-γ antagonist	Inverse agonist PPAR-β/γ
GSK3787		PPAR-γ antagonist	Inverse agonist PPAR-β/γ
BADGE		PPAR-γ selective	Antagonist for roziglitazone
LG-100641		PPAR-γ selective	Blocks TZDs antagonist

PPAR ligands, and the combinations in which they occur depending on physiological (e.g., abundance and composition of food, physical activity) and pathophysiological conditions (e.g., hyperlipidemia, hypertension, diabetes, chronic inflammation, cancer, and atherosclerosis), and it is difficult to carefully evaluate the roles of each PPAR ligand in a given cell at a fixed time-point, and this remains a major challenge in the field of investigation.

But it is tempting to speculate that the diversity of PPAR functions has been acquired in association with the rich variety of ligands. With the development and clinical use of PPAR ligands in the past decade have greatly advanced our understanding of the physiological and pathophysiological roles of PPAR and therapeutic implications of modulating these receptors (see Table 1).

4. PPAR-γ

PPAR-γ is most widely expressed in adipose tissue but is also expressed in immune/inflammatory cells (e.g., monocytes, macrophages), mucosa of the colon and cecum, and the placenta. Its expression is the lowest in skeletal muscle and liver. PPAR-γ is essential for the differentiation and functioning of brown and white adipocytes and promotes the accumulation of lipids in the adipocytes [49]. There are three isoforms of PPAR-γ well identified, namely, PPAR-γ1, PPAR-γ2, and PPAR-γ3 and are derived from the same gene by the use of alternative promoters [50, 51]. PPAR-γ1 differs by the presence of 30 amino acids extras in the N-terminus region. PPAR-γ1 and γ3 RNA transcripts translate into the identical PPAR-γ1 protein. From animal studies, in knock-out mice it was evident that PPARs-γ is specific for adipocytes by expressing hypocellular adipocytes, developing insulin resistance in liver but not in muscles [52]. However, since PPAR-γ expression is tissue dependent, PPAR-γ1 is found in a broad range of tissues; whereas PPAR-γ2 is restricted to adipose tissue and PPAR-γ3 is abundant in macrophages, large intestine, and white adipose tissue [51–54]. Several investigations have elucidated that adipogenesis, glucose homeostasis, and lipid metabolisms are the major mechanisms of PPARs-γ, and it is also involved in the improvement of insulin resistance and plays a key role in inflammation and neoplastic growth [55–59].

4.1. Implication of PPAR-γ Agonist in Lipid Metabolism and Insulin Sensivtity. Thiazolidinediones (TZDs) are known for anti-diabetics (troglitazone, pioglitazone, ciglitazona, and englitazone) (see Table 1). These are a class of synthetic agonists that activate PPAR-γ, whose properties are to improve insulin resistance and lower blood glucose levels in patients with T2DM60. Even with low concentration, PPAR-γ it exhibits its effect in adipose tissue, liver, and muscle [60]. Several clinical studies have evaluated the efficacy of PPAR-γ agonists (troglitazone, pioglitazone, and rosiglitazone) in the management of insulin resistance and T2DM [61–65]. A number of studies have assessed the effects of PPAR-γ agonists in the prevention of onset of T2DM.

Genomics studies have shown that PPAR-γ is associated with several genes that affect insulin action. This receptor causes suppression of insulin action and promotes insulin resistance. In particular, PPAR-γ appears to be associated with genes involved in FA transport, lipid droplet formation, and TAG synthesis and breakdown. Furthermore, activation of PPAR-γ increases adipocyte insulin sensitivity and this may be mediated in part by direct activation of genes encoding factors of the insulin signaling pathway [66, 67]. Synthetic agonists are successful in insulin sensitivity by blocking the interaction of PPAR-γ with co-repressors as a result there is an increase in the accumulation of lipids in the adipose tissue and a decrease in the release of free fatty acids [68]. The benefits of TZDs are attributed to direct effects on lipid metabolism in adipose tissue and secondary effects on lipid and glucose metabolism in liver and skeletal muscle. As elucidated, TZD treatment leads to adipocyte remodeling as a result of selective preadipocyte differentiation in subcutaneous depots and apoptosis of older and larger insulin-resistant visceral adipocytes [69]. The generated new adipocytes are smaller in size and are more sensitive to insulin [70]. Furthermore activation of PPAR-γ by TZDs enhances lipolysis of circulating TGs and their storage in adipose tissue. In addition, there are also numerous studies that show a direct effect of these drugs on the pancreatic β-cells through a reduction of the lipotoxicity on the islets of pancreas, and the mechanism of action is not yet clear, but the transcriptional repressor B-cell lymphoma-6 (BCL-6) was found to play a crucial role in reducing lipotoxicity [71, 72]. However, PPAR-γ activation by TZDs agonists modulates the insulin signal transduction pathway by increasing the expression of intracellular proteins such as c-Cbl-associated protein (CAP), which is predominant in insulin-sensitive tissues and correlates well with insulin sensitvity [73]. Moreover, PPAR-γ triggers an increase in plasma concentrations of adiponectin, a hormone secreted from adipose tissue that is found at low levels in plasma of patients with T2DM. Overall functions of adiponectin are to increase FA oxidation in liver and skeletal muscle, improve insulin sensitivity in skeletal muscle and liver, and decrease glucose production in liver, resulting in decreased circulating FFAs and TG and glucose levels in liver [74]. In addition, PPAR-γ affects insulin sensitivity by regulating adipocyte hormones, cytokines, and proteins that are involved in insulin resistance. In fact, the TZDs agonists are well known to have a greater effect on the secretion of adipokines. Where the action of insulin is suppressed in the adipocytes and macrophages, both produce inflammatory cytokines such as TNF-α and IL-6, and express TLRs. Since free FA levels are elevated in obesity, their effects may be mediated by TLRs which are thought to connect metabolism to innate immunity by secretion of inflammatory cytokines and chemokines. Other adipokines are overregulated, particularly adiponectin and resistin which are known to be potential insulin sensitizers in liver and skeletal muscle [75, 76].

A recent study showed that metabolic syndrome is associated with an increased risk of all-cause cancer mortality in men [77]. A possible explanation for the increased cancer risk connected to metabolic syndrome, elevated nocturnal free-fatty acids and hyperinsulinemia, which were found in obese animals [78]. Additional pathophysiological mechanisms, which link metabolic syndrome to cancer, are elevated

adipokines levels, IGF, the mitogenic action of insulin, and increased levels of reactive oxygen species [79]. This pathological state can be regulated with TZDs agonists (see Table 1).

4.2. Implication of PPAR-γ in Glucose Metabolism. As well known insulin is the most potent physiological anabolic agent, promoting the storage and synthesis of lipids, protein, and carbohydrates and inhibiting their breakdown and release into the circulation [80]. The first step by which insulin increases energy storage or utilization involves the regulated transport of glucose into the cell, mediated by the facilitative glucose transporter Glut4. Insulin increases glucose uptake mainly by enriching the concentration of Glut4 proteins at the plasma membrane, rather than by increasing the intrinsic activity of the transporter [81, 82]. PPAR-γ activation by rosiglitazones was found to increase the expression and translocation of the glucose transporters GLUT1 and GLUT4 to the cell surface, thus increasing glucose uptake in adipocytes and muscle cells and reducing glucose plasma levels [83, 84]. Activation of PPAR-γ by TZDs decreases glycated hemoglobin (HbA1c) and fasting and postprandial glucose and lowers circulating insulin levels in patients with T2DM, largely as a consequence of the improvement in insulin sensitivity. Furthermore, TZDs stimulate the use of glycerol for TG production, thereby reducing FFA release from adipocytes and this reduction in FFAs alleviates lipotoxicity in skeletal muscle, liver, and pancreas, leading to a reduction in hepatic glucose production and improved glucose utilization in skeletal muscles, resulting in the hypoglycemic effects of TZDs. The changes in glucose homeostasis can also be partly attributed to a direct action of PPAR-γ activation on insulin-stimulated glucose disposal. Moreover, T2DM is associated with a state of chronic inflammation of fat cells that secrete elevated levels of cytokines; agonists of PPAR-γ were shown to inhibit the expression of cytokines such as resistin, tumor necrosis factor a (TNFα), and interleukin-6 which promote insulin resistance. PPAR-γ agonists trigger an increase in plasma concentrations of adiponectin, a hormone secreted from adipose tissue that is found at low levels in plasma of patients with T2DM. Adiponectin increases FA oxidation in liver and skeletal muscle. Overall, adiponectin improves insulin sensitivity in skeletal muscle and liver and decreases glucose production in liver, resulting in decreased circulating FFAs and TG and glucose levels [85].

4.3. Implications of PPAR-γ in Atherosclerosis and Inflammation. Interestingly PPAR-γ was the first reported to undergo agonist-dependent simulation, which promotes binding to nuclear receptor co-repressor-1 protein (NCoR) and stabilizes association with promoter-bound NF-κB, thereby leading to the transrepression of inflammatory genes in macrophages [86–88]. Other beneficial and inhibitory effects of PPAR-γ agonists on inflammation were reduction in the production of proinflammatory molecules in T lymphocytes, promotion of the expression of anti-inflammatory mediators in the innate immune system, reduce cytokines (TNF-α, IL-1, and IL-6) production by inhibition of genes encoding pro-inflammatory molecules, and reduction of transcriptional activities Nuclear Factor-κB (NF-κB), AP-1, and STAT [89, 90]. PPAR-γ also reduces vascular smooth muscle cell proliferation, increases monocyte apoptosis, and suppresses metalloproteinase-9 expression in atherotic plaques [91–94]. During atherogenesis PPAR-γ is expressed by monocytes and macrophages, a strategy behind inflammation homeostasis. First, monocytes are attracted to the vascular wall of large arteries by adhering to them through integrins l and integrins endothelial cell activation. Monocytes infiltrate to the subendothelial space by a chemokine gradients, such as IL-8, which originates from the source of infection, where they will be differentiated to macrophages. This key step is already altered in response to activation of PPAR-γ. Thus, troglitazone (TZD) inhibits the early formation of injury atherosclerotic, decreasing the accumulation of macrophages in the intimate through the inhibition of monocyte transendothelial migration. In addition, PPAR-γ agonists may indirectly suppress systemic production of a proinflammatory milieu mainly by inhibiting TNF-α, plasminogen activator inhibitor-1, and IL-6 expression in adipose tissue [95, 96]. Elevated levels of HDL cholesterol and reduced triglyceride levels may also contribute to the beneficial effect of PPAR-γ agonists in atherosclerosis [97]. On the basis of these data, it seems likely that TZD PPAR-γ agonists will have beneficial effects on atherosclerosis and provide a promising therapy for the metabolic syndrome and its cardiovascular complications.

Novel antagonist and partial agonists of PPAR-γ have recently been identified; triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a partial agonist with anti-inflammatory properties [98] and bisphenol diglycidyl ether (BADGE) and LG-100641 have been identified as antagonists for PPAR-γ (see Table 1) [99, 100]. Even though these compounds have little clinical significance, they can be used to understand the physiology of the PPAR-γ and for the identification of new ligands. In addition to synthetic chemical methods, research in natural products has also yielded potent PPAR-γ agonists from several medicinal plants. Saurufuran A from *Saururus chinensis* (Saururaceae) [101], flavonoids such as chrysin, apigenin and kaempferol [102], and phenolic compounds from *Glycyrrhiza uralensis* (Fabaceae) [103] are recently identified PPAR-γ agonists to treat risk factors of metabolic syndrome.

5. PPAR-α

PPAR-α belongs to the nuclear receptor superfamily and was the first to be identified as PPAR receptor. It was named based on the ability to be activated by substances leading to the proliferation of peroxisomes in rodents. PPAR-α is expressed in numerous tissues, both in rodents and in humans, for example, in liver, kidneys, heart, skeletal muscle, and Brown fat [104, 105], and in a wide range of vascular cells such as endothelial cells, vascular smooth muscle cells (VSMCs), and monocytes/macrophages [106–109]. Accumulating evidence demonstrates that PPAR-α is an important modulator of the metabolic syndrome and may be a therapeutic target for treating some of its features, especially cardiovascular complications. PPAR-α has been identified as a key regulator of the genes involved in fatty acid oxidation, which occurs in mitochondria, peroxisomes, and microsomes in the liver

[110]. Transcription and protein levels of critical enzymes in β-oxidation and ω-oxidation pathways are direct targets of PPAR-α, including acyl CoA oxidase, carnitine palmitoyl transferase I, mitochondrial hydroxymethylglutaryl CoA synthase, and cytochrome P450 4A enzymes (CYP4A) [111]. By increasing the expression of these genes, PPAR-α ligands significantly activate hepatic fatty acid oxidation, whereas genetic inactivation of the PPAR-α gene results in massive accumulation of lipids in the liver, severe hypoketonemia, hypoglycemia, hypothermia and elevated plasma free fatty acid levels [112]. These data clearly indicate that PPAR-α is a key factor in governing metabolic adaptation to increased fatty acids. PPAR-α is also known to regulate the expression of genes for the transport of proteins and enzymes that are involved in the processes of inflammation. Many lines of study show that PPAR-α regulates lipid homeostasis. By increasing β-oxidation of fatty acids and providing energy to the cell, it also cuts the long-chain fatty acids, thereby preventing the accumulation and toxicity of lipids in cells [113]. In addition, they also stimulate the cellular uptake of fatty acids by increasing the expression of the fatty acid transport protein (FATP) and fatty acid translocase (FAT) [114].

5.1. Implication of PPAR-α in Lipid Metabolism. PPAR-α plays a critical role in lipid metabolism. Its known target genes are involved in almost all aspects of lipid metabolism, including uptake, binding, and oxidation of fatty acids; lipoprotein assembly; and lipid transport [115, 116]. Synthetic PPAR-α ligands, such as gemfibrozil, fenofibrate, and clofibrate (see Table 1), have been used in clinical practice as hypolipidemic agents for more than 3 decades. Notably, activation of PPAR-α with synthetic agonists not only increases hepatic FAO, but it also increases lipogenesis and FA chain elongation, in a sterol regulatory element binding protein (SREBP)-1c dependent manner. This indicates that PPAR-α induces the entire FA handling regimen in liver to either catabolize it or store it, thereby diminishing cytotoxic effects of free FA. As evident PPAR-α is highly expressed in liver and activation of this receptor promotes the expression of cytochrome P4504A (CYP4A). The CYP4A is a subclass of cytochrome P450 enzyme that catalyzes the ω-hydroxylation of fatty acids [111] which is beneficial in reducing the synthesis of triglycerides (TGs). In addition, PPAR-α agonists are used for the treatment of dyslipidemia, which is characterized by decreased triglycerides levels and increased HDL-c levels in plasma [117]. This state can be achieved by increasing the production of the major component of HDL-c, called Apolipoprotein A-I and A-II (APO A-I & APO A-II) [118, 119] which plays a vital role in reverse cholesterol transport (RCT) pathway from peripheral cells. Moreover, PPAR-α PPAR-activation further decreases TG levels by amplifying the expression of lipoprotein lipase (LPL) [120] and inhibiting APO C-III in the liver. The biological mechanism of PPAR-α is activated under nutrient-deficient conditions and is necessary for the process of ketogenesis, a key adaptive response to prolonged fasting. In an experiment that pharmacologically blocked transport of long-chain fatty acids into mitochondria, knockout mice developed hepatic steatosis, severe hypoketonemia, hypoglycemia, and hypertriglyceridemia [121]. This indicates that genetic inactivation of PPAR-α gene results in massive accumulation of lipids in the liver.

5.2. Implication of PPAR-α in Glucose Metabolism and Insulin Sensitivity. Metformin is most commonly used in the initial management of T2DM, but its mechanisms by which it lowers glucose levels are not completely known. T2DM patients with metformin treatment reduce the production of hepatic glucose via gluconeogenesis decrease. It is believed that metformin exerts its action via incretins, increasing levels of GLP-1 (glucagon-like peptides) via PPAR-α dependent or independent mechanism [122]. Altered PPAR-α has also been implicated in the pathogenesis of obesity and insulin resistance [123]. Activation of PPAR-α reduces weight gain in rodents, and inactivation of PPAR-α results in a late onset of obese phenotype [124, 125]. Treatment of PPAR-α null mice with a high-fat diet leads to a more dramatic increase in body weight [126], further suggesting that PPAR-α may be involved in the genesis of obesity. Evidence has recently emerged suggesting that PPAR-α is an important regulator of insulin sensitivity. Treatment with PPAR-α activators dramatically improved insulin resistance and glycemic control in type 2 diabetic db/db mice and OLETF rats [127–129]. Similarly, the PPAR-α agonist bezafibrate markedly improved glucose intolerance and insulin resistance in a lipoatrophic diabetic patient [130]. More importantly, recently it was reported that the PPAR-α agonist fenofibrate prevents the development of diabetes in insulin-resistant obese OLETF rats. Although the downstream mechanisms underlying these observations are not clear, they are consistent with the idea that PPAR-α plays a critical role in regulating insulin sensitivity *in vivo* and that its activation may lead to the delay of onset of type 2 diabetes [128].

5.3. Atherosclerotic Lesions Effect and Myocardial Regulation. Activation of PPAR-α in endothelial cell receptor will block the cellular adhesion induced by cytokines and increase the expression of the HDL in CLA-1/SR-BI receptor [131] and the cholesterol efflux pump ATP binding cassette A-1 (ABCA1) transporter in macrophages [132]. Some studies suggest that activation of PPAR-α increased the expression of ABCA1 by enhancing the expression of the liver X receptor, LXR-α [133]. Thus, PPAR-α and LXR-α agonists may induce positive effects on atherosclerotic lesions. Finally, fibrate PPAR-α activators have been reported to potently reduce atherosclerosis both in apoE$^{-/-}$ mice and in human ApoAI transgenic apoE$^{-/-}$ mice [134]. In heart, PPAR-α supplies energy to the myocardium by regulating the genes responsible for fatty acid uptake and β-oxidation [135]. This function is achieved by decreasing fatty acid oxidation and inhibiting lipoprotein lipase (LPL) [136]. Consequently all these mechanisms have been reducing progression of atherosclerosis and decreasing the incidence of coronary events in several clinical studies. More important, fibrate treatment of patients who exhibit more than three features characteristic of the metabolic syndrome (diabetes, glucose intolerance or high fasting insulin, hypertension, obesity and high triglycerides or low HDL cholesterol) was associated with a significant 35% risk reduction in the rate of coronary artery disease death, nonfatal myocardial

infarction, or definite stroke [137–139]. These data support the concept that fibrate PPAR-α agonists may be particularly effective agents for the cardiovascular complications of the metabolic syndrome.

5.4. Implication of PPAR-α in Anti-Inflammation. PPAR-α agonist's exhibit anti-inflammatory effect in vascular cells by inhibiting the production of some inflammatory cytokines such as TNF-α, IL-6 in blood, and decreased the expression of cyclooxygenase-2 in VSMCs by NF-κB signaling repression in cardiac myositis [140] and induced expression of VACAM-1 [141] and increased expression of endothelial nitric oxide synthase (eNOS) [142]. Moreover, WY14.643, a potent agonist of PPAR-α, can directly increase the expression of adiponectins, antidiabetics, antiatherosclerosis, and anti-inflammatory effects [143].

6. PPAR-β/-δ

PPAR-β/-δ has important functions in the skin, gut, placenta, skeletal and heart muscles, adipose tissue, and brain [144]. Contrary to PPAR-α and PPAR-γ, PPAR-δ is expressed all over the body, but its pharmacology is less understood than the other subtypes [145]. It is the most abundant isoform among the three PPARs in skeletal muscle. Since skeletal muscle accounts for about 50% of whole body mass, and more than 50% of metabolism occurs in it. Therefore, activities involved in muscle contraction may significantly increase energy expenditure and result in the usage of glucose or breakdown of fat as fuel. PPAR-δ encourages skeletal muscle to burn stored fat as fuel. It can be greatly beneficial because it decreases triglycerides and LDL-cholesterol (bad cholesterol) levels and increases insulin sensitivity and HDL-cholesterol (good cholesterol) levels. Since metabolic syndrome is a problem caused by too much fat stored in the body, PPAR-δ has been recognized to be a possible solution because it makes the body burn more fat. Fat is the main source of fuel for endurance exercise; therefore, PPAR-δ is produced for increasing the breakdown of body fat to generate energy. Indeed, the increase of PPAR-δ helps to lose fat and exercise longer because it facilitates the use of fat. So far reported candidates for endogenous activators of PPAR-β/-δ are fatty acids, triglycerides, and prostacyclin. In addition a number of synthetic compounds including L-165,041, GW501516, GW0742, and GW610742 (see Table 1) [144, 146–149] have also been designed as selective PPAR-β/-δ ligands. Unlike PPAR-α (fibrates) or -γ, (glitazones) there are no PPAR-β/-δ drugs in clinical use yet, though lead compounds such as GW501516 are in phase II clinical trials for dyslipidaemia. As yet only one selective PPAR-β/-δ antagonist has been described GSK0660. In skeletal muscle myoblast cells in culture, GSK0660 inhibits GW0742 induction of established PPAR-β/-δ target genes (carnitine palmitoyltransferase 1A, angiopoietin-like 4 protein, and pyruvate dehydrogenase kinase-4), along with the concurrent PPAR-β/-δ induced increase in fatty acid oxidation [150]. As yet this is the only report on GSK0660, so still nothing is known regarding its long-term effects *in vivo*.

6.1. Implication of PPAR-β/δ in the Regulation of Lipid Metabolism. PPAR-β/-δ is the most abundant isoform among the three PPARs in skeletal muscle; it acts as a central regulator of fatty acid catabolism in skeletal muscle by controlling expression of proteins directly implicated in this metabolic pathway and also by increasing the intrinsic oxidative capability of the tissue. Since skeletal muscle accounts for about 50% of whole body mass and more than 50% of metabolism occurs in it. Therefore, activities involved in muscle contraction may significantly increase energy expenditure and result in the usage of glucose or breakdown of fat as fuel. PPAR-β/-δ encourages skeletal muscle to burn stored fat as fuel. And this is greatly beneficial because it decreases triglycerides and LDL-cholesterol (bad cholesterol) levels and increases insulin sensitivity and HDL-cholesterol (good cholesterol) levels. Since metabolic syndrome is a problem caused by too much fat stored in the body, PPAR-β/-δ has been recognized to be a possible solution because it makes the body burn more fat. Since fat is the main source of fuel for endurance exercise; therefore, PPAR-β/-δ is produced for increasing the breakdown of body fat to generate energy. Indeed, the increase of PPAR-β/-δ helps to lose fat and exercise longer because it facilitates the use of fat. Synthetic PPAR-β/-δ ligands are considered as effective compounds to improve metabolic syndrome. An experimental study using obese diabetic db/db mice as a model was examined to see the effect of a PPAR-β/-δ specific agonist L-165041 on plasma lipid profile [151]. L-165041 treatment significantly increases HDL cholesterol levels, possibly associated with a decreased lipoprotein lipase activity in the white adipose tissue. Confirmatory results were recently obtained with a more potent and selective PPAR-β/-δ agonist, GW501516 (Ki = 1.1 ± 0.1 nM) in insulin-resistant middle-aged obese rhesus monkeys [152]. These results showed that GW501516 causes a dramatic dose dependent increase in serum HDL cholesterol and a reduction in LDL and triglyceride, suggesting that activation of PPAR-β/-δ is associated with a less atherogenic lipid profile and antidiabetic action [152–154]. PPAR-β/-δ has also exhibited a potential role in placentation, adiposity, colorectal cancer, and diabetic factors. GW0742 is a closely related analog of GW501516 and shows equivalent potency and selectivity for PPAR-β/-δ [152].

6.2. Implication of PPAR-δ in the Regulation of Insulin Sensitivity. The role of PPAR-β/-δ in the regulation of glucose homeostasis has emerged with the findings that PPAR-β/-δ agonists reduce adiposity and improve glucose tolerance and insulin sensitivity in different mouse models of obesity [153]. In addition, PPAR-β/-δ null mice display glucose intolerance when fed a chow diet. GW501516 treatment reduced insulin levels in obese insulin-resistant monkeys [152]. The use of PPAR-β/-δ tissue-specific transgenic mice, in adipose tissue or skeletal muscle, has shown that activated PPAR-β/-δ induces the expression of genes involved in FA oxidation and in energy expenditure through the induction of uncoupling proteins in brown adipose tissue and in skeletal muscle [154–156]. As a consequence, substrate supply for lipid storage in white adipose tissue is decreased, resulting in the

reduction of adiposity. It is also believed that PPAR-β/-δ induces fat burning in muscle, which together with an overall improvement in systemic lipid metabolism is responsible for lowering fat overload in insulin-sensitive tissues, thereby reducing insulin resistance.

In a primate model of the metabolic syndrome, the PPAR-β/-δ selective agonist GW501516 dose-dependently lowered plasma insulin levels, without adverse effects on glycemic control [152]. Similarly, in ob/ob mice, a model of the metabolic syndrome, a PPAR-β/-δ specific agonist markedly improved glucose tolerance and insulin resistance [153]. Although the underlying mechanism is unclear, activation of PPAR-β/-δ in skeletal muscle, which has a significant role in insulin sensitivity, has been proposed to account for the beneficial metabolic effects of PPAR-β/-δ agonists on lipid profile and insulin resistance, possibly as a result of increased fatty acid catabolism, cholesterol efflux, energy expenditure [153–157], and oxidative capability [155] in the muscle. Recently it was described that lipid peroxidation and the consequent production of 4-HNE in β-cells stimulate the secretion of insulin via a dependent mechanism, and these results were confirmed by treating these cells with an antagonist of this nuclear factor that resulted in blocking of the effect [158]. Activation of PPAR-β/-δ with agonist prevents induction of the transcription factor STAT3 by inhibiting the activation of ERK and inhibiting the interaction of STAT3 and Hsp90; this translates into the prevention of insulin resistance in adipose tissue [159]. In PPAR-β/-δ knockout mice it showed an obese phenotype when they were fed a diet rich in fats. The overexpression of PPAR-β/-δ or activation by the ligand GW501516 shows induction of muscle fiber type I, cell type rich in mitochondria that allow mice to undertake large periods of aerobic activity, and for this reason they are called mouse marathon runner. These mice were also resistant to diet-induced obesity and insulin resistance [160]. Finally, with the availability of three synthetic ligands (GW501516, GW0742, and L-165041) that activate PPAR-β/-δ at very low concentrations both *in vivo* and *in vitro* with high selectivity over other PPAR isotypes [161] had led to a huge increase in experimental studies on the role of PPAR-β/-δ in cellular processes. The IC_{50} for these compounds assessed with recombinant human PPAR-β/-δ were 1.0 nM for GW0742, 1.1 nM for GW501516, and 50 nM for L-165041 [161, 162].

6.3. Implication of PPAR-β/δ in Regulating Cardiovascular Complications and Atherosclerotic Lesions. Chronic low-grade inflammation plays a role in cardiac hypertrophy and heart failure [163]. Ongoing basic studies have demonstrated the role of PPAR-β/-δ in ameliorating cardiovascular complications. Few studies have shown that PPAR-β/-δ ligands have the potential to inhibit cardiac hypertrophy due to their inhibitory activity on NF-κB transcription factor which produces inflammatory cytokines such as TNF-α, MCP-1, and IL-6, and these are secreted by cardiac cells under various pathophysiological stimuli which may participate in myocardial inflammation [163]. Activated PPAR-β/-δ also dampens LPS-induced TNF-α inflammation signaling in cultured cardiomyocytes and blocks palmitate-induced inflammatory pathways in mouse heart and human cardiac cells through protein-protein interaction between PPAR-β/-δ and p65, suggesting inhibition of NF-κB [164, 165]. From these biological effects (Figure 3), PPAR-β/-δ may serve as a potential therapeutic target to prevent cardiac hypertrophy and heart failure in metabolic disorders. PPAR-β/-δ also attenuates progressive cardiac fibrosis occurring in diabetic cardiomyopathy. In null mice, PPAR-β/-δ specific cardiomyocyte and macrophage were infused with angiotensin II to trigger cardiac fibrosis, and then treated with pioglitazone; it is observed that the macrophage and not cardiomyocyte PPAR-β/-δ that attenuates fibrosis [156].

Finally, PPAR-β/-δ has recently been proposed as a potential target for modulating foam cell and macrophage activation in atherosclerosis. *In vitro* studies suggested that PPAR-β/-δ activation in cultured macrophage results in increased expression of the reverse cholesterol transporter ATP-binding cassette A1 and enhances efflux of cholesterol [152]. PPAR-β/-δ also participates in cellular VLDL sensing and mediates VLDL-triglyceride-driven transcription events in macrophage [157]. VLDL-triglyceride treatment results in triglyceride accumulation and the induction of adipocyte phenotype, which can be blocked by disruption of the PPAR-β/-δ gene. Furthermore PPAR-β/-δ also attenuates atherogenic inflammation. Its synthetic ligands GW0742 and GW501516 reduce atherosclerosis in low-density lipoprotein receptor (LDLR) null mice, possibly by decreasing monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1, and TNF-α expression [166, 167]. These new observations suggest that agonists for PAR-β/-δ may be effective agents to reverse cholesterol deposition in foam cells in atherotic lesions and therefore decrease cardiovascular disease associated with the metabolic syndrome. Taken together, PPAR-β/-δ is a critical player in the pathogenesis of the metabolic syndrome, and its ligands may provide useful agents for treating dyslipidemia, obesity, insulin resistance, and atherosclerosis.

7. Other Biological Mechanisms

The role of PPAR ligands has been well established in some very important therapeutic areas such as diabetes, obesity, cardiovascular diseases, and inflammation. But, more recently, it is becoming clear that they are also involved in antithrombosis. Diabetes mellitus is associated with a heightened risk of developing atherosclerotic vascular disease and its acute thrombotic complications, such as myocardial infarction [168]. Interestingly platelets play an important role in hemostasis and thrombosis, and there is an increasing evidence showing they are involved in mechanisms of inflammation and host defense, contributing to the pathogenesis and progression of vascular complications in the T2DM [169]. Therefore, treatment with antiplatelet agents may be a beneficial strategy to prevent and improve thrombosis-related cardiovascular diseases [170].

Platelets are enucleated cells derived from megakaryocytes; they contain transcription factors, notably the peroxisome proliferator-activated receptors (PPARs). So far, three PPAR isoforms (PPAR-α, PPAR-β/-δ, and PPAR-γ) have been found in human platelets, and upregulation of PPARs

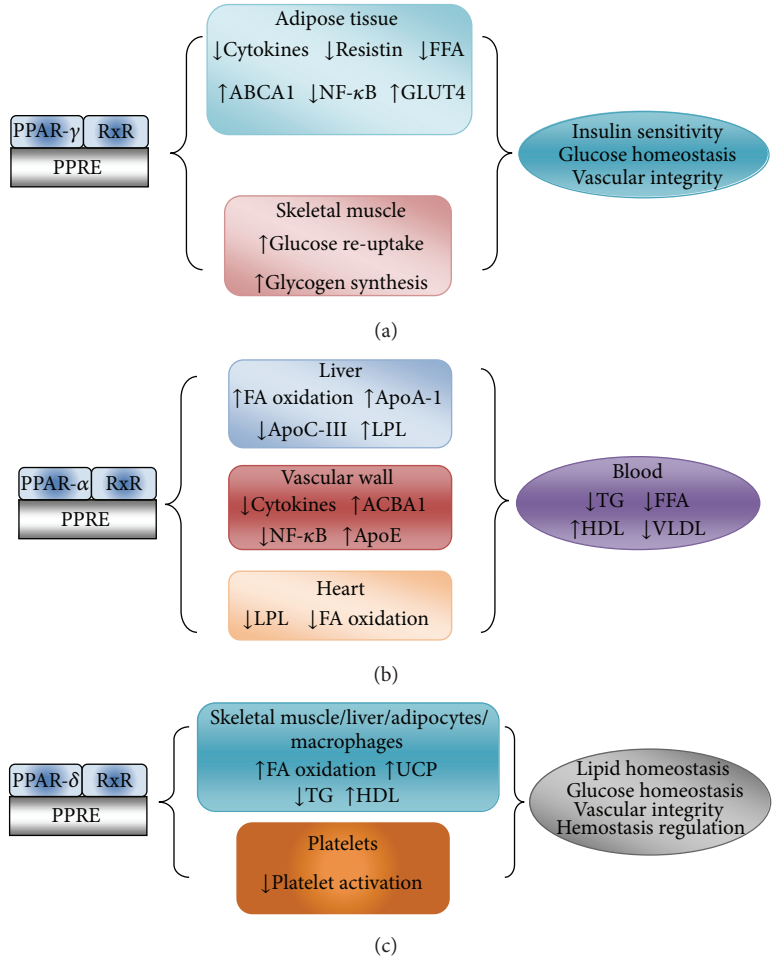

FIGURE 3: Mechanism of transcription and biological effects in different organs. (a) PPAR-γ exhibits anti-diabetic and atherosclerotic effects in adipocytes and skeletal muscle. (b) PPAR-α has multiple effects on liver, heart, and vascular wall. (c) PPAR-δ is expressed widely throughout the body and its gene expression is involved with the metabolism of lipids and glucose, as well as in decreasing the platelet activation. (Modified from [87].)

inhibits platelet activation through a nongenomic mechanism [171]. Platelet activation is associated with signaling that affects cell shape and spreading, secretion, and the release of multiple prothrombotic factors; all through the binding of plasma fibrinogen and von Willebrand factor (VWF) to integrin $\alpha_{IIb}\beta_3$, this leads to the formation of a stable platelet thrombus [172–174]. Many findings suggest that agents with PPARs-activating effect may exert an anti-platelet activity. A recent study was done by Ching-Yu Shih and group to prove that PPARs-mediated pathways contribute to the anti-platelet activity [175]. They used a natural product, magnolol, extracted from Chinese medicinal herbs, which has demonstrated multiple pharmacological effects, including antiatherosclerosis, antioxidative, anti-inflammatory, and anti-bacterial, even anti-platelet activity [176, 177]. Magnolol is a PPAR-γ agonist through direct binding to the PPAR-γ ligand binding domain it exhibit the anti-platelet activity and inhibits various important mediator formation and signaling pathways related to platelet activation. In the presence of selective PPAR-β antagonist (GSK0660) or PPAR-γ antagonist (GW9662), the inhibition of magnolol on collagen-induced platelet aggregation and intracellular Ca^{2+} mobilization was significantly reversed. These show that the excellent anti-platelet and antithrombotic activities of magnolol are modulated by upregulation of PPAR-β/-γ-dependent pathways [178].

Other findings of anti-platelet activity are lipid-lowering agents such as fibrates and statins that reduce thrombotic and cardiovascular risk. These are hypolipidemic drugs which decrease cardiac events in individuals without raised levels of cholesterol. In platelets, PKC_α activation facilitates platelet aggregation [179, 180]. Simvastatin and fenofibrate drugs inhibit platelet activation by inhibiting PKC_α which are associated with PPARs. Selective PPAR-γ antagonist GW9662, and PPAR-α antagonist GW6471 showed inhibition on effects of simvastatin and fenofibrate on platelet function which are mediated by PPAR-γ and PPAR-α, respectively, and the aggregation effects of α-lipoic acid can be attributed to the activation of PPAR-α/-γ [172]. In another study they demonstrate the ability of PPAR-γ ligands to modulate

collagen stimulated platelet function and suppress activation of the glycoprotein VI (GPVI) signaling pathway. PPAR-γ ligands inhibited collagen-stimulated platelet aggregation that was accompanied by a reduction in intracellular calcium mobilization and P-selectin exposure. PPAR-γ ligands inhibited thrombus formation under arterial flow conditions. The incorporation of GW9662 antagonists reversed the inhibitory actions of PPAR-γ agonists, implicating PPAR-γ as modulator. Furthermore, PPAR-γ ligands were found to inhibit tyrosine phosphorylation levels of multiple components of the GPVI signaling pathway. PPAR-γ was also found to associate with Syk and LAT after platelet activation. All this association was prevented by PPAR-γ agonists, indicating a potential mechanism for PPAR-γ function in collagen-stimulated platelet activation [181].

8. Conclusions and Future Prospects

Treatment and prevention of metabolic syndrome require lifestyle changes, including weight reduction, increased physical activity, and better diet. However, as many patients cannot control the pathology with lifestyle modification, there is a need for drugs to manage the metabolic syndrome. As discussed in this paper, that PPARs are transcription regulators which are involved in different metabolic pathways, they are able to interfere with many normal cellular processes as well as in altered processes that ultimately lead to pathology. This nuclear hormone receptor is believed to originate from a common ancestral receptor of 600 million years old. Furthermore it has managed to differentiate into several subtypes that can adapt to different ligands [182]. Its large capacity and strict control location allow these nuclear regulators to control complex processes such as inflammation or control energy homeostasis [183]. They are also attributed for their ability to regulate cellular differentiation in numerous cell lines such as keratinocytes, adipocytes, or immune system cells [182, 183]. Another factor which makes them particularly interesting is its ability to be activated or repressed by internal or natural ligands and synthetic or exogenous ligand; this capability opens thousands of possibilities in the treatment of various pathologies by numerous processes that are capable of controlling genes [184–186].

The pharmacological race generated new and increasingly potent agonists or antagonists, and it has prompted pharmaceutical companies to invest large sums of money on science, but nothing had been positive; unfortunately adverse effects have been reported for many synthetic compounds. For instance, one of the most important problems of PPAR agonists is cardiac toxicity which changes in the QT segment of the ECG, although the results obtained for this were controversially fair. Now recently it was confirmed that therapeutic concentrations of aleglitazar (dual agonist) show no evidence of changes in the QT segment of ECG. Consequently, the new dual agonist of PPAR alpha and gamma shows great advantages over its predecessors because the results obtained in phase I and II show a clear balance between patient safety and efficacy in improving metabolic parameters [187–191] and are extremely encouraging to start phase III. Clinical and basic sciences are on a quest to find double or triple agonists (PAN) in which the unwanted effects of agonists can be decreased significantly, and as a result individuals get a better quality of life with more potent drugs that result in increasingly lower doses (IC_{50} under toxic levels). Furthermore, with the development of PPAR delta targets, it has been shown that this nuclear regulator has become a therapeutic target in combating cardiovascular diseases that is more common in industrialized countries. PPAR delta targets will regulate the level of lipid oxidation in skeletal muscle and regulate the ratio of LDL and high density lipids [192, 193]; it is even able to inhibit the formation of foam cells induced by the small, dense LDL [194]. Recent studies have used GW501516 (a potent agonist of PPAR delta) which show a change in the lipoprotein profile, and highly atherogenic profile was passed to onefold less, proving to be a powerful cardiovascular protector for individuals with MS [193]. It is evident that platelets are extremely important in ischemic cardiovascular diseases, and it has been demonstrated that the greater number of active circulating platelets or a greater number of platelet-leukocyte complexes predict larger plates with greater lipid accumulation [195]. This fact evidences that rosiglitazone is capable of reducing the amount of circulating activated platelets and makes these compounds an ideal therapeutic target to control atherogenesis [196–198]. Above all it is tempting to speculate that the diversity of PPAR functions has been acquired in association with the rich variety of ligands. With the development and clinical use of PPAR ligands in the past decade have greatly advanced our understanding of the physiological and pathophysiological roles of PPAR and therapeutic implications of modulating these receptors. Surely the solution to metabolic diseases can be found in a drug response with super powers or with a combination of drugs that individually present positive effects and enhance the whole colud open Pandora's box, which could result in a real treatment.

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Research Article

Contribution of CFTR to Alveolar Fluid Clearance by Lipoxin A_4 via PI3K/Akt Pathway in LPS-Induced Acute Lung Injury

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The lipoxins are the first proresolution mediators to be recognized and described as the endogenous "braking signals" for inflammation. We evaluated the anti-inflammatory and proresolution bioactions of lipoxin A_4 in our lipopolysaccharide (LPS-)induced lung injury model. We demonstrated that lipoxin A_4 significantly improved histology of rat lungs and inhibited IL-6 and TNF-α in LPS-induced lung injury. In addition, lipoxin A_4 increased alveolar fluid clearance (AFC) and the effect of lipoxin A_4 on AFC was abolished by $CFTR_{inh\text{-}172}$ (a specific inhibitor of CFTR). Moreover, lipoxin A_4 could increase cystic fibrosis transmembrane conductance regulator (CFTR) protein expression *in vitro* and *in vivo*. In rat primary alveolar type II (ATII) cells, LPS decreased CFTR protein expression via activation of PI3K/Akt, and lipoxin A_4 suppressed LPS-stimulated phosphorylation of Akt. These results showed that lipoxin A_4 enhanced CFTR protein expression and increased AFC via PI3K/Akt pathway. Thus, lipoxin A_4 may provide a potential therapeutic approach for acute lung injury.

1. Introduction

Acute lung injury (ALI) is a critical illness syndrome characterized by an increased permeability of the alveolar-capillary barrier resulting in impairment of alveolar fluid clearance (AFC). A major cause for development of ALI is sepsis, wherein Gram-negative bacteria are a prominent cause. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, was the one of mainly pro-inflammatory reaction factor in lung injury, leading to neutrophil recruitment and pulmonary edema [1]. Alveolar fluid clearance in patients with ALI and the acute respiratory distress syndrome (ARDS) is impaired in the majority of patients, and maximal alveolar fluid clearance is associated with better clinical outcomes [2].

AFC depends on active ion transport, which leads to an osmotic gradient that drives the movement of fluid from the alveolar space back into the interstitium and eventually to the blood circulation [3]. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel expressed in both alveolar type I (ATI) and alveolar type II (ATII) cells [4–6]. More recent studies have shown that upregulation of alveolar CFTR function in rat and mouse lungs speeds clearance of excess fluid from the airspace and that CFTRs effect on active Na^+ transport requires the beta-adrenergic receptors, which can be blocked by nonspecific chloride channel inhibitors [7]. In addition, absence or inhibition of CFTR leads to lack of increase in alveolar liquid clearance in response to beta adrenergic agonist [8, 9]. This evidences suggests that CFTR chloride channel contributes to AFC.

Although significant efforts have been made to pharmacologically upregulate alveolar fluid clearance to reverse the progression of lung injury, these approaches have not been proven effective. Previous studies have demonstrated that

β-adrenergic receptor agonists, glucocorticoids, and several growth factors stimulated alveolar fluid clearance in animal models of ALI [10–12]. Based on *in vivo* and *in vitro* work, we demonstrated that salbutamol stimulated alveolar epithelial repair and reduced extravascular lung water [13, 14]. However, in a multicentre, randomized controlled trial, we found that intravenous salbutamol increased 28-day mortality in patients with ARDS [15]. Thus, new insights are needed to provide novel therapeutic approaches for ALI and ARDS.

The lipoxins are the first proresolution mediators to be recognized and described as the endogenous "braking signals" for inflammation [16]. Lipoxins elicit distinct antiinflammatory and proresolution bioactions, including inhibition of neutrophil functional responses, T-cell activation, and pro-inflammatory cytokines release [17]. We have previously demonstrated the biphasic role of lipoxin A_4 on expression of cyclooxygenase-2 (COX-2) in LPS-stimulated lung fibroblasts and the therapeutic effect of lipoxin A_4 in LPS-induced ALI [18, 19]. However, whether lipoxin A_4 can increase AFC stimulated by LPS and, if so, what the underlying mechanisms are, remain unclear.

In the present study, we examined the effects of lipoxin A_4 on AFC in LPS-induced ALI rats. Additionally, we also investigated its effects on the CFTR protein expression in the rat lungs and primary ATII cells. Finally, to gain a better understanding of the mechanisms, we investigated the signaling pathways which regulated the effects of lipoxin A_4.

2. Materials and Methods

2.1. Reagents. Lipoxin A_4, from Cayman Chemical Company, was stored at −80°C before use. LPS (*E. coil* serotype 055: B5), Evan's blue, $CFTR_{inh\text{-}172}$, LY294002, U0126, and deoxyribonuclease I were purchased from Sigma. ELISA kits of IL-6 and TNF-α were purchased from R&D Systems. DMEM, FCS, and Trypsin EDTA were purchased from Gibco. Penicillin and streptomycin in saline citrate buffer were from Invitrogen. Anti-CFTR and anti-β-actin were purchased from Santa Cruz. Anti-phospho-ERK and anti-phospho-Akt were purchased from Cell Signaling Technology.

2.2. Animal and Preparation. Male Sprague-Dawley rats (200–300 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All Animal experiments were permitted by the Animal Care Committee of Wenzhou Medical College.

The rats were divided into four groups ($n = 10$): (1) control group, in which the rats were treated with 0.1% ethanol (vehicle for lipoxin A_4, 5 mL/kg, iv) 6 h after they were treated with 0.9% saline (vehicle for LPS, 4 mL/kg, iv); (2) LPS group was identical to the control group except that LPS (20 mg/kg, iv) was administered instead of its vehicle; (3) lipoxin A_4 treatment group was identical to the LPS group except that lipoxin A_4 (2 μg/kg, iv) was administered instead of its vehicle; (4) $CFTR_{inh\text{-}172}$ group was identical to the lipoxin A_4 treatment group, but $CFTR_{inh\text{-}172}$ (1 uM, tracheal instillation) was administered after lipoxin A_4.

For the experiment, different concentrations of LPS including 10 mg/kg, 15 mg/kg, 20 mg/kg and lipoxin A_4 including 1 μg/kg, 1.5 μg/kg, 2 μg/kg, 2.5 μg/kg, 3 μg/kg were treated to measure the AFC.

2.3. Measurement of AFC in Live Rats. Preparation of the alveolar instillate was as follows: a 5% albumin instillate solution was prepared by dissolving 50 mg/mL of bovine serum albumin in modified lactated Ringers solution: 137 mM NaCl, 4.67 mM KCl, 1.82 mM $CaCl_2 * 2H_2O$, 1.25 Mm $MgSO_4 * 7H_2O$, 5.55 mM Dextrose, and 12 mM HEPES. The pH was adjusted to 7.4 at 37°C. The albumin solution was labeled with 0.15 mg/mL Evans Blue. AFC was assessed in living rats as previously described [12, 20, 21] with some modifications. Briefly, at 6 h after injection of LPS or saline by tail vein, the rats were anesthetized with 2% sodium pentobarbital (50 mg/kg, ip). A endotracheal tube was inserted through a tracheotomy. The rats were ventilated with a constant volume ventilator (model TKF-200c; TeLi anesthesia breathing equipment company, Jiangxi, China) with an inspired oxygen fraction of 100%, peak airway pressures of 8–10 cm of H_2O and positive end expiratory pressure of 3 cm of H_2O during the baseline period. Following surgery the rats were allowed to stabilize for 10 min. The animals were then placed in the left lateral decubitus position and instillation tubing (16 G Epidural catheter) was gently passed through the tracheotomy tube into the left lung. Then 1.5 mL (5 mL/kg) of the instillate solution with or without $CFTR_{inh\text{-}172}$ was instilled at a rate of 0.08 mL/min using a syringe pump. After instillation was complete 0.2 mL of air was injected to achieve complete deposition of all fluid into the alveolar space. The instillate solution remaining in the syringe was collected as the initial sample. Following instillation, the catheter was left in place for the duration of 60 min. The final alveolar sample was collected via the instillation catheter. The concentrations of Evans Blue labeled albumin in the instilled and aspirated solutions were measured by a spectrophotometer at a wavelength of 621 nm. AFC was calculated using the following equation: $AFC = 1 - (C_i/C_f)$ [12], where C_i is the protein concentration of the instillate before instillation and C_f is the protein concentration of the sample obtained after 60 min of mechanical ventilation. AFC was expressed as a percentage of total instilled volume (%/60 min).

2.4. Measurement of IL-6 and TNF-α in Lung Tissue Homogenate. Right lung tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 12,000 rpm for 20 min at 4°C, then aliquoted and stored at −80°C. IL-6 and TNF-α were measured by ELISA kits. All procedures were done in accordance with the manufacturer's instructions.

2.5. The Haematoxylin-Eosin (H&E) Staining Analysis of the Lung. For histological examination, the right lung tissues were fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin wax, sectioned (5 μm thicknesses). The pulmonary tissue slides were stained with hematoxylin and eosin and were examined using a light microscope (Nikon eclipse 90i, Tokyo, Japan). Analyses of lung tissue slides were

carried out by blinded observation to evaluate (a) alveolar congestion; (b) hemorrhage; (c) infiltration or aggregation of neutrophils in airspace or vessel wall; and (d) thickness of alveolar wall/hyaline membrane formation. The results were graded from 0 to 4 for each item, as described previously [22]. The four variables were summed to represent the lung injury score (total score, 0–16).

2.6. Immunohistochemistry. After paraffin removal in xylene, the sections were rehydrated and were placed in a pressure cooker with citrate buffer, pH 6.0, for 1 min after reaching boiling temperature to retrieve antigenic sites masked by formalin fixation. After quenching of endogenous peroxidase with 3% of H_2O_2 for 15 min, the sections were incubated with rabbit polyclonal antibody to CFTR (1 : 100 dilution) or with the preimmune serum as a negative control stain overnight at 4°C, and subsequently were incubated for 1 h at room temperature with the biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin, with diaminobenzidine (DAB) as the substrate. The slides were counterstained for 30 seconds with hematoxylin. The slides were observed and photographed with microscopy (Nikon eclipse 90i, Tokyo, Japan).

2.7. Isolation and Culture of Primary ATII Cell. ATII cells were isolated from Sprague-Dawley male rats weighing 200–300 g as previously reported with a slight modification [23, 24]. Rats were anesthetized with 2% sodium pentobarbital (50 mg/kg, ip), and then inferior aorta and vena cava were cut. The lungs were perfused with solution A (133 mM NaCl, 5.2 mM KCl, 2.59 mM phosphate buffer, 10.3 mM HEPES buffer, 1 mg/mL glucose, pH 7.4) using a 20-mL syringe fitted with a 23-gauge needle through the right ventricle until the lungs were perfectly turned to white. The lungs were excised with trachea, and were lavaged ten times with solution B (solution A plus 1.89 mM $CaCl_2$ and 1.29 mM $MgSO_4$) through trachea to remove macrophages, and then completely filled with 0.25% trypsin for 20 min at 37°C. The trachea, main bronchi and large airways were discarded and the lungs were placed onto the sterile petri dish. Each lung was minced into 1-2 mm within 10 min. 5 mL FBS was added to stop trypsin reaction and the tissue suspension was prepared to a final volume of 20 mL by HBSS containing deoxyribonuclease I (250 U/mL). The minced lung tissue was transferred to a 50-mL centrifuge tube in a water bath for 8 min at 37°C. The suspension was filtered through 150 and 75 μm stainless steel meshes and then centrifuged at 1000 rpm for 8 min. The layer containing ATII cells suspended in DMEM containing 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin. The cells were plated into 75-cm^2 flask and incubated for 2-3 h at 37°C in an atmosphere of 95% air-5% CO_2 to allow fibroblasts to adhere. Nonadherent cells were collected and enriched for ATII cells by differential adherence to IgG-coated plastic dishes for 1 h. At last, unattached cells resuspended in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at a density of 2×10^6 cells/mL and cultured on plastic six-well plates. The culture period was limited to 48–72 h so as to minimize dedifferentiation.

2.8. Immunofluorescence. Rat primary ATII cells were grown on coverslips incubated with DMEM stimulated by LPS in the presence or absence of lipoxin A_4 for 6 h at 37°C. The cells were fixed for 5 min with 4% paraformaldehyde in PBS, and were washed three times with PBS. Fixed cells were permeated with 1% Triton X-100 for 5 min, and were washed three times with PBS. Nonspecific binding of antibodies was prevented by the addition of 5% bovine serum albumin in PBS for 30 min at 37°C. The cells were then incubated with antibody against CFTR (1 : 100) overnight at 4°C. Following three PBS washes, cells were incubated for 2 hours with fluorescein-conjugated goat anti-rabbit IgG (1 : 500), in 5% BSA/PBS at room temperature. After washing three times with PBS, cell nuclei were counter stained with Hoechst (1 : 1000) for 15 min, followed by three PBS washes. Cells were then mounted on a slide and visualized using microscopy.

2.9. Western Blotting Analysis. Western blotting analysis from frozen lungs and cells homogenates were performed as described previously [25]. After equal amounts of protein were loaded in each lane and separated by 10% SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h with 5% skimmed milk, which was also used as primary and secondary antibodies incubation buffer. The primary antibodies were used at dilutions of 1 : 1,000 or 1 : 2,000, and incubated overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies, which were either goat anti-mouse or goat anti-rabbit, were used at 1 : 2,000 dilution and imaged with the Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Quantification was performed with the AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

2.10. Measurement of cAMP in ATII cells. cAMP levels were measured in triplicate by a commercially available enzyme-linked immunosorbent assay (ELISA; from R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer's protocol.

2.11. Statistical Analysis. All values were reported as means ± SEM. Data were analyzed by a one-way ANOVA, followed by a Student Newman-keuls post hoc test and $P < 0.05$ defined as statistically significant. Statistical analysis and graphs were done with GraphPad Prism 5.0 (GraphPad, San Diego, CA).

3. Results

3.1. The Beneficial Effects of Lipoxin A_4 on LPS-Induced ALI. The control group had normal pulmonary histology (Figure 1(a)). In contrast, the lung tissues from the LPS group were significantly damaged with alveolar disarray and severe inflammatory cell infiltration (Figure 1(a)). All indicated that there was ALI in this model. Mild histological changes were observed in the rats lung tissues after treatment with lipoxin A_4 (Figure 1(a)). Consistent with these histopathological observations, the lung injury score in LPS group was significantly higher than those in control group and LPS + LXA_4 group (Figure 1(b)). TNF-α (Figure 1(c)) and

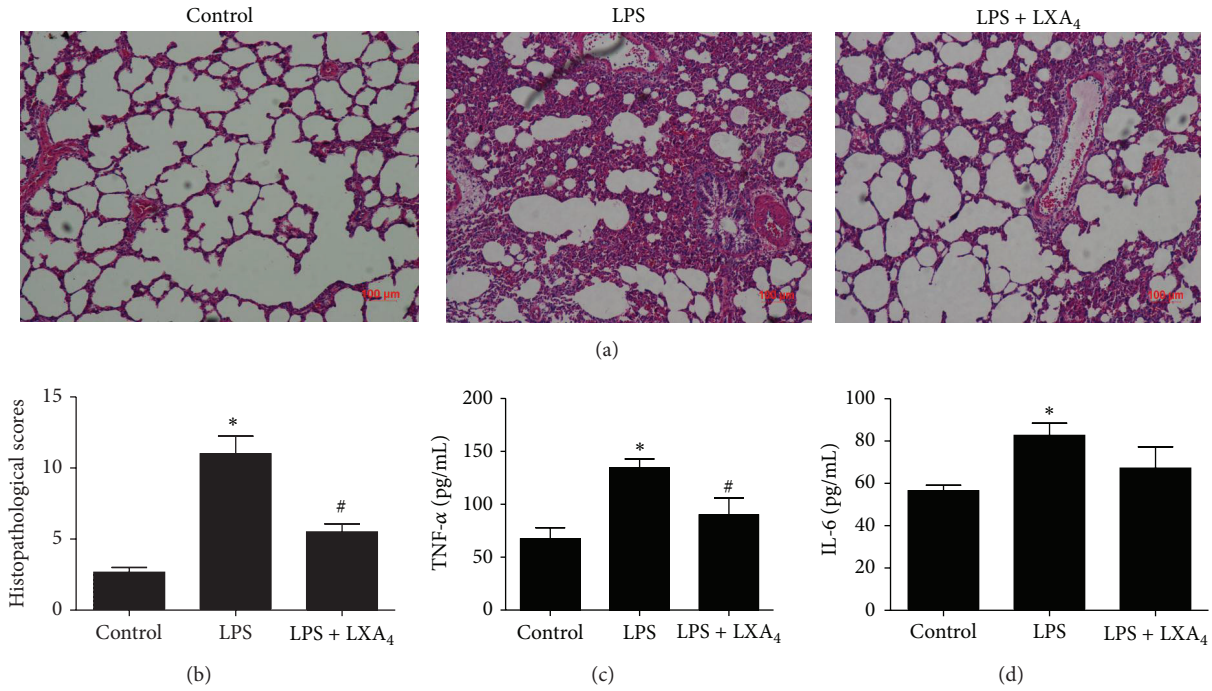

Figure 1: Effects of lipoxin A_4 on LPS-induced ALI. Lipoxin A_4 (2 μg/kg) was administered intravenously to Sprague-Dawley (SD) rats 6 h after LPS (20 mL/kg) stimulation through the tail vein, and intratracheal instillation of 5% albumin solution containing Evans Blue-labeled albumin through a tracheostomy to the left lung, and ventilating for 1 hour. The right lung was isolated. The effect of lipoxin A_4 was assessed by (a) histology in hematoxylin and eosin-stained sections, (b) histopathological scores, (c) the lung tissues homogenate TNF-α protein expression, and (d) the lung tissues homogenate IL-6 protein expression. Data were expressed as mean ± SEM for each group. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus LPS group, $n = 6$ for each group. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4.

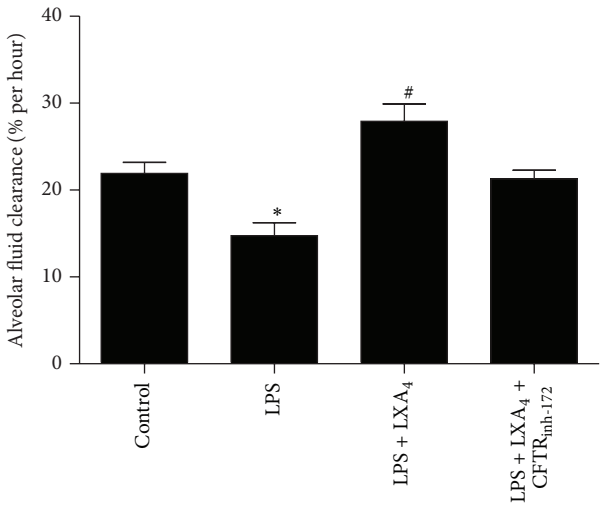

Figure 2: Effects of lipoxin A_4 on AFC in LPS-induced ALI. Lipoxin A_4 (2 μg/kg) was administered intravenously to Sprague-Dawley (SD) rats 6 h after LPS (20 mL/kg) stimulation through the tail vein, and intratracheal instillation of 5% albumin solution containing Evans Blue-labeled albumin through a tracheostomy to the left lung, ventilating for 1 hour. $CFTR_{inh\text{-}172}$ was instilled with albumin solution. Data were expressed as mean ± SEM for each group. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus LPS group, $n = 8$ for each group. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4.

IL-6 (Figure 1(d)) concentration increased significantly in the LPS group compared with control group ($P < 0.05$). This increase in TNF-α was significantly ($P < 0.05$) reduced in the lipoxin A_4 group. Concentration of IL-6 in lipoxin A_4 group (67.24 ± 24.56) was lower than LPS group (82.74 ± 14.04), although the different was not significant ($P > 0.05$).

3.2. The Effect of Lipoxin A_4 on LPS-Stimulated AFC. Different concentrations of LPS were used in the experiments. We found the effect of 10 mg/kg LPS on AFC is unstable in rats. However, 15 mg/kg LPS could inhibit AFC, and reached the significant effect at 20 mg/kg, so we chose 20 mg/kg LPS in our experiments. We also have performed different concentrations of lipoxin A_4 including 1 μg/kg, 1.5 μg/kg, 2 μg/kg, 2.5 μg/kg, 3 μg/kg in our experiments. We found that 1 μg/kg lipoxin A_4 had no influence on improving the AFC reduced by LPS; 1.5 μg/kg lipoxin A_4 improved the AFC reduced by LPS, and reached the maximal effect at 2 μg/kg, the effect of lipoxin A_4 was similar between 2 μg/kg and 2.5 μg/kg, 3 μg/kg, so 2 μg/kg lipoxin A_4 was chosen in our experiments (data not shown).

AFC was found to be markedly decreased in the LPS (20 mg/kg) group as compared with control group ($P < 0.05$). The decrease was significantly ($P < 0.05$) reduced in the lipoxin A_4 (2 μg/kg) group (Figure 2). Next, we tried to block chloride ion channel using $CFTR_{inh\text{-}172}$ (1 uM), a specific

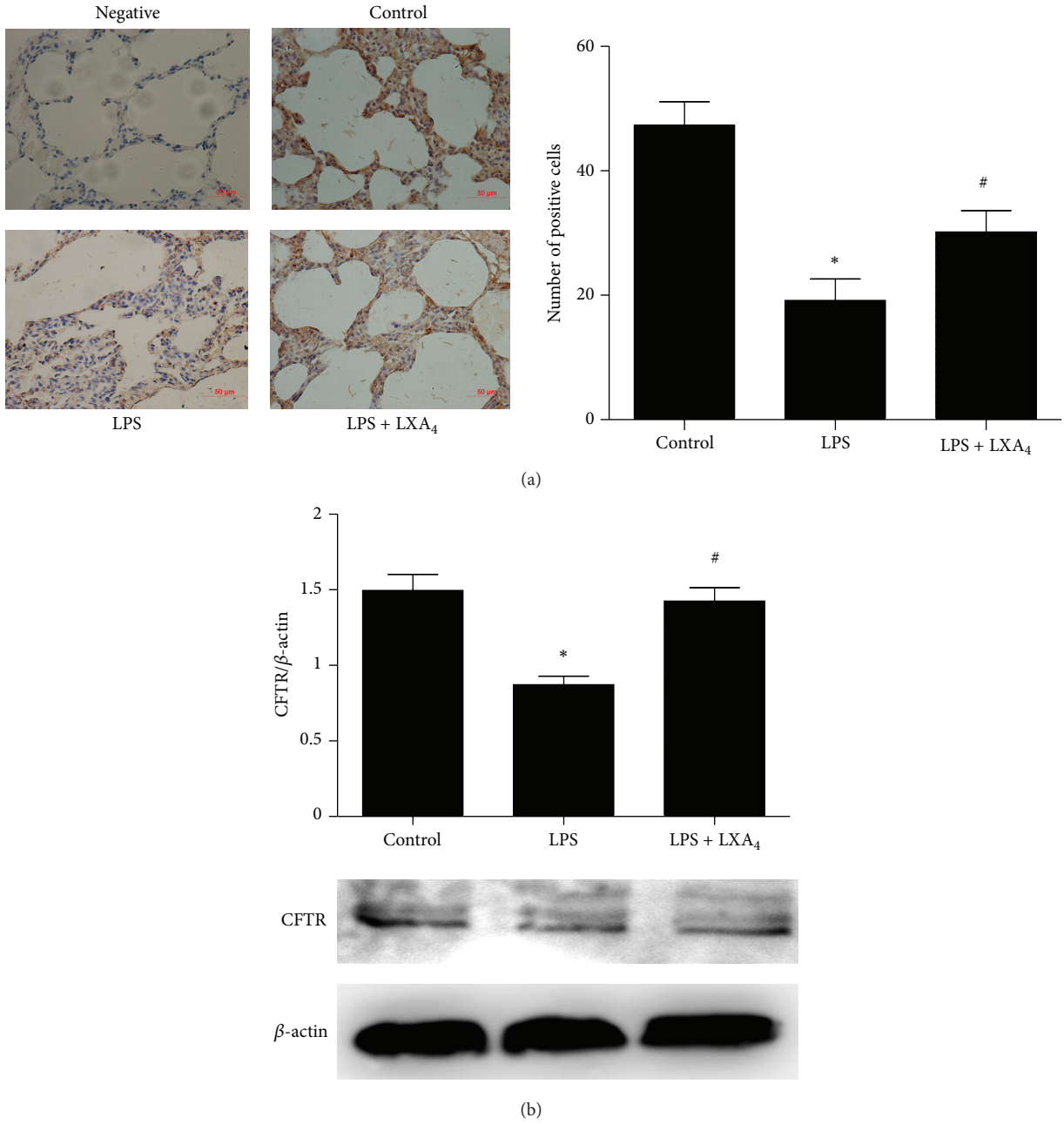

FIGURE 3: Effects of lipoxin A_4 on protein expression of CFTR *in vivo*. Lipoxin A_4 (2 μg/kg) was administered intravenously to Sprague-Dawley (SD) rats 6 h after LPS (20 mL/kg) stimulation through the tail vein, and intratracheal instillation of 5% albumin solution containing Evans Blue-labeled albumin through a tracheostomy to the left lung, ventilating for 1 hour. The right lung was isolated. (a) The protein expression of CFTR was assessed by immunohistochemistry. Immunopositive cells were counted in five randomly selected nonoverlapping fields of three separately immunostained lung sections per group. (b) The right lung was also homogenized for western blotting. Data were expressed as mean ± SEM for each group. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus LPS group. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4, CFTR = cystic fibrosis transmembrane conductance regulator.

inhibitor of CFTR. We found that the effect of lipoxin A_4 on AFC was abolished by the treatment with $CFTR_{inh\text{-}172}$ (Figure 2).

3.3. The Effect of Lipoxin A_4 on Protein Expression of CFTR In Vivo and In Vitro Experiments. *In vivo*, the expression of CFTR protein in the lung tissue homogenate was detected by immunohistochemistry assay, which was decreased by LPS stimulation, but enhanced by lipoxin A_4 treatment (Figure 3(a)). The change in western blotting analysis was similar to the result in immunohistochemistry assay (Figure 3(b)). CFTR protein expression was significantly

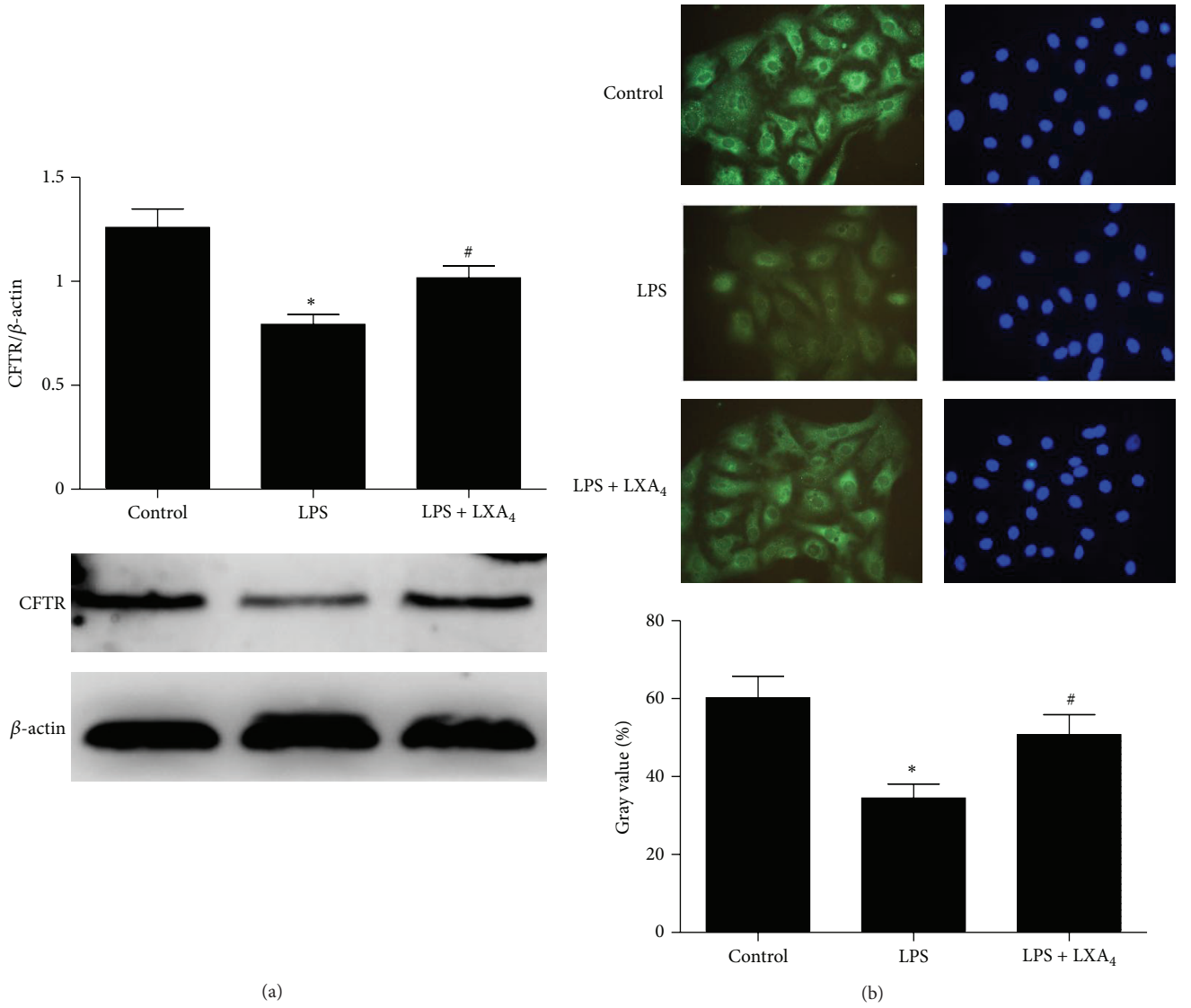

Figure 4: Effects of lipoxin A_4 on protein expression of CFTR in primary ATII cells. The cells were treated with lipoxin A_4 (100 nM) in the presence of LPS (1 μg/mL) for 6 hours. (a) After incubation, the cells were harvested, sonicated and CFTR protein detected by western blotting. (b) The protein expression of CFTR also was detected by immunofluorescence assay. Data were expressed as mean ± SEM for each group. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus LPS group, $n = 4$ for each group. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4, CFTR = cystic fibrosis transmembrane conductance regulator.

increased in the lipoxin A_4 treatment group compared with the LPS group in rat primary ATII cells (Figure 4(a)). Result in immunofluorescence assay was consistent with that in western blotting analysis (Figure 4(b)).

3.4. LPS Decreases Protein Expression of CFTR via PI3K/Akt Signaling Pathway in Primary ATII Cells. To investigate which signaling pathway was involved in the regulation of LPS on CFTR protein expression, firstly, we tested the phosphorylation of Akt and ERK after rat primary ATII cells were stimulated by LPS. The phosphorylation of ERK and Akt reached a peak within 30 min (Figure 5(a)). Secondly, ATII cells were pretreated with PI3K/Akt kinase inhibitor (LY294002) and ERK inhibitor (U0126) for 30 min. Only the addition of LY294002 abrogated LPS-induced downregulation of CFTR protein expression (Figure 5(b)).

3.5. Lipoxin A_4 Suppresses LPS-Stimulated Phosphorylation of Akt. Phosphorylation of Akt was significantly reduced in lipoxin A_4 treatment group compared with LPS group. Phosphorylation of Akt was also decreased in LY294002 administrated cells (Figure 6). The experiment was repeated four times with similar results.

3.6. The Effect of Lipoxin A_4 on Intracellular cAMP in LPS-Stimulated ATII Cells. We measured the intracellular cAMP

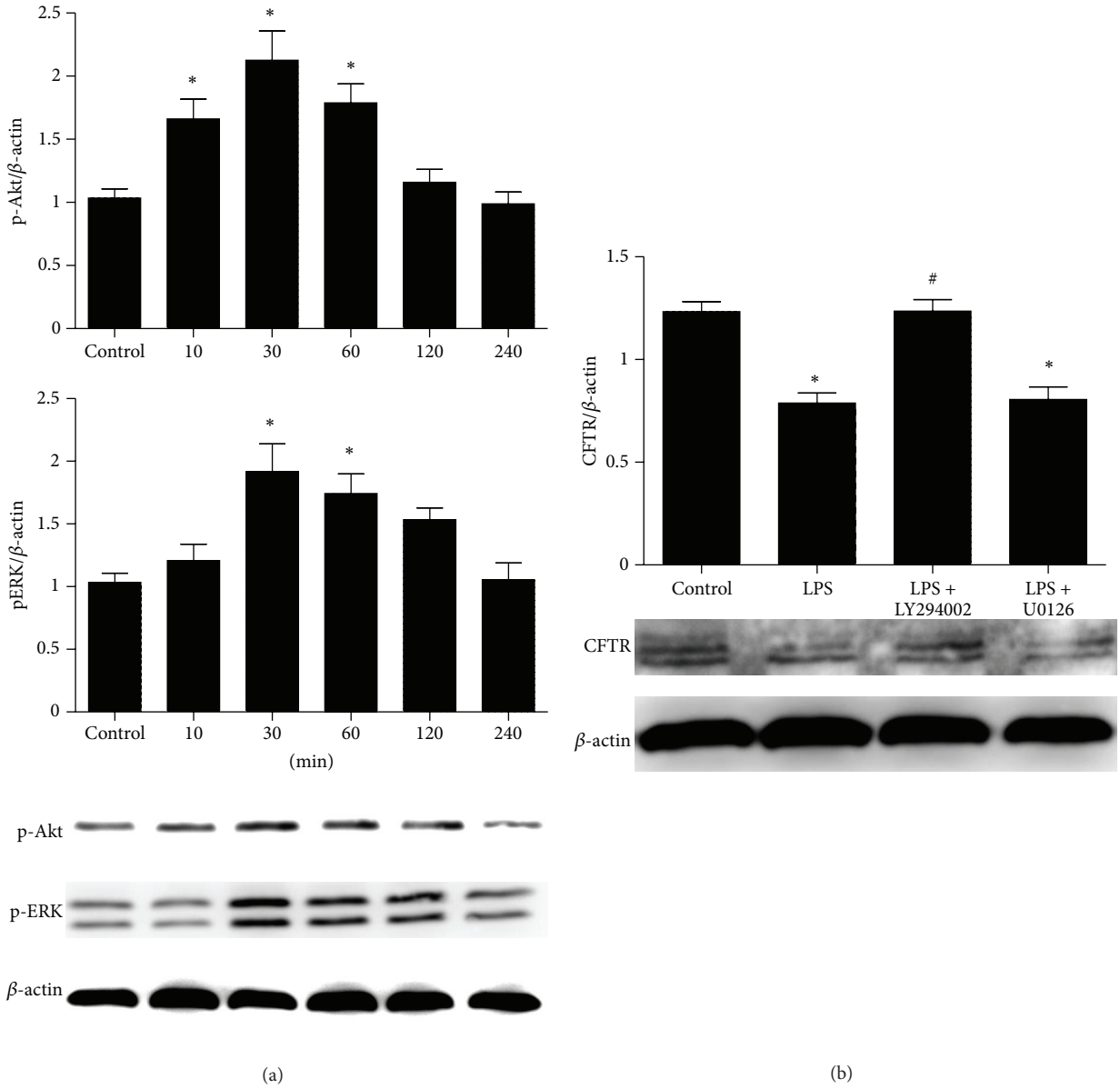

FIGURE 5: LPS decreases protein expression of CFTR via PI3K/Akt signaling pathway in ATII cells. The cells were incubated with LPS (1 μg/mL) for 10, 30, 60, 120, and 240 min. After incubation, the cells were harvested and sonicated. (a) Phosphorylation of Akt and ERK in the cell lysates were assessed by western blotting. (b) The rat primary ATII cells were pretreated with LY294002 (10 μM, PI3K/Akt inhibitor) or U0126 (20 μM, ERK inhibitor) for 30 min and then incubated with LPS (1 μg/mL) for 6 h. After incubation, the cells were harvested, sonicated and CFTR protein detected by western blotting. Data were expressed as mean ± SEM for each group. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus LPS group, $n = 4$ for each group. LPS = lipopolysaccharide, CFTR = cystic fibrosis transmembrane conductance regulator.

levels in ATII cells after 1 h of exposure to LPS or LPS plus lipoxin A_4 (Figure 7). cAMP level was decreased in LPS group compared with control group ($P < 0.05$), but LPS + LXA_4 group increased the cAMP level compared with LPS group ($P < 0.05$).

4. Discussion

ALI and its more severe form, the acute respiratory distress syndrome (ARDS), are relatively common syndromes in critically ill patients associated with high morbidity and mortality [26]. No specific therapy is currently available to modulate this inflammatory response and protect the lung. Experimental strategies to block prophlogistic mediators have not proven successful [27] because of the multiple, redundant pathways that initiate inflammation. These strategies also decrease the host's ability to adequately deal with infection, given that the innate inflammatory response is a beneficial defensive event [28]. Traditionally, it was argued that pro-inflammatory mediator catabolism was sufficient for

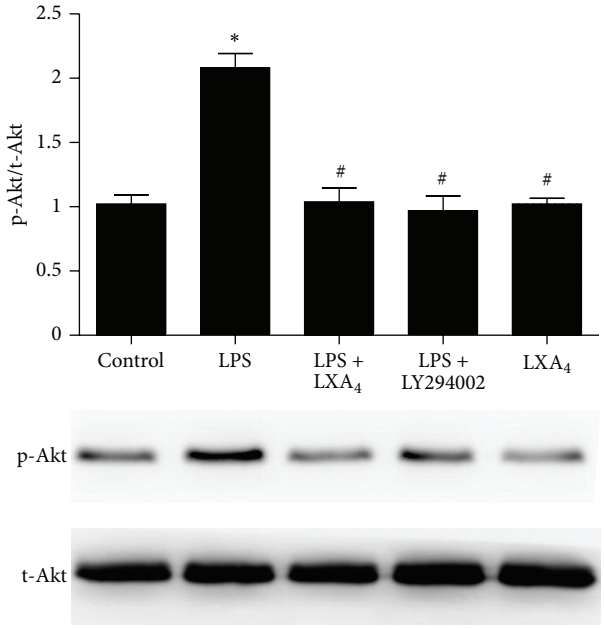

Figure 6: Lipoxin A_4 reverses LPS-induced phosphorylation of Akt. ATII cells were treated with LY294002 (10 μM, PI3K/Akt inhibitor) or lipoxin A_4 (100 nM) in the presence of LPS (1 μg/mL) for 30 min. After incubation, the cells were harvested and sonicated. Phosphorylation of Akt in the cell lysates was assessed by western blotting. Data were expressed as mean ± SEM for each group. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus LPS group, $n = 4$ for each group. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4.

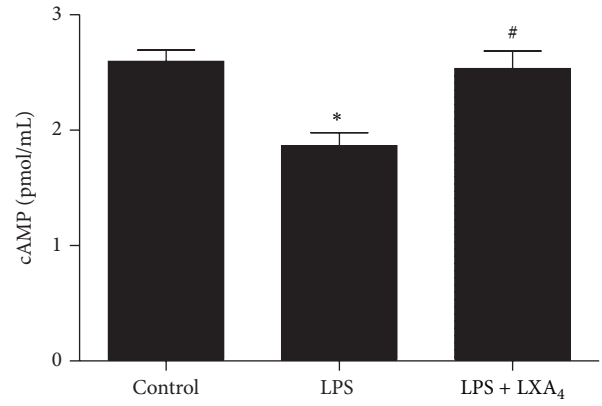

Figure 7: The effect of lipoxin A_4 on cAMP in ATII cells. The cells were treated with lipoxin A_4 (100 nM) in the presence of LPS (1 μg/mL) for 1 hour. After incubation, the cells were harvested and intracellular cAMP was detected by Elisa Kits. Data were expressed as mean ± SEM for each group. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus LPS group. $n = 6$. LPS = lipopolysaccharide, LXA_4 = lipoxin A_4.

inflammation to switch off, with the subsequent responses ending passively. New evidence indicates that resolution of inflammation and the return to homeostasis is not a passive, but an actively regulated process [29]. Specifically in ALI, resolution is characterized by clearance of PMN from the lung and reabsorption of alveolar fluid [30]. Within the resolution phase, endogenous proresolving lipid mediators, such as lipoxins, can counter-regulate host inflammation responses and promote resolution [31]. Taking these data together, our purpose was to evaluate whether lipoxin A_4 have protective action against LPS-induced ALI and promote reabsorption of alveolar fluid.

Our data clearly demonstrated that treatment of rats with lipoxin A_4 significantly inhibited IL-6, TNF-α production and increased AFC with the outcome of decreased pulmonary edema in lung tissue. The inhibitor of CFTR was able to abolish the beneficial effects of lipoxin A_4. Furthermore, we demonstrated that treatment with lipoxin A_4 upregulated the CFTR protein expression *in vivo* and primary ATII cells. Finally, our data provided evidence that LPS decreased CFTR protein expression via PI3K/Akt signaling pathway and the lipoxin A_4 can suppress LPS-stimulated phosphorylation of Akt.

LPS stimulates macrophages, neutrophils, and other immune cells to produce different mediators including cytokines such as TNF-α, IL-6 that recruits polymorphonuclear neutrophils into the injured site [32]. In addition, activated neutrophils transmigrate across the endothelial surface into lung by release of reactive oxygen species, resulting in alveolar capillary barrier leakage, interstitial and alveolar edema [33]. Accumulating data indicated that upregulation of CFTR increased AFC [7, 34]. The lack of functional CFTR in ΔF508 mice could limit their capacity to remove alveolar edema [8]. Transforming growth factor β_1 inhibited AFC via downregulation of CFTR protein expression [35].

CFTR protein expression is regulated by a complex network of signaling pathways, including the Akt and ERK pathways [35, 36]. Akt is a key mediator of signal transduction in protein synthesis and is a downstream kinase of PI3K [37]. The PI3K/Akt pathway has been shown to mediate TGF-β_1 induced decrease of CFTR protein expression in primary ATII cells [35]. It is known that LPS can bind to toll-like receptor 4 (TLR4), which acts as receptor that activates downstream signaling pathways including PI3K/Akt [38]. The present study revealed that culture of primary ATII cells with LPS resulted in a rapid phosphorylation of Akt and ERK. The phosphorylation of ERK and Akt reached a peak within 30 min. Only the addition of LY294002 (PI3K/Akt inhibitor) abrogated LPS-induced down-regulation of CFTR protein expression. These findings suggest that LPS inhibits CFTR protein expression via activation of the PI3K/Akt pathway.

We found that lipoxin A_4 downregulated the LPS-stimulated phosphorylated Akt in primary ATII cells. Previous studies have showed that lipoxin A_4 inhibited connective tissue growth factor (CTGF)-induced proliferation of human lung fibroblasts via down-regulation of PI3K/Akt [39]. Aspirin-triggered lipoxin A_4 also inhibited myeloperoxidase (MPO) suppression of neutrophil apoptosis via down-regulation of PI3K/Akt [40]. In the current study, the effects of lipoxin A_4 mimicked the inhibition of PI3K/Akt with LY294002, suggesting that the PI3K/Akt pathway also regulates the effect of lipoxin A_4 on LPS induced decrease of CFTR protein expression.

cAMP is a ubiquitous second messenger regulating a majority of intracellular functions. Extracellular signals interact with GPCRs to activate adenylate cyclase and increase

the intracellular cAMP levels. Experiments on duodenal epithelial cells suggested that cAMP not only increased the activity of CFTR but also shifted CFTR proteins to the plasma [41]. Previous study reported that pretreatment of the bronchial epithelial cells with either MDL hydrochloride (adenylate cyclase inhibitor) or (Rp)-cAMP (cAMP-dependent protein kinase inhibitor) inhibited the Ca^{2+} response to lipoxin A_4. Pertussis toxin treatment completely abolished the Ca^{2+} response induced by lipoxin A_4 in 16HBE14o$^-$ cells [42]. These found suggested that lipoxin A_4 up-regulated CFTR expression may through the cAMP pathway.

In conclusion, this study demonstrates that administration of lipoxin A_4 systemically increases AFC in LPS injured rat lungs. We also demonstrate that augmented AFC is associated with enhanced CFTR protein expression after treatment with lipoxin A_4. The mechanism may be through PI3K/Akt signaling pathway. Our findings reveal a novel mechanism for pulmonary edema fluid reabsorption and lipoxin A_4 may provide new opportunities to design "reabsorption targeted" therapies with high degree of precision in controlling ALI/ARDS.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

Protective Effect of Short-Term Genistein Supplementation on the Early Stage in Diabetes-Induced Renal Damage

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Hyperglycemia-induced oxidative stress has been concerned in the development of diabetic nephropathy (DN), which may cause kidney damage associated with inflammation and fibrosis. This study has been conducted to investigate the role of genistein supplementation in an acute DN state. Mice with FBG levels more than 250 mg/dL after alloxan injection (single i.p., 150 mg/kg) were considered as diabetic. Diabetic mice (DM) were further subdivided according to their FBG levels, medium-high FBG (DMMH < 450 mg/dL) and high FBG (DMH; 450 mg/dL) and were administrated by an AIG-93G diet supplemented with different doses of genistein (0, 0.025 or 0.1%). After 2 weeks' treatment, the levels of kidney malondialdehyde (MDA), blood urea nitrogen (BUN), and plasma creatinine and lipid profiles, as well as oxidative stress and inflammation-related markers, were measured ($P < 0.05$). Genistein supplementation improved levels of FBG in the DMMH groups, but not in the DMH group, regardless of the treatment dose. Moreover, the supplementation attenuated kidney oxidative stress indicated by MDA, BUN, and plasma creatinine. In addition, genistein treatment decreased inflammatory markers such as nuclear factor kappa B (p65), phosphorylated inhibitory kappa B alpha, C-reactive protein, monocyte chemotactic protein-1, cyclooxygenase-2, and tumor necrosis factor-alpha and improved oxidative stress markers (nuclear-related factor E2, heme oxygenase-1, glutathione peroxidase, and superoxide dismutase isoforms) in treatment groups, regardless of the genistein treatment dose. Furthermore, genistein supplementation inhibited the fibrosis-related markers (protein kinase C, protein kinase C-beta II, and transforming growth factor-beta I) in the DN state. However, 0.1% genistein supplementation in diabetes with high FBG levels selectively showed a preventive effect on kidney damage. These results suggest that genistein might be a good protective substance for DN through regulation of oxidative stress and inflammation. In particular, genistein is more efficient in diabetes patients with medium-high blood glucose levels. Finally, it is required to establish the beneficial dosage of genistein according to blood glucose levels.

1. Introduction

Diabetes mellitus (DM) is a major endocrine-metabolic disorder that is associated with chronic hyperglycemia by disturbance in carbohydrate, protein, and lipid metabolism. According to the WHO (World Health Organization), the world prevalence of diabetes has been increasing explosively from 171 million in 2000 to an assumed 366 million in 2030 [1]. As DM have severe health consequences, it gives rise to diabetic complications including retinopathy, neuropathy, and nephropathy. About 20%–40% of diabetic patients suffer from diabetic nephropathy (DN), which is characterized by end-stage renal disease [2]. DN has been implicated in several mechanisms by hyperglycemia, which may simulate overproduction of reactive oxygen species (ROS). ROS play a crucial role in generation of oxidative stress and several inflammatory responses [3, 4] that trigger cellular dysfunction and progression of kidney fibrosis. Indeed, the response may be upregulated by ROS-related activation of transcription factors and their downstream genes. This fact suggests that the mechanism of several transcription factors is implicated in hyperglycemia-mediated expression of genes involved in DN [5]. Recently, it has become increasingly acknowledged that NFκB generally works with other transcription factors [6, 7], such as nuclear related factor E2 (Nrf2) [8]. DN condition is expected to bring out diverse synergistic effects at the transcriptional level [6]. NFκB induced by oxidative stress is one of the most critical transcriptional regulatory factors that control the expression of a large number of genes involved in inflammatory response, including cytokines,

chemokines, growth factors, and adhesion molecules [9]. It mediates damages in extracellular matrix, glomerulosclerosis, and renal failure, thus stimulating the development of DN. Recently, an increase in NFκB activation has been observed in DM patients [10, 11] and in DN animals [12]. In contrast to the inflammatory action of NFκB, Nrf2 is responsible for the defense system against oxidative stress [13, 14] and inflammation [8] by regulation of phase II detoxifying enzymes and redox-related antioxidant proteins [15]. It is known that activation of Nrf2 and upregulation of its downstream antioxidant genes in hyperglycemic condition were found not only in the cultured cells, but in DN patients [16]. Therefore, Nrf2 may contribute to the improvement of inflammatory conditions such as DN.

With the onset of ROS production in diabetic kidneys, fibrosis is stimulated by increases in oxidative stress and inflammation. Protein kinase C (PKC) is associated with phosphorylation of serine/threonine residues in insulin receptors and is generated due to the synthesis of diacylglycerol (DAG) under the high intracellular concentration of glucose [17, 18]. In particular, PKC-βII, as one of the various isoforms of PKC, is well known to accelerate the pathogenesis of hyperglycemic kidney injuries, and it leads to insulin resistance as well as to dysfunction of various cells through the reduction of insulin receptor substrate- (IRS-) 2 tyrosine phosphorylation, resulting in defected insulin stimulation and intracellular accumulation of diacylglycerol in various organs [19, 20]. Thus, excessive production of PKC-βII in diabetic kidneys may induce formation of advanced glycation end products (AGE), as well as production of growth factors, such as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), and vascular endothelial growth factor (VEGF) [21, 22].

Genistein, a class of phytoestrogens known as isoflavones, is mostly found in legumes. It has attracted attention because of its beneficial effects on prevention of metabolic disorders related to cardiovascular disease (CVD), obesity, cancer, and diabetes [23–26]. Thus, genistein has been extensively established as a multifunctional agent through enhancing the antioxidant defense system and anti-inflammation response. Recently, a study focused on the protective role of genistein on renal malfunction in rats fed a fructose rich diet, through the modulation of insulin resistance-induced pathological pathways [27]. Furthermore, Yuan et al. have noted that high doses of genistein ($\geq 5\ \mu mol \cdot L^{-1}$) protected renal mesangial cells against a hyperglycemic condition, which increased fibrosis through induction of fibrosis related genes, such as extracellular matrix (ECM) and TGF-β [28]. Another study has shown that genistein injections (10 mg/kg via i.p.) reduced urinary TBARs excretion and renal gp91phox expression, as well as decreased production of inflammatory markers, including p-ERK, ICAM-1, and MCP-1, in DN mice [29]. However, the efficacy of genistein on the connection of complex responses associated with oxidative stress and inflammation in DN is very uncertain. Moreover, little research has focused on the role of genistein in the development of DN in accordance with the degree of fasting blood glucose levels. In this study, we hypothesized that short-term genistein supplementation protects against diabetic kidney damage, depending on fasting blood glucose levels, through enhancement of hyperglycemia-induced oxidative stress, inflammation, and fibrosis in DN.

2. Materials and Methods

2.1. Animals. 5.5-week-old female ICR mice were obtained from Daehan Biolink Co., LTD (Eumseong, Choungcheongbuk-do, Republic of Korea). Mice were individually housed in cages and acclimated for a week in animal facility conditions ($22 \pm 1^{\circ}C$ and $50 \pm 1\%$ humidity with a 12 h in the light/dark). Diabetes was induced with a single intraperitoneal (i.p.) injection of 150 mg/kg alloxan monohydrate (Sigma-Aldrich Co., St Louis, MO, USA) in saline. On the other hand, nondiabetic control mice were injected with only saline in the same manner as the diabetic mice were treated. After a 1-week treatment, the induction of diabetes was confirmed by measuring fasting blood glucose levels. Fasting blood glucose levels from the mouse tail vein were measured by using a one-touch blood glucose meter (LifeScan Inc., Milpitas, USA). Fasting blood glucose levels ≥250 mg/dL were considered as diabetes. All mice care and experiments were approved by the Animal Care Institutional Committee of Kyung Hee University, Seoul, Republic of Korea.

2.2. Experimental Design. Diabetic mice were subdivided into two groups in accordance with fasting blood glucose (FBG) levels: medium high FBG (DMMH; 250 mg/dL ≤ FBG levels ≤ 450 mg/dL) and high FBG (DMH; 450 mg/dL ≤ FBG levels ≤ 600 mg/dL). Mice were treated with different diets and divided into the following groups (n = 9-10 per group): non-diabetic mice (CON) and diabetic-control mice (DMC; DMMH-C, DMH-C) mice were fed an AIN-93G diet without genistein supplementation (0%). DM-0.025% (0.025% genistein; DMMH-0.025%, DMH-0.025%) mice were fed 0.025% genistein (LC Laboratories, Woburn, MA) supplementation. DM-0.1% (0.1% genistein; DMMH-0.1%, DMH-0.1%) mice were fed 0.1% genistein supplementation. More details are shown in Table 1. At the end of the treatment (2 weeks), body weight, food consumption, and fasting blood glucose levels were measured once a week. Mice were fasted 8 h and anesthetized with isoflurane. Blood samples were collected by cardiac puncture, and then they were centrifuged at 3000 rpm for 10 min at $4^{\circ}C$. The kidneys were washed in saline and frozen immediately in liquid nitrogen. All samples were stored at $-80^{\circ}C$ until subsequent analysis.

2.3. Measurement of Serum Biochemical Analysis (Lipid Profile). Blood samples were collected in heparin pretreated-tubes and centrifuged at 3000 rpm for 15 min to obtain plasma. The concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein (HDL) cholesterol in plasma were assayed using the enzymatic method. Briefly, 20 μL of plasma was mixed with an enzymatic kit (Bio-Clinical System, Gyeonggi-do, Republic of Korea) and incubated at $37^{\circ}C$ water bath for 10 min. Concentrations were determined at 505 nm, 550 nm, and 500 nm, respectively.

Table 1: Classification of experimental groups.

Group	Treatment
CON	Nondiabetic mice were fed AIN-93G diet without genistein supplementation
DMMH-C	Diabetic-control mice with the level of medium-high FBG between 250 and 450 were fed AIN-93G diet without genistein supplementation
DMMH-0.025%	Diabetic mice with medium high FBG levels between 250 and 450 were fed 0.025% genistein supplementation
DMMH-0.1%	Diabetic mice with medium high FBG levels between 250 and 450 were fed 0.1% genistein supplementation
DMH-C	Diabetic-control mice with the level of high FBG between 450 and 600 did not receive genistein supplementation
DMH-0.025%	Diabetic mice with high FBG levels between 450 and 600 were fed 0.025% genistein supplementation
DMH-0.1%	Diabetic mice with high FBG levels between 450 and 600 were fed 0.1% genistein supplementation

The atherogenic index (AI) of plasma was calculated by the following ratio: (TC/HDL-C)/HDL-C.

2.4. Renal Function Monitoring

2.4.1. Blood Urea Nitrogen (BUN) Measurement. Kidney function was measured by BUN. Specimens were examined by a commercially available kit (Asan pharmaceutical, Seoul, Republic of Korea) and incubated in a 37°C water bath for 5 min. Then, concentrations were determined at 580 nm using an ELISA reader (BIO-TEK instruments, Winooski, VT, USA).

2.4.2. Plasma Creatinine. Plasma creatinine levels were examined by a creatinine assay kit (Bio-Clinical System, Gyeonggi-do, Republic of Korea) according to the manufacturer's protocol. Briefly, a mixture of plasma and picric acid were centrifuged at 3000 rpm for 10 min. Supernatant was reacted by an NaOH reagent at room temperature for 20 min and determined at 515 nm using an ELISA reader.

2.5. Malondialdehyde (MDA) Measurement in Kidneys. Malondialdehyde (MDA) measurement was usually used for estimation of lipid peroxidation levels [30]. Briefly, kidney homogenates were prepared in 0.15 M KCl buffer. A total of 200 μL of homogenated kidney tissues were mixed with 200 μL of 8.1% SDS and incubated at room temperature for 10 min. A total of 3 mL of 20% acetic acid-0.8% thiobarbituric acid (TBA) mixture (1 : 1, v/v) and 600 μL of distilled water were added to make a total volume of 4 mL. The mixture was heated for 1 h in a 95°C water bath. After cooling in ice water, 1 mL distilled water and a 5 mL mixture of n-butanol and pyridine (15 : 1, v/v) were added to each tube. After centrifuging at 4000 rpm for 10 min, the upper layer was measured at 532 nm using an ELISA reader. Concentrations were determined using a 1,1,3,3-tetramethoxypropane (TMP, sigma-Aldrich, St. Louis, MO., USA) as a standard.

2.6. Preparation of Western Blot. For extraction of whole protein, 0.1 g of kidney tissues was homogenated at 4°C in lysis buffer (containing 20 mM Tris-HCl, 150 mM NaCl, pH7.5, 1% NP40, 0.5% Na-deoxycholate stock, 1 mM EDTA, 0.1% SDS) with a protease inhibitor (Sigma Aldrich) and centrifuged at 14,000 rpm for 30 min. The resulting supernatants were frozen at 80°C until western blot analysis. Nuclear extracts were prepared from 0.25 g of kidney tissue and homogenated in 5 mL of buffer A (0.6% NP40, 150 mM NaCl, 10 mM HEPES (pH7.9), 1 mM EDTA, 0.5 mM PMSF, Leupeptin, Pepstatin, and Aprotinin). After centrifugation (2,000 rpm, 4°C, 30 sec), the supernatants incubated on ice for 5 min, centrifuged at 5,000 rpm for 5 min, and discarded the supernatant. 200 μL of buffer B (25% Glycerol, 20 mM HEPES (pH7.9), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, Benzamidine, Leupeptin, Pepstatin, and Aprotinine) was added to the resulting pellet and shacked on ice for 20 min. The resulting suspensions were frozen at 80°C until western blot analysis. Protein concentration was measured using the NanoPhotometer (Implen, Germany). For gel electrophoresis, an equal amount of cytosolic and nuclear protein extracts (50 μg and 25 μg of total protein) was loaded in each lane. Proteins were separated by 10% SDS-PAGE and then transferred to the PVDF membrane (Millipore, Marlborogh, MA, USA). The membrane was blocked with 5% nonfat milk or 3% BSA in PBS containing Tween 20 (PBST) and probed overnight at refrigeration temperature with primary antibodies against Nrf2 (dilution 1 : 1000; Abcam), HO-1 (dilution 1 : 1000; Stressgen), GPx (dilution 1 : 16000; Abcam), CuZn-SOD (dilution 1 : 1000; Santa Cruz Biotechnology), MnSOD (dilution 1 : 1000; Stressgen), p65 (dilution 1 : 200; Santa Cruz Biotechnology), pIκBα (dilution 1 : 200; Santa Cruz Biotechnology), TNF-α (dilution 1 : 200; Santa Cruz Biotechnology), CRP (dilution 1 : 200; Abcam), MCP-1(dilution 1 : 1000; Cell Signaling), COX-2 (dilution 1 : 200; Stressgen), PKC (dilution 1 : 200; Santa Cruz Biotechnology), PKC-βII (dilution 1 : 200; Santa Cruz Biotechnology), TGF-β1 (dilution 1 : 200; Santa Cruz Biotechnology), and β-actin (dilution 1 : 1000; Santa Cruz Biotechnology). The membrane was washed with PBST and incubated with an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h. The target proteins were detected and visualized by enhanced chemiluminescence western blotting agents (Elpis Biotech, Republic of Korea) on an Image Analyzer (G box, Syngene, UK). The quantitation of each protein expression compared to the β-actin protein expression level was performed.

2.7. Statistical Analysis. All data are presented as mean ± SD. Sample normality was tested for primary outcomes (body weight, food intake, and fating blood glucose level). Statistical differences of variables (body weight, food intake, and fasting blood glucose level) between CON and DMH-C

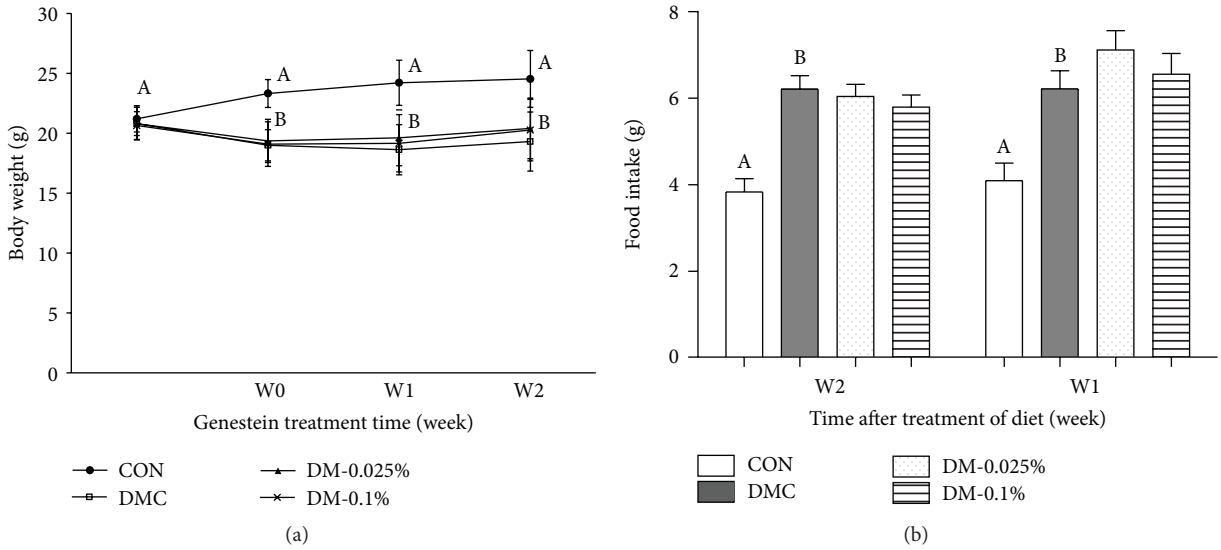

Figure 1: Effect of genistein supplementation on body weight (a) and food intake (b) in experimental mice. Data are presented as means ± SD (n = 9-10/group). Mean values with different letters were significantly different, $P < 0.05$. Statistical differences of variables between CON and DMH-C analyzed by unpaired t-test were shown in capital letters. CON, control mice; DMC, diabetic control mice; DM-0.025%, diabetic mice supplemented with 0.025% genistein; DM-0.1%, diabetic mice supplemented with 0.1% genistein.

were analyzed by unpaired t-test. The effects of DM severity (normal control, DMMH, and DMH) and/or genistein supplemented diet (0, 0.025, and 0.1%) were analyzed by one-way analysis of variance (ANOVA). Two-way ANOVA was used to analyze the effects of the genistein supplemented diet and DM severity and their interaction on outcomes followed by post hoc test (Tukey HSD) using SPSS (20.0 K for Windows) statistical analysis program. For all outcomes, a value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of Genistein Supplementation on Body Weight and Food Intake. Changes in body weight and food intake during the experimental period are shown in Figure 1. After 1 week of alloxan injection to induce diabetes, body weight in diabetic mice was significantly lower than that of CON (Figure 1(a)). However, genistein supplementation, regardless of dose, did not prevent the decrease in body weight. Food intake was significantly increased in diabetic mice, regardless of genistein supplementation compared with the CON group (Figure 1(b)).

3.2. Effect of Genistein Supplementation on Changes in Fasting Glucose Level. Levels of fasting blood glucose were significantly higher in all the diabetic groups compared to the CON group. The 0.025% genistein supplementation in DMMH significantly decreased FBG levels, but 0.1% genistein in DMMH did not significantly reduce FBG levels (Figure 2(a)). In Figure 2(b), genistein supplementation in DMH did not show a difference in FBG levels.

3.3. Effect of Genistein Supplementation on Biochemical Markers

3.3.1. Lipid Profiles. To examine the effect of genistein supplementation on lipid profiles, we measured plasma lipid profiles. As shown in Table 2(a), plasma levels of total cholesterol (TC) and triglycerides (TG) were elevated in the DMC more than in the CON, but there were no significant differences between the DMC groups and genistein supplementation groups. Moreover, the plasma level of high density lipoprotein cholesterol (HDL-C) did not differ among the groups. Thus, the DMC groups were characterized by a markedly elevated atherogenic index (AI) as compared to the CON group, but genistein supplementation did not effectively decrease AI.

3.3.2. Blood Urea Nitrogen (BUN). As shown in Table 2(b), the concentration of BUN was significantly increased in the DMMH-C and DMH-C groups compared to that of the CON group ($P < 0.05$). BUN concentrations of 0.025% DMMH and 0.1% DMMH were decreased by 47% and 43%, respectively, as compared to the DMMH-C group, BUN concentrations of 0.025% DMH and 0.1% DMH groups were significantly decreased by 52% and 51%, respectively, as compared to the DMH-C group.

3.3.3. Plasma Creatinine. As shown in Table 2(c), plasma creatinine levels were significantly elevated in the DMMH-C and the DMH-C groups compared with the CON group ($P < 0.05$). The concentration of plasma creatinine was much higher in the DMH-C group than in the DMMH-C group. Genistein supplementation, regardless of dose, in the DMMH group ameliorated plasma creatinine levels. However, although the plasma creatinine level of the DMH group

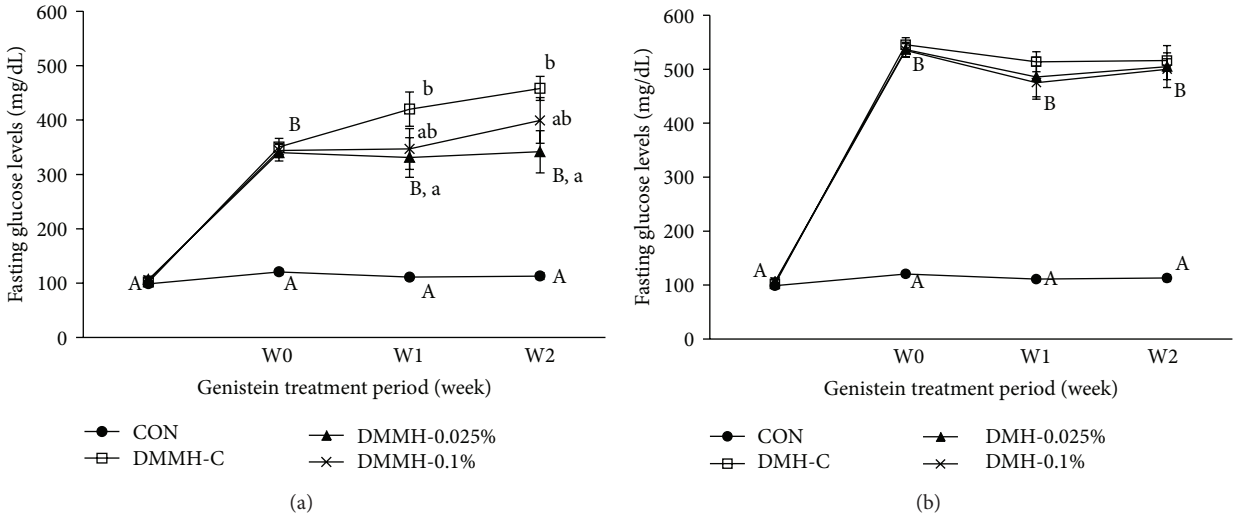

Figure 2: Effect of genistein supplementation on fasting blood glucose levels in experimental mice. Fasting blood glucose levels in DMMH group (a) and fasting blood glucose levels in DMH group (b). Data are presented as means ± SD (n = 9-10/group). Mean values with different letters were significantly different, $P < 0.05$. Statistical differences of variables between CON and DMH-C analyzed by unpaired t-test were shown in capital letters and effects of the genistein supplemented diets on body weight and food intake in diabetic mice using one-way ANOVA was represented by small letters.

Table 2: Effect of genistein supplementation on biochemical markers.

Group	Lipid profiles			AI	Kidney function		Oxidative stress
	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)		BUN (mg/dL)	Creatinine (mg/dL)	MDA (nM)
CON	121.72 ± 12.83^{A}	87.03 ± 19.35^{A}	77.74 ± 13.45	0.57 ± 0.23^{A}	17.76 ± 3.04^{A}	0.57 ± 0.08^{A}	16.87 ± 4.28^{A}
DMMH-C	187.98 ± 21.15^{B}	119.89 ± 26.92^{AB}	70.24 ± 11.61	0.97 ± 0.2^{B}	46.99 ± 21.49^{BCb}	0.81 ± 0.13^{Bb}	25.82 ± 6.23^{Bb}
DMMH-0.025%	171.65 ± 21.47	96.19 ± 22.39	75.93 ± 5.62	0.80 ± 0.14	24.93 ± 10.99^{a}	0.62 ± 0.10^{a}	17.23 ± 3.01^{a}
DMMH-0.1%	178.56 ± 39.68	109.65 ± 14.96	73.81 ± 5.36	0.88 ± 0.24	26.78 ± 5.56^{a}	0.64 ± 0.09^{a}	17.65 ± 2.97^{a}
DMH-C	206.64 ± 43.56^{B}	132.21 ± 20.88^{B}	64.05 ± 8.78	0.98 ± 0.22^{B}	67.35 ± 58.51^{Cb}	0.94 ± 0.25^{Cc}	25.90 ± 3.57^{Bb}
DMH-0.025%	172.29 ± 45.89	107.71 ± 29.20	73.83 ± 15.64	0.93 ± 0.23	32.30 ± 10.68^{a}	0.72 ± 0.11^{ab}	18.33 ± 5.26^{a}
DMH-0.1%	173.32 ± 46.33	121.43 ± 35.15	71.10 ± 29.38	0.94 ± 0.41	32.78 ± 7.95^{a}	0.79 ± 0.21^{bc}	20.04 ± 4.94^{ab}

Abbreviations: TC: total cholesterol, TG: triglyceride, HDL: high density lipoprotein cholesterol, AI: atherogenic index, BUN: blood urea nitrogen, and MDA: malondialdehyde.

Mean values with different letters were significantly different ($P < 0.05$). Statistical differences of variables among CON, DMMH-C, and DMH-C analyzed by one-way ANOVA were shown in capital letters and effects of the genistein supplemented diet and/or DM severity using two-way ANOVA were represented by small letters.

reached more than 1.5-fold compared to the CON group, only the 0.025% genistein supplementation significantly decreased plasma creatinine levels in DMH.

3.3.4. MDA. To examine the effect of genistein supplementation on oxidative stress in kidneys, kidney MDA levels were measured. MDA levels were significantly elevated in both the DMMH-C and DMH-C groups (1.5-fold above CON, $P < 0.05$). On the other hand, genistein supplementation in the DMMH-C groups reduced the level of MDA concentration to the normal level. The supplementation of 0.025% genistein significantly decreased the kidney MDA levels (DMMH-L; 33.26%, DMH-H; 29.22% compared to DMC), but the supplementation of 0.1% genistein did not significantly reduce it in the DMH mice (Table 2(d)).

3.4. Effect of Genistein on Protein Expression Levels of Oxidative Stress Markers in Diabetic Kidneys. We performed western blot analysis to determine whether genistein supplementation declined the activation of Nrf2-linked oxidative stress proteins in DN. The levels of cytosolic Nrf2 protein expression decreased in the DMMH-C and DMH-C groups ($P < 0.05$, Figure 3(a)). We found that the reduction of cytosolic Nrf2 protein levels in DMMH was effectively restored by genistein supplementation regardless of dose. The 0.025% genistein supplementation in DMH significantly raised the cytosolic Nrf2 levels, more than the DMH-C ($P < 0.05$), but 0.1% genistein in DMH did not significantly affect cytosolic Nrf2 expression. The expression of HO-1 levels, a representative target gene in the Nrf2 pathway, was significantly increased in the DMC as compared with the CON ($P < 0.05$). In addition, the expression of

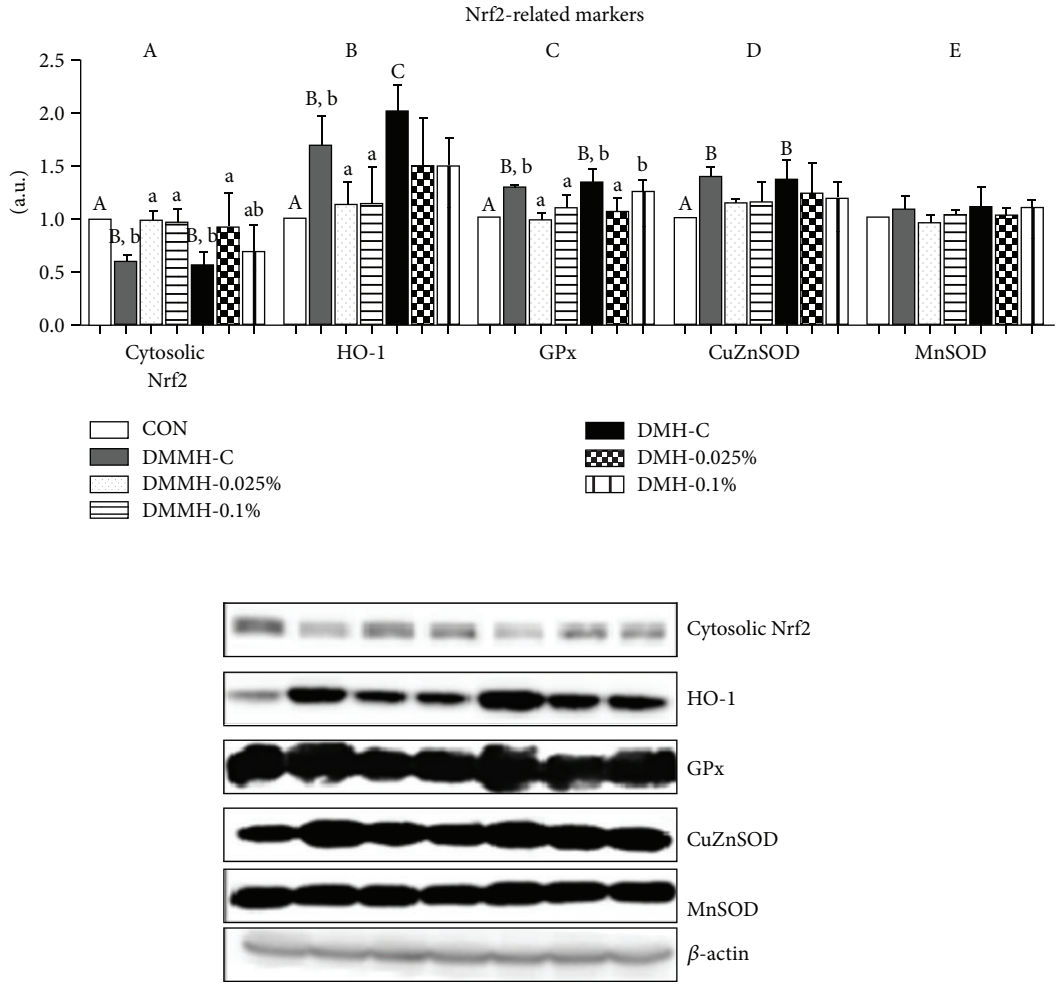

Figure 3: Effect of genistein supplementation on the kidney protein levels of cytosolic Nrf2 (a), HO-1 (b), GPx (c), CuZnSOD (d), and MnSOD (e) in experimental mice. All results were conducted at least three times. Data are presented as means ± SD (n = 9-10/group). Mean values with different letters were significantly different, $P < 0.05$. Statistical differences of variables among CON, DMMH-C, and DMH-C analyzed by one-way ANOVA were shown in capital letters and effects of the genistein supplemented diet and/or DM severity using two-way ANOVA were represented by small letters.

HO-1 was much higher in the DMC-C group than in the DMMH-C group. Genistein supplementation, regardless of dose, completely reduced the expression of HO-1 levels in DMMH and DMH (Figure 3(b)). Furthermore, GPx levels were significantly increased in DMC mice, more so than in CON mice (Figure 3(c)). GPx expression in the DMMH group was normalized by genistein supplementation independently of the dose. In the DMH group, 0.025% genistein supplementation significantly reduced GPx expression, while 0.1% genistein supplementation was not changed. As shown in Figure 3(d), the expression of CuZnSOD levels was higher in the DMMH-C and the DMH-C than in the CON. Genistein supplementation relatively decreased the CuZnSOD levels, although the difference was not statistically significant. Unfortunately, the expression of MnSOD levels did not significantly differ among the groups (Figure 3(e)).

3.5. Effect of Genistein on Protein Expression Levels of Inflammation Markers in Diabetic Kidneys. We tested to elucidate whether the genistein supplementation reduced the expression of NFκB-related inflammatory proteins in DN. The levels of NFκB (p65) and pIκBα, an indirect marker for measuring the activation of NFκB, were significantly increased in the DMC as compared to the CON (Figures 4(a) and 4(b)). Genistein supplementation, regardless of dose, significantly decreased the levels of cytosolic pIκBα and nuclear NFκB in DMMH and DMH compared to DMMH-C and DMH-C ($P < 0.05$). Next, we measured the expression of CRP, which was increased by alloxan-induced diabetes (Figure 4(c)). Genistein supplementation, regardless of dose, significantly inhibited increased CRP levels in the DMMH and DMH groups ($P < 0.05$). As shown in Figure 4(d), MCP-1 levels were higher in DMMH-C and DMMH-C than in CON, and the levels in DMMH and DMH were markedly lower by 0.025% genistein. However, 0.1% genistein showed no significant inhibitory effects on the MCP-1 levels in DMMH and DMH. The protein expression of COX-2, as a representative marker of the NFκB-pathway, was significantly

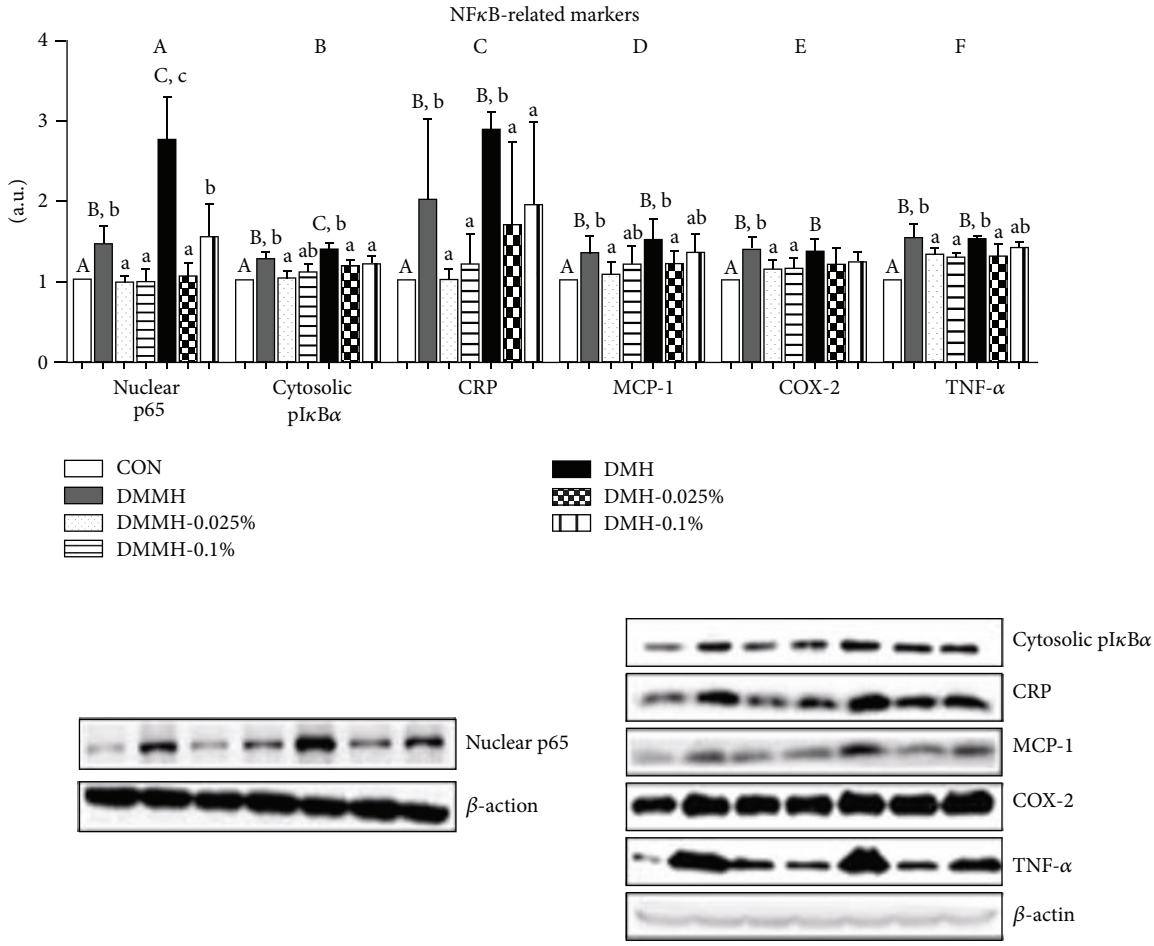

Figure 4: Effect of genistein supplementation on the kidney protein levels of p65 (NFκB) (a), pIκBα (b), CRP (c), MCP-1 (d), COX-2 (e), and TNF-α (f) in experimental mice. All results were conducted at least three times. Data are presented as means ± SD (n = 9-10/group). Mean values with different letters were significantly different, $P < 0.05$. Statistical differences of variables among CON, DMMH-C, and DMH-C analyzed by one-way ANOVA were shown in capital letters and effects of the genistein supplemented diet and/or DM severity using two-way ANOVA were represented by small letters.

elevated in DMC ($P < 0.05$). Genistein supplementation in DMMH suppressed the upregulation of COX-2 levels, while there was no difference in the DMH groups (Figure 4(e)). Additionally, TNF-α levels were significantly higher in all diabetic mice than in CON mice (Figure 5(f)). However, the genistein supplementation groups exhibited a remarkable reduction in the expression of TNF-α in comparison with the DMC groups, excluding DMH-0.1% ($P < 0.05$).

3.6. Effect of Genistein on Protein Expression Levels of Fibrosis-Mediated Markers in Diabetic Kidneys. We examined the question as to whether genistein supplementation contributed to enhancing an antidiabetic kidney fibrosis pathway in the experimental mice. Our data showed a significant increase in PKC and PKC-βII protein expression in the DMC groups, regardless of FBG levels (Figures 5(a) and 5(b)). The levels of PKC and PKC-βII protein expression in DMMH were effectively decreased by genistein supplementation regardless of dose ($P < 0.05$). The level of PKC expression in DMH was reduced by genistein supplementation, regardless of dose, but there was not a significant difference (Figure 5(a)). The 0.025% genistein supplementation in DMH was more effective at reducing the level of PKC-βII protein expression than the 0.1% genistein in DMH (Figure 5(b)). To further investigate the mechanism of an antifibrosis effect of genistein, we tested the expression of TFG-βI. TFG-β1, as one of the most potent fibrogenic response markers, was greater in DMC than those of CON. However, as contrasted with nontreated genistein supplementation, genistein supplementation groups experienced significant decreased expression of TFG-βI, except DMH-0.1% ($P < 0.05$).

4. Discussion

The present study provides some good evidence that genistein has an ability to protect kidneys from hyperglycemia-induced oxidative stress, inflammation, and fibrosis in alloxan-induced diabetic mice. Although genistein has beneficial influences with respect to both antioxidative stress and anti-inflammation [31], there is no clear underlying mechanism by

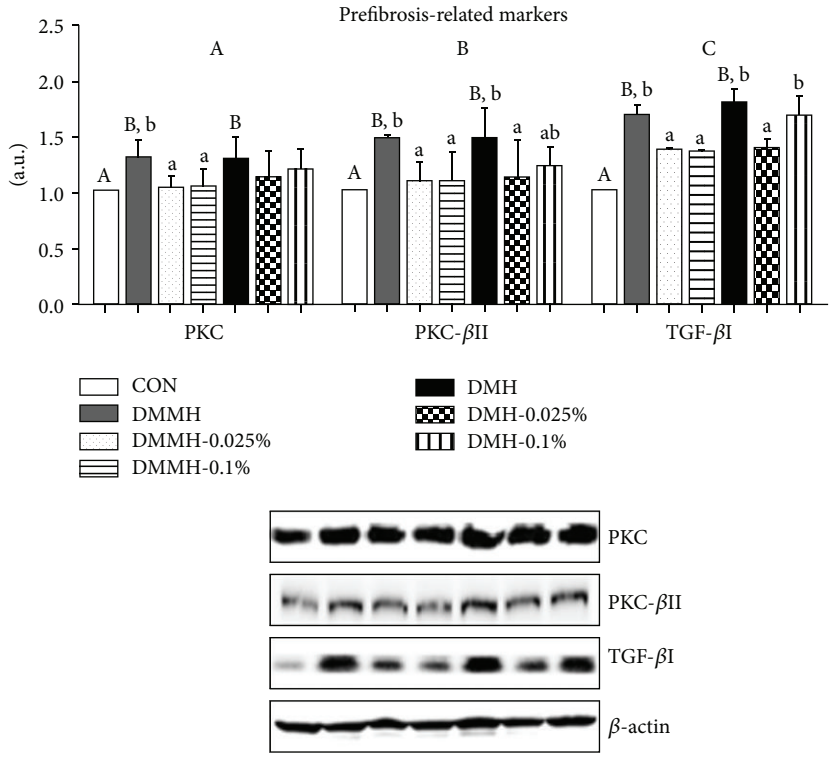

Figure 5: Effect of genistein supplementation on the kidney protein levels of PKC (a), PKC-βII (b), and TFG-βI (c) in experimental mice. All results were conducted at least three times. Data are presented as means ± SD (n = 9-10/group). Mean values with different letters were significantly different, $P < 0.05$. Statistical differences of variables among CON, DMMH-C and DMH-C analyzed by one-way ANOVA were shown in capital letters and effects of the genistein supplemented diet and/or DM severity using two-way ANOVA was represented by small letters.

which genistein can boost a protective role in DN progression in accordance with blood glucose levels. A severe loss of body weight (B.W.) and an increase of food intake generally occur in diabetic conditions [32–34]. We also examined the question as to whether genistein supplementation did not improve body weight loss and food intake as shown in the previous studies [35, 36].

An ultimate treatment goal of diabetes and its complications is the control of the FBG levels [37]. According to previous studies, a glucose level with 250–450 mg/dL was diagnosed as mild hyperglycemia [38], and a glucose level above 450 mg/dL was considered as severe hyperglycemia [39]. In this study, diabetes with different FBG levels, in a range from 250 mg/dL to 600 mg/dL (maximum read by commercial glucometer), and FBG levels of 450 mg/dL were used as the criteria of hyperglycemia classification. Our data found that genistein supplementation decreased the FBG level in DMMH mice, but it did not affect blood glucose levels in DMH mice. Previously, genistein has been shown to have an effect on the modulation of blood glucose levels *in vivo*, regardless of the manner of genistein administration and treatment period and dose, which have included short-term (for 16 days) i.p. injection of genistein (1 mg/kg B.W./day) in rats fed a fructose rich diet [27] and long-term (for 9 weeks) dietary supplementation of genistein (0.02% w/w) in nonobese diabetic (NOD) mice [35]. In other findings [40], it was discovered that not a low dose (under 15 mg/kg B.W.) but a high dose (15–30 mg/kg B.W.) of genistein supplementation markedly reduced blood glucose levels in alloxan-induced diabetes mice. However, researchers have not proven a potential benefit of genistein on diabetic animals with different levels of FBG. Collectively, the results suggested that short-term supplementation of genistein possesses the capacity to reduce hyperglycemia in the DMMH group without insulin treatment but not in DMH group.

Impairment of insulin secretion in diabetes increases the release of free fatty acids (FFA) into the liver, and it may cause an increase in triglyceride production [41]. It promotes diabetic dyslipidemia, which may worsen the interplay of inflammation and intrarenal fibrosis [42, 43]. A previous study reported that genistein supplementation (600 mg/kg diet) for 3 weeks improved plasma lipid profiles (TC, TG, and HDL) in diabetic mice [44], whereas another study confirmed that genistein supplementation (250 mg/kg diet) for 4 weeks did not improve the plasma lipid profiles in diabetic mice [45]. Our data showed that genistein supplementation, regardless of supplementation doses (0.025% in a 250 mg/kg diet or 0.1% in a 1000 mg/kg diet), did not show a lowering effect on dyslipidemia. These results suggest that diabetes-related dyslipidemia is controlled by a relatively high concentration of genistein supplementation for longer than 3 weeks of treatment.

BUN and plasma creatinine, as waste products of metabolism, preannounce damage in kidney function [46].

We observed that BUN and plasma creatinine levels were increased in DMC mice, especially in DMH-C. Sung et al. [47] reported that the genistein addition (10 mg/kg B.W.) for 3 days significantly reduced BUN and serum creatinine levels in cisplatin-induced acute renal injury. We also observed that genistein supplementation decreased BUN levels in DMMH group and DMH group. BUN is usually done together with a plasma creatinine, which is a more sensitive marker of kidney damage. Genistein supplementation in DMMH alleviated plasma creatinine levels to normal levels and significantly reduced the levels in DMH-0.025%, but not in DMH-0.1%. Thus, our data demonstrated that genistein, regardless of supplemented dose, could prevent an impairment of kidney function in DMMH, and only the 0.025% genistein supplementation may have beneficial effects on kidney damage when the FBG level is very high.

In diabetic conditions, a continuous overproduction of ROS and an antioxidant defense system may cause mitochondrial impairment [48]. Thus, oxidative stress is considered as a mediator in tissue injury, including liver, brain, and kidney. The kidney is known as a highly sensitive organ in oxidative stress conditions because lipid composition in kidneys comprises long-chain polyunsaturated fatty acids [49]. In our experiments, the MDA accumulation was increased by consequences of oxidative stress, such as diabetes [50], but genistein (6 mg/kg/B.W.) decreased the MDA levels in the brain and liver of STZ-induced diabetic mice [51]. Our study also observed that genistein supplementation significantly lowered kidney MDA levels in diabetic mice, except in DMH-0.1%. A previous study demonstrated that a high dose of genistein can have adverse actions as a prooxidant, depending on the status of oxidative stress [52]. Therefore, the results suggest that 0.1% genistein supplementation may act as a prooxidant in the DMH group, which is considered as possessing higher oxidative stress status compared to the DMMH group.

Nrf2 is normally combined with its repressive protein Keap1 (Kelch-like ECH-associated protein-1) in cytoplasm [53]. In an oxidative stress state, Nrf2 is separated from Keap1 and translocated to the nucleus. It activates antioxidant enzymes such as HO-1, GST, NADH(H) quinoline oxidoreductase-1 (NQO1), and glutathione peroxidase (GSH-Px) [54, 55]. Therefore, Nrf2 and its downstream genes play a crucial role in defense of cellular damage against oxidative stress, but its overproduction may lead to paradoxical effects in connection with a disturbance in the protection of cells from oxidative damage [56]. Previous studies reported that expression of Nrf2 and antioxidant genes, such as HO-1, SOD, catalase (CAT), and GPx, was increased in diabetes [57–59]. The results suggest that excessive production of oxidative stress seems to stimulate increases in antioxidant enzyme production in order to eliminate oxidative stress agents in DM. It is known that genistein has cytoprotective effects on Nrf2 activation and its downstream antioxidant enzymes, including HO-1, SOD, CAT, and GSH [60]. Our data showed that cytosolic Nrf2 was decreased in diabetes, which may lead to an increase in nuclear Nrf2 activation as a consequence of the activation of a cellular antioxidant defense with increased transcription of antioxidant genes. The results were reversed by the genistein supplementation, and this outcome supports the hypothesis that genistein supplementation was able to reestablish the cell homeostasis. However, 0.1% genistein in DMH did not markedly change Nrf2 levels. These findings indicate that 0.1% genistein may be not enough to provide beneficial effects on the Nrf2-mediated oxidative stress pathway in diabetic mice with high FBG levels.

HO-1, a representative marker of an Nrf2-related stress response, has been found to increase in pathological conditions such as diabetes [61–63]. The present study demonstrated that genistein supplementation, regardless of dose, tends to reduce the expression of HO-1 levels in DMMH and DMH. Moreover, protein levels of GPx and SOD isoforms are associated with oxidative damage and mitochondrial dysfunction through hydrogen peroxide (H_2O_2) production by the glucose oxidase system [64–66]. A previous study [67] demonstrated that GPx activity was increased in diabetic mice organs including the liver, pancreas, and kidney. Our findings proved that genistein supplementation reduced GPx levels, except the DMH-0.1% group. However, genistein did not significantly reduce CuZnSOD levels and did not change MnSOD levels among the groups. These results suggest that genistein supplementation selectively alleviated oxidative stress through the regulation of Nrf2 levels and its consequent events. Moreover, the DMH group with a high dose supplementation of genistein may have more oxidative stress.

Nrf2-mediated interplay has two sides of action as either a regulator of antioxidant response or a reactive promoter of oxidative stress in abnormal conditions [68]. On the basis of our results, we proposed that an overproduction of reactive oxygen species (ROS) in diabetes can trigger activation of nucleus Nrf2 and transcription of its downstream target enzymes. On the other hand, oxidative stress leads to activation of the inflammatory-mediated transcription factor, NFκB. Thus, we identified the fact that genistein supplementation attenuated the hyperglycemia-induced inflammatory responses through the regulation of the NFκB pathway. Many studies have reported experimental evidence showing that NFκB was activated in diabetic kidneys [69, 70], and genistein supplementation (1 mg/kg/B.W.) attenuates NFκB (P65) activation in kidneys of rats fed a fructose rich diet [71]. We have investigated the pIκBα level in cytosol and NFκB (p65) level in nucleus to identify NFκB activation. pIκBα level is a representative of NFκB activation in cytosol because pIκBα after phosphorylation of IκBα is subsequently ubiquitinated and degraded via the proteasome pathway [72]. NFκB, p65 and p50 heterodimer, separated from IκBα is translocated into the nucleus and activates the expression of inflammatory genes. Our data showed that the protein levels of pIκBα in cytosol and NFκB in nucleus as increased in DMC and lowered in genistein supplementation. These results imply that genistein supplementation blocked NFκB activation by reduction of pIκBα.

Activation of the NFκB signaling pathway is known to enhance inflammatory cytokines (IL-1β, TNF-α) and activate fibrosis markers (AGE, RAGE) in diabetic mice [73]. Among them, TNF-α (tumor necrosis factor-α) is the main proinflammatory cytokine, which acts toward the progression of diabetic kidney disease through recruitment of macrophages

and neutrophils into the kidney [74]. An increased renal TNF-α level is correlated with indicators of renal failure in DM animals [75] and patients [76]. The present study confirmed that TNF-α levels in genistein supplementation groups were even lower than those in DMC groups. However, DMH with 0.1% genistein did not show significant differences, which means that 0.025% genistein is more effective than 0.1% genistein for DN with high FBG levels. CRP is generally increased in inflammatory conditions, such as those found in DN patients and animals [77, 78]. Dietary isoflavone, including genistein, has a capacity to decrease the concentration of CRP in human plasma [79, 80]. Similarly, the expression of CRP levels in DN mice was significantly reduced in all diabetic mice supplemented with genistein, more so than those of DMC. In addition, expression of MCP-1 and COX-2 is relevant to NFκB-mediated modulation of an inflammatory cascade, which contributes to endothelial dysfunction [81–83]. Genistein (10 mg/kg via i.p., three times a week) as a tyrosine kinase inhibitor has been shown to reduce significantly the excretion of urinary MCP-1 in STZ-induced diabetic mice [29]. Our results showed that the production of MCP-1 significantly decreased in the 0.025% genistein supplementation groups, whereas the 0.1% genistein supplementation groups did not reduce MCP-1 production. Thus, the results suggest that a relatively low dose of genistein may reduce MCP-1 protein via inhibition of NFκB activation. Moreover, genistein, as an inhibition agent of cell proliferation, inhibited COX-2 protein in cancer cells [84], a result that improved the balance of angiogenesis and apoptosis. In our findings, overproduction of COX-2 in DMMH was attenuated by genistein supplementation at both 0.025% and 0.1% levels, but not in DMH. In other words, 0.025% genistein supplementation in diabetes with medium high FBG may control vascular homeostasis through suppression of NFκB-mediated inflammation.

Moreover, the findings indicate that Nrf2 activation and its downstream signalling pathway interact with the activation of NFκB-mediated inflammatory responses in diabetes, and genistein supplementation might reduce activation of antioxidant defence systems and inflammatory responses by regulation of Nrf2 and NFκB interactions.

Nrf2 and NFκB interactions may play a serious role in fibrosis in diabetic kidneys, which corresponds with increased PKC-mediated pathways in hyperglycemic conditions. This conclusion has been supported by several *in vitro* experiments [85], which demonstrated that genistein (40 μM) blocked PKC activation in VEGF-stimulated endothelial cells [86]. However, there is no research focusing on the effect of genistein on PKC inhibition in diabetic animals. The PKC-β isoform is mainly responsible for hyperglycemia-induced fibrosis in DN [87]. Several reports have provided evidence that genistein attenuated the levels of PKC isoenzymes, such as PKC-βI, in rat ventricular monocytes [88], as well as levels of PKC-βII in rats fed a fructose rich diet, an experiment that constitutes a hypertension mouse model [89]. Our data showed the different effects of genistein on prefibrosis-related markers, both PKC and PKC-βII, in DMH depending on their treatment dose. The 0.1% genistein supplementation in the DMH group did not significantly reduce the levels of both PKC and PKC-βII. This result suggests that 0.1% genistein supplementation may not have beneficial effects on fibrosis in diabetes with high FBG. Continuous exposure of ROS in hyperglycemia may also lead to changes in cell membrane structure. The transforming growth factor βI (TFG-βI), a family of fibrogenic cytokine, has been generally known to induce deposition of matrix components, such as ECM, as well as synthesis of glomerulosclerosis in DN rats [90]. A hyperglycemic condition induces an increase in TGF-βI levels, and it stimulates fibrosis of numerous organs, such as the kidney [91]. Thus, inhibition of TGF- βI is a key player in protection of diabetic kidneys. Genistein has been proven effective in the prevention of hyperglycemia-induced fibrosis by inhibiting the expression of TGF- βI [28] and TGF- βII [92]. In particular, the data showed that genistein was able to inhibit TGF-βI production, not at a low concentration ($\leq 5\,\mu\text{mol}\cdot\text{L}^{-1}$), but at a high concentration ($\geq 5\,\mu\text{mol}\cdot\text{L}^{-1}$) [28], and to reduce TGF-βII production at the high concentration level (5 μg/mL) [92]. However, our results demonstrated that 0.1% genistein supplementation did not protect against the fibrosis process, represented by TGF-βII, from a severe hyperglycemic condition in DMH. The results might be associated with the prooxidant effect of genistein at high doses on severe hyperglycemia as a promotor of prefibrosis in DN.

Taken together, our data evidenced that genistein supplementation inhibited hyperglycemia-induced fibrosis pathways as well as the activation of the transcription factors, Nrf2 and NFκB. Moreover, we found that genistein supplementation has selective effects on diabetic kidney damage in accordance with FBG levels. In previous studies, genistein has been shown to have adverse effects in pathogenetic conditions, which may act as prooxidants and accelerate the progression of disease [93]. However, this study has several limitations. Only the short-term effects of dietary genistein supplementation have been investigated with respect to diabetes induced kidney damage. Long-term supplementation protocols may be helpful to verify the role of genistein in the DMH group (>450 mg/dL) because the DMH group may need a longer time to control inflammation, oxidative stress, and fibrosis processes. Moreover, short-term supplementation at different doses did not change plasma lipid profiles. This result might be associated with supplemented doses and periods, as well as FBG levels. In addition to treatment regimens, histological analysis of kidneys may be more helpful to investigate fibrosis process in this study.

In conclusion, understanding the molecular mechanisms that regulate oxidative stress, inflammation and fibrosis is critical not only in diabetic kidney damage, but also in other diabetic complications. Hence, the results of this study may provide critical insight into future nutritional intervention strategies, with or without insulin treatment, designed to prevent diabetic complications according to FBG levels.

Abbreviations

DN: Diabetic nephropathy
FBG: Fasting blood glucose
ROS: Reactive oxygen species

BUN: Blood urea nitrogen
NFκB : Nuclear factor kappa B
Nrf2: Nuclear related factor E2
HO-1: Heme oxygenase-1
CRP: C-reactive protein
MCP-1: Monocyte chemotactic protein-1
COX-2: Cyclooxygenase-2
GPx: Glutathione peroxidase
MDA: Malondialdehyde
CuZnSOD: Copper zinc superoxide dismutase
MnSOD: Manganese superoxide dismutase
pIκBα: Phosphorylated inhibitory kappa B alpha
TNF-α: Tumor necrosis factor-alpha
PKC: Protein kinase C
PKC-βII: Protein kinase C-beta II
TGF-βI: Transforming growth factor-beta I.

Conflict of Interests

The authors and manufacturers disclose no actual potential conflict of interests.

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Review Article

Vitamin D Receptor Agonists Target CXCL10: New Therapeutic Tools for Resolution of Inflammation

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Understanding the many biological extraskeletal actions of vitamin D has increased in the past decades. Indeed, vitamin D and analogue molecules, besides the classical actions on bone metabolism, exert several beneficial effects on metabolic homeostasis, heart-cardiovascular, brain, and muscle physiological functions, throughout the interaction with the specific vitamin D receptor (VDR). In particular, VDR agonists powerfully control innate and adaptive immune system with favorable effects on human health. VDR ligands act as immunomodulators that are potent enough to retain anti-inflammatory effects, even though the mechanism underlying those effects is not yet fully elucidated. VDR agonists exert a significant suppression of inflammatory processes switching the immune response from T helper 1 (Th1) to T helper 2 (Th2) dominance and counteracting the self-enhancing inflammatory loop between immune and resident cells, especially by cytokine release impairment. Those molecules are able, indeed, to reduce the release of the interferon (IFN)γ-induced 10 kDa protein IP-10/CXCL10, a powerful chemokine driving Th1-mediated inflammation. Based on their features, VDR ligands show the potentiality to be included in immunosuppressive regimens, aimed to control auto- and alloimmune Th1-driven overreactivity, occurring, for example, in autoimmune disease or graft rejection.

1. Introduction

The concept that vitamin D, classically categorized as a regulator of calcium/phosphorous balance and bone metabolism, is able to act on the immune system has emerged more than 20 years ago [1, 2]. Since then, accumulating evidence confirmed that those pharmacologic effects on the immune system are suppressive enough to retain therapeutic potentials for the management of immune-related inflammatory diseases. Furthermore, epidemiological studies show that circulating altered levels of vitamin D are associated with a higher susceptibility to immune-mediated disorders and inflammatory diseases [3].

The pleiotropic activities in immune regulation by VDR agonists rely on their ability to interfere with maturation/differentiation/activation of the majority of the immune system cells which express VDR, such as monocytes, macrophages, B and T lymphocytes, neutrophils, and dendritic cells (DCs). Vitamin D, for example, is able to suppress cellular immune response by inhibiting the proliferation of T cells and the maturation of DCs, the most potent antigen-presenting cells (APCs) [4]. Hence, vitamin D can polarize Th1 immune response, which dominates in inflammation, toward a more regulatory Th2 phenotype, which dominates in tolerogenicity, by specifically repressing Th1 cytokine gene transcription in immune cells. Notably, VDR agonists are able to inhibit cytokine expression and release also in tissue resident cells, with a definite anti-inflammatory effect.

In particular, we have previously reported on the ability of two less or nonhypercalcemic VDR ligands, BXL-01-0029 and elocalcitol, to counteract in lymphocytes and different human resident cell types the release of CXCL10 [5–7]; this is a key chemokine triggering Th1 inflammatory molecular processes, in auto- or alloimmune response, secreted by several types

of resident cells (skeletal muscle cells, thyrocytes, cardiomyocytes, tubular renal cells, and human adrenal cells) [7–12].

In the present paper we intend to offer an overview on VDR agonists as new pharmacological tools with anti-inflammatory properties on immune and tissue resident cells, with particular attention to CXCL10, as a new biomolecular target for resolution of inflammation.

2. Vitamin D

The pleiotropic hormone vitamin D, also known as vitamin D_3 or calcitriol, is known since almost 90 years ago to prevent rickets in children, osteomalacia in adults, and hypocalcemic tetany [13, 14]; for long time, its function has been considered to be exerted exclusively on calcium, phosphorus, and bone metabolism. With the years, beside the other vitamin D functions—for example, on metabolism, cardiovascular system, muscle and brain functions, and cell growth/differentiation—important effects have been documented on the immune system. In fact, more than 25 years ago, the immunomodulatory role of vitamin D emerged [3] after the observation that monocytes/macrophages from patients affected by granulomatous disease sarcoidosis constitutively synthesize the active form of vitamin D [4]. Vitamin D is synthesized through a multistep process, which begins in the skin. The ultraviolet light (appropriate wavelength: 270–300 nm) photocatalyzes the conversion of the precursor 7-dehydrocholesterol to previtamin D_3 or cholecalciferol, without any significant biological activity until its conversion to the hormonally active form, 1,25-dihydroxycholecalciferol. This conversion occurs in two steps. Within the liver, cholecalciferol is hydroxylated to 25-hydroxycholecalciferol [$25(OH)D_3$] by the enzyme 25-hydroxylase (CYP2R1); within the kidney, 25-hydroxycholecalciferol serves as a substrate for 1-alpha-hydroxylase (CYP27B1), yielding 1,25-dihydroxycholecalciferol or calcitriol [$1,25(OH)_2D_3$], the biologically active form. Each form of vitamin D is hydrophobic and transported throughout the body by the specific vitamin D binding proteins (DBP). Vitamin D action is limited by catabolism—mainly by a 24-hydroxylase (CYP24A1)—which results in 1,24,25-trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$], a compound with substantially lower affinity for the VDR; this compound is further metabolized to calcitroic acid and secreted in urine. The metabolism of vitamin D is complex and tightly regulated [15]. Rate limiting steps in the metabolism of vitamin D are the activity of CYP2R1, induced by low $25(OH)D_3$ levels, and the activity of CYP24A1, induced by high levels of $25(OH)D_3$, and $1,25(OH)D_3$ to avoid vitamin D toxicity. While liver and kidney are the main sites for vitamin D synthesis and degradation, many other tissues (colon, prostate, breast, lung, pancreas, brain, and endothelium) can synthesize and degrade the active form of vitamin D. Vitamin D biologic effects are exerted throughout the interaction with VDR, which is known to be present in over 30 human target tissues, as reported in Table 1 [16, 17].

Such a diffuse expression in several different tissues, together with metabolic enzyme presence, suggests paracrine/autocrine mechanisms of actions, in addition to the classical endocrine effect of the hormone.

Table 1: VDR is almost ubiquitary expressed in humans. Many of human tissues and organs express VDR: upon ligand-receptor interaction genomic and nongenomic action likely occur by endocrine, paracrine, and autocrine mechanisms.

Human organs and tissues expressing vitamin D receptor (VDR)	
Adipose	Pancreatic β-cell
Adrenal	Parathyroid
Bone	Parotid
Brain	Pituitary
Breast	Placenta
Cartilage	Prostate
Colon	Retina
Hair follicle	Skin
Heart	Sperm
Intestine	Stomach
Kidney	Testis
Liver	Thymus
Lung	Thyroid
Immune cells	Tonsils
Muscle, smooth and skeletal	Uterus
Ovary	

The dominant genomic pathway, by which vitamin D mediates its biologic effects, involves the regulation of target genes by ligand-receptor complex in the nucleus of target cells [18]. Summarizing, upon ligand-nuclear VDR interaction, vitamin D forms heterodimers with the retinoid X receptor (RXR) and its ligand (9 cis-retinoic acid); these dimers subsequently occupy specific binding sites on DNA, the vitamin D response elements (VDREs). In conjunction with other transcription factors, this complex induces the transcription of vitamin D responsive genes [19, 20]. Besides the well-characterized nuclear VDR, a less clearly defined cell membrane receptor, which mediates rapid nongenomic actions, has been hypothesized [21, 22]. Rapid nongenomic actions of vitamin D do not affect the nuclear transcriptional activity. Even if those vitamin D rapid mechanisms are still unclear, evidence suggests that the initiation of the fast nongenomic signal may involve the engagement of either a novel membrane receptor [23] or the nuclear VDR translocation to the cell surface [24].

Vitamin D classical genomic and "new" nongenomic actions are involved in the regulation of several critical functions, such as immunity, angiogenesis, differentiation, apoptosis, and cell growth. The inhibition, for example, by VDR ligands of prostate cell growth, either growth factor induced or neoplastic, is exerted throughout a rapid mechanism that blocks the phosphorylation/activation of growth factor receptors [25, 26]. In particular, due to their antiproliferative and prodifferentiation properties, known since quite ago [27], VDR ligands control tumoral cell growth in different models of cancer, such as prostate, breast, and colon [28–30]. Moreover, a strong epidemiological association between

prostate, breast, colon cancer, and vitamin D deficiency has been documented [31].

Interestingly, vitamin D plays a pivotal role also in immune system cell control and differentiation, with important effects on the immune-mediated response.

3. Protolerogenic Effects of VDR Agonists

3.1. Immune System Cells. VDR ligands usually exert their antiproliferative, prodifferentiation, and immunomodulatory effects throughout the activation of VDR—either constitutively present or induced—in the majority of the immune cells [32, 33]. Figure 1 summarizes the effects of vitamin D in different immune cell types. One of the first evidence for the immunoregulatory role of vitamin D was proven by the vitamin D-induced differentiation of monocyte precursors into mature macrophages [34]; the VDR high expression in monocytes has been hypothesized to be responsible for an autocrine mechanism for cell maturation, which, in fact, is impaired by vitamin D deficiency [35]. Monocytes from blood mononuclear cells (PBMCs) are able to synthesize vitamin D under inflammatory stimuli, such as interferon (IFN)γ or bacterial antigens [36, 37]; macrophage inflammatory response is modulated by vitamin D throughout the regulation of the release of critical inflammatory mediators, such as cytokines and chemotactic cytokines or chemokines. In both monocytes and macrophages, vitamin D regulates its own effects by controlling VDR and CYP27B1 expression and activity; signaling throughout Toll-like receptors (TLRs) is also engaged in association with VDR expression increase. In human monocytes treated with vitamin D, the expression of TLR2, TLR4, and TLR9 is inhibited, and TLR9-dependent interleukin (IL)-6 secretion is altered [38]. The observation that vitamin D, while promoting antimicrobial activity in myeloid cells, also inhibits TLR2 and TLR4 expressions in monocytes, suggested a feedback mechanism to prevent inflammatory overresponses by TLR activation at later stage of infection [39]; this downregulatory effect in APC might be one of the key mechanisms by which vitamin D is able to attenuate excessive Th1-driven inflammation and avoid downstream potential autoimmunity consequence [40]. Some stimulatory effects have been also shown on innate immunity, such as the increase of monocyte proliferation *in vitro* or IL-1 and cathelicidin (a bactericidal peptide) release by monocytes and macrophages [41, 42]; however, vitamin D effects on the adaptive immune response are predominantly suppressive.

In T cells, vitamin D inhibits not only proliferation but also IL-2 and IFNγ gene and protein expressions [43–46], likely through VDR-RXR complex interaction with VDREs in the promoter of the genes [47, 48]; it inhibits IL-17 and IL-2 expressions in $CD4^+$ T cells and decreases $CD8^+$ T cell-mediated cytotoxicity [49], with an overall effect towards a block of Th1-mediated response. Th2-type tolerogenic response is also promoted by a direct enhancement of IL-4 production [4]. Although vitamin D is known to stimulate the development and differentiation of regulatory T cells (Treg) enhancing their suppressive function [50–52], the direct effect on T cell differentiation and function is still unknown, since naïve T cells—differently from effector/memory T cells—express VDR at very low level [42]. However, it is quite clear that Treg cell differentiation is a key event connecting vitamin D with adaptive immunity, with potential beneficial effects for autoimmune diseases and host-graft rejection [3, 42, 53, 54]. It is widely accepted that those immunosuppressive functions are substantially driven by vitamin D induction of tolerogenic DCs [54–56]. In DCs, vitamin D inhibits differentiation and function as well, throughout a decrease in the expression of major histocompatibility complex (MHC) class II molecules and CD40, CD80, and CD86 [4, 57–59] costimulatory proteins; it decreases IL-6, IL-23, and IL-12 [60] while simultaneously increases IL-10 production. Those events also mirror a net decrease in Th1 cell response in favor of Th2-mediated events. By reducing IL-6 and IL-23 production, vitamin D likely inhibits also Th17 cells, another T cell subset deeply engaged in inflammatory responses; although the precise mechanism of vitamin D on Th17 regulation is still unclear [3], it seems that vitamin D-mediated Th1 and Th17 suppression occurs throughout Forkhead box protein 3 ($Foxp3^+$) Treg cells expansion [3]. Furthermore, B cell proliferation, plasma-cell differentiation, and immunoglobulin (IgG) secretion are also affected by VDR ligands [1, 61], maybe throughout their effect on APC or T cells [62]. Vitamin D is likely to play a pivotal role in the maintenance of B cell homeostasis by regulating autoantibody production; notably, the correction of vitamin D deficiency might ameliorate B cell-mediated autoimmune disorders [63]. Finally, it is likely that endogenous production of vitamin D by macrophage, DCs, and T cells physiologically regulates both innate and adaptive immune responses [64–67]. Immune cells, indeed, seem to be not simple targets of VDR agonists but responsible for activation/inactivation of vitamin D metabolites [68].

3.2. Organ/Tissue Resident Cells. The ability of VDR agonists to modify the function of T cells and DCs depends not only on VDR expression in both cell types, but also on the presence of common targets in their signal transduction pathways, such as the nuclear factor κB (NF-κB) [69]. NF-κB is a transcription factor well known to play a pivotal role in proinflammatory cytokine and chemokine production and release not just by immune system cells [70] but, remarkably, by different tissue resident cells, as previously reported [7–10, 12]. Based on VDR agonist capacity to inhibit NF-κB activation in tissue resident cells, a strong reduction in local release of potent chemotactic factors by organ/tissue cells occurs; this, in turn, mirrors a reduced recruitment of Th1 cells, macrophages, and DCs to the site of inflammation. That feature of VDR agonists is particularly relevant for the treatment of inflammation involved in both auto- or alloimmune response, since it counteracts the mechanisms underlying the self-enhancing inflammatory loop between immune and resident cells. The potential therapeutic application of the less-hypercalcemic VDR agonist BXL-219 for autoimmune type 1 diabetes (T1D) has been highlighted in nonobese diabetic (NOD) mice, which retain a pathogenesis

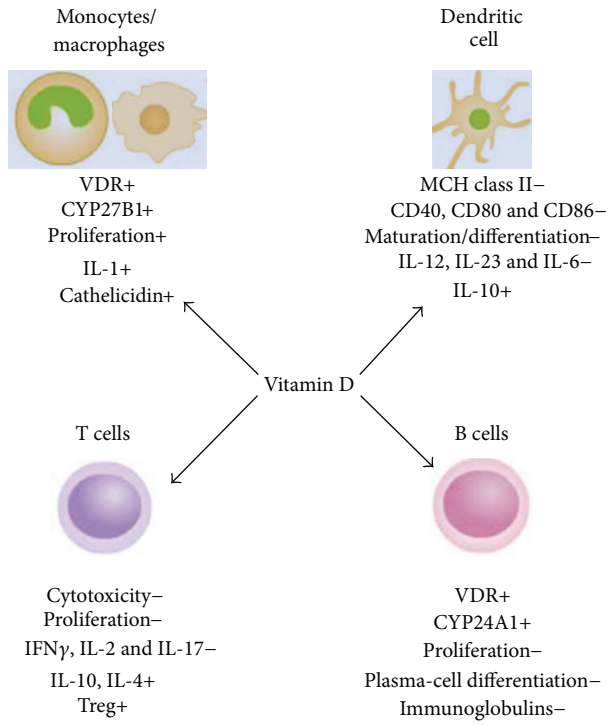

Figure 1: Effects of vitamin D on different immune system cells. Vitamin D regulates several immune system cell functions. It stimulates monocyte proliferation and differentiation towards macrophage-like cells, by self-increasing VDR, CYP27B, and IL-1 expressions; this "fast-forward" autocrine mechanism seems to be the basis for the subsequent maturation into macrophages, which does not take place in vitamin D deficient conditions; macrophage specific surface antigen expression is also enhanced. Vitamin D prevents T cells from proliferation, maturation, and releasing Th1-type molecules, such as IFNγ, IL-2, and IL-17, whereas it promotes Treg development. Vitamin D treatment prevents DCs from maturation and differentiation as well, by MHC class II, costimulatory factors, and interleukin downregulation. Vitamin D-induced protolerogenic DCs seem to be the key event for suppressive effects on immune system cells. Downregulation of B cell proliferation and maturation seems to be an indirect consequence of the suppressive effect exerted by vitamin D on T cells and APC. (+) and (−) indicate induction or inhibition.

similar to the human disease. In this model, BXL-219-induced block of NF-κBp65 nuclear translocation is associated with decreased CXCL10 production by pancreatic islets, even in presence of restimulation with TLR agonists; this is reflected in a significant decrease in Th1 cell organ infiltration [71]. Similarly, we have reported that elocalcitol or BXL-628, a nonhypercalcemic VDR agonist, impairs NF-κBp65 and STAT1 nuclear translocation directly in human thyrocytes in association with a significant decrease in cytokine-induced CXCL10 release. This effect, in addition to a decreased Th1- and Th17-cytokine secretion by $CD4^+$ T cells, makes elocalcitol to be a potential pharmacological tool in the treatment of autoimmune thyroid diseases [5].

4. CXCL10

Chemokines and their receptors are so far critical during inflammation to become novel targets for immunointervention [72, 73].

Among chemokines, CXCL10 plays a critical role in the initiation and maintenance of Th1-polarized response in autoimmune diseases or in graft injury; it appears to be directly linked to the disease pathogenesis and not related to a generic inflammatory status [11]. CXCL10 belongs to CXC chemokine subfamily and modulates innate and adaptive immune responses by controlling leukocyte trafficking [11, 74]. Under proinflammatory conditions, CXCL10 is secreted by several types of immune cells and by different resident cell types, as well [11]. CXCL10 exerts its action by binding the receptor CXCR3. Remarkably, local tissue secretion of CXCL10 represents the driving force for the recruitment of cytotoxic immune CXCR3-positive cells, such as T, natural killer (NK), B cells, macrophages, and DCs [75–77]. In particular, subtype A receptor activation leads to a potent CXCL10-induced chemotaxis for Th1 cell recruitment into inflammation sites [78], while the activation of subtype B, selectively expressed in human microvascular endothelial cells, is essentially involved in angiogenesis inhibition [79].

Hence, local CXCL10 production in inflammation sites is responsible for a positive feedback loop between IFNγ-producing Th1 cells and resident cells that, in turn, release CXCL10 upon IFNγ stimulation, as summarized in Figure 2 [80]. By those mechanisms a dominance of Th1-type cytokines and inflammatory response occurs together with a simultaneous Th2-type response downregulation.

The induction of CXCL10, important to protect against bacteria and some viruses infections, is described to be associated with inflammation processes engaged either in

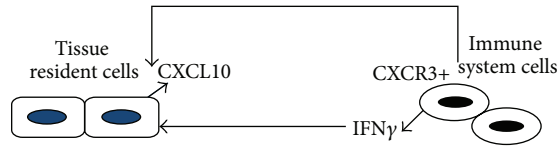

Figure 2: CXCL10-CXCR3 axis. CXCL10 secreted by different types of organ-resident cells under IFNγ induction is a potent chemoattractant for CXCR3-positive immune cells, in particular for activated T cells. T cell recruitment to sites of inflammation leads to an enhanced local increase of IFNγ, which, in turn, stimulates CXCL10 secretion by tissue cells; thus, a self-promoting inflammatory loop is established between resident and immune cells, making CXCL10-CXCR3 axis a therapeutic target for resolution of inflammation.

allo- or autoimmune response. Concerning the latter ones, different autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), multiple sclerosis (MS), autoimmune thyroid diseases, Addison's disease, and T1D, are associated with an enhanced tissue expression of CXCL10, not only with increased circulating levels [81–85].

CXCL10-CXCR3 axis plays a pivotal role in the pathogenesis of graft failure and organ rejection, as well. In fact, CXCL10 is critical in promoting and amplifying host alloresponses responsible for acute allograft rejection [86–91]: CXCL10- or CXCR3-gene-deficient mice show permanent engraftment of cardiac transplants [86, 92]. In cardiac and small bowel models of allograft rejection, CXCL10 neutralization with monoclonal antibodies prolongs the allograft survival [86, 87]. CXCL10 intragraft expression level is associated with human renal [79], lung [89], and cardiac [90, 91] allograft rejections. In addition, intragraft CXCL10 expression correlates with damage degree, rejection, and even loss of the organ [11].

Circulating levels of CXCL10 are also increased and associated with the rejection rate in human recipients undergoing transplantation of organs (such as kidney, heart, lung, and liver), cardiopulmonary bypass, and allogeneic stem cell transplantation (SCT) [11, 93]. Importantly, CXCL10 high pretransplant serum levels may predict the risk for the development of acute rejection and chronic allograft vasculopathy (CAV) in different human organ rejection settings [11]. In particular, pretransplant CXCL10 serum assessment may be helpful in the prospective determination of the use of immune suppression therapy, in both renal and heart transplantations [11].

5. VDR Agonists and CXCL10

The protolerogenic properties of VDR agonists, as discussed above, render them suitable candidates as immunosuppressants for either autoimmune diseases or graft rejection, as clearly summarized by Mathieu and Adorini, since quite ago [94]. The *in vivo* effect of some VDR agonists on inflammatory mediators/processes involved in different diseases is depicted in Table 2 [95–102].

VDR agonists, besides their ability to switch the immune system cell balance from Th1 to Th2 dominance, are able to counteract CXCL10 production and release by several resident cell types [5–7]. Indeed, as previously mentioned, VDR ligands can prevent CXCL10 release by human thyrocytes or murine pancreatic cells, with potential benefits in autoimmune diabetes or thyroiditis [5, 53]. Those data are particularly intriguing in the light of the results from epidemiologic studies which underline an inverse correlation observed between vitamin D level and some autoimmune diseases, as SLE, RA, MS or SSc, in which chemokines seem to be engaged [103]; in addition, the vitamin D intake in early life of animal models prone to common autoimmune disorders (RA, MS, and autoimmune prostatitis) successfully prevents disease occurrence [4, 96, 104]; similarly, vitamin D supplementation in early childhood seems to be able to protect against T1D development [105]; accordingly, vitamin D serum levels are often decreased in patients with T1D [106], and subjects with a vitamin D deficiency are predisposed toward developing the disease [4, 107].

It is noteworthy that VDR agonist beneficial effects have been shown in different models of experimental organ transplantation as well—heart [98, 108–110], kidney [111, 112], liver [113, 114], pancreatic islets [71, 115–117], skin [99], and small bowel allografts [98]—since they delay acute and chronic allograft rejection. The latter effect, probably the most interesting in terms of potential clinical application, involves also the reduction of vascular intimal thickening—for example, due to vascular smooth muscle and endothelial cells hyperplasia—in association with a lesser extent of immune cell infiltration, after the treatment with VDR agonists [118]. Remarkably, many of the immunoregulatory properties of VDR agonists favorable in acute and chronic allograft rejection likely rely on their capacity to inhibit CXCL10 production by organ target cells, that is, β cells in mouse model of pancreatic islet transplantation [54] or human cardiomyocytes and renal tubular cells [6, 7] in heart or kidney transplantation. Of interest, in our hands, elocalcitol and BXL-01-0029 significantly decreased CXCL10 secretion, without cytotoxic effects neither in resident nor immune cells, differently from the majority of current immunosuppressants. Indeed, both VDR ligands left unchanged cardiac, renal, and $CD4^+$ T cell viability, acting specifically on CXCL10 release [6, 7]. This effect is in line with the concept that vitamin D *in vivo* plays a pivotal role in immune homeostasis maintenance without strict immunosuppressants effects [68].

This observation could be of particular relevance as VDR agonists might serve as dose-reducing agents to add to conventional immunosuppressants in organ rejection management or autoimmune disease. Vitamin D analogues successfully decreased the doses of conventional immunosuppressive drugs in experimental autoimmune encephalomyelitis (EAE) model [4]; furthermore, in EAE, the addition of the vitamin D analogue TX527, with reduced calcemic activity,

Table 2: *In vivo* effect of some VDR agonists. Vitamin D analogs suppress inflammatory mediators and processes resulting in disease prevention.

Disease	Analogs	Main in vivo effects	Reference
Type 1 diabetes	KH1060	Type I diabetes prevention without significant effects on calcium or bone metabolism	[95]
Autoimmune prostatitis	BXL-628	Inhibition of the intraprostatic inflammatory response	[96]
Interstitial cystitis	BXL-628	Reduction of mast cell degranulation	[97]
Heart and small bowel graft	MC-1288	Delay/prevention of graft rejection	[98]
Skin allograft	KH1060, CB966	Skin allograft survival prolongation	[99]
Collagen-induced arthritis (CIA)	MC-1288	CIA prevention and suppression	[100]
Inflammatory bowel disease	TX527	Reduction in mucosal damage and crypt loss and suppression of the infiltration of immune cells	[101]
Experimental autoimmune encephalomyelitis (EAE)	TX527	EAE prevention	[102]

empowered the protective effect of IFN-β and CsA regimens, suggesting that this compound could be considered for clinical intervention in MS [102]; it is of interest that an association between CXCL10 and subjects affected with MS has been previously shown [119].

In organ transplantation, additive or synergistic effects are reported with VDR ligands and cyclosporine A (CsA), tacrolimus (FK-506) and sirolimus [120]. The combination of low-dose CsA with VDR agonists results in a significant decrease of IL-2 and IL-12 expressions and increased IL-10 in kidney allografts [112], likely by a reduction of renal bioactive transforming growth factor (TGF)-β. Furthermore, paricalcitol, a VDR activator, combined with trandolapril, an angiotensin-converting enzyme inhibitor, ameliorates obstructive nephropathy in a mouse model [121]. In humans, a retrospective study reports that vitamin D administration is able to delay renal graft loss in patients receiving conventional immunosuppressive drugs [122]. In isolated human tubular renal cells, the addition of BXL-01-0029 allows to lower FK-506 doses to reach the same inhibitory effect on cytokine-induced CXCL10 secretion [7].

6. Remarks and Conclusions

Given the pleiotropic effects of VDR agonists, based on their multifaceted interaction with immune and resident cells, it seems mandatory to encourage the research on those molecules, which, in light of their properties—due to their features to balance immune system homeostasis without being classical "immunosuppressants"—appear optimal candidates as novel therapeutic agents for Th1-driven inflammatory disease resolution. Large placebo-controlled, randomized-controlled trials should be encouraged since some discrepancies have been reported between *in vivo* and *in vitro* effects of vitamin D and its analogues, depending on both chemical structure (side chain configuration) and target cells [123]. Many clinical trials have been or are currently conducted by several investigators to test the therapeutic application of vitamin D or its derivatives in inflammatory processes underlying different pathologic conditions and diseases, that is, metabolic and/or kidney diseases (ClinicalTrial.gov, NCT01752244 and NCT00656032), inflammatory bowel diseases (Crohn's disease or ulcerative colitis, ClinicalTrial.gov NCT00122184 and NCT01426724), musculoskeletal diseases (ClinicalTrial.gov NCT01417923 and NCT01400009), and cardiovascular diseases (ClinicalTrial.gov NCT01331317). In this scenario, it is of particular interest a clinical trial evaluating vitamin D repletion, inflammation, and CXCL10 (ClinicalTrial.gov NCT01570309) in coronary artery disease.

Notably, VDR ligands are able to block CXCL10, that is a potential biomarker to monitor the inflammatory status but, more importantly for the topic of this paper, it represents a novel therapeutic target by which; that is, it could be feasible to fine-tune therapy for patients undergoing organ transplantation [11].

Furthermore, another benefit associated with the addition of VDR ligands to standard immunosuppressive regimens is related to their protective effects on bone loss [124]. Indeed, immunosuppressive agents are often associated with detrimental effects on bone. Together with bisphosphonates, vitamin D metabolites are the more extensively used molecules for bone-loss treatment. At variance with the first ones [118, 125], vitamin D analogues are indicated also in patients with adynamic bone disease.

Finally, it is known since long time ago that vitamin D does not significantly interfere with protective immune response against infective pathogens [126] and displays antineoplastic properties [127], both quite relevant benefits in order to avoid opportunistic infection or tumor development, often associated with immunosuppression.

Despite the many advantages so far summarized, the use of VDR agonists in clinics is until now limited to calcipotriol, a vitamin D analogue topically applied for the treatment of psoriasis [128].

The limit in therapeutic applications of vitamin D undoubtedly relies on the systemic toxicity associated with long-term intake of this hormone; in fact, the supraphysiological doses of vitamin D necessary to reach the low local effective concentration (about 10^{-10} M) are associated with the undesirable risk of hypercalcemia [3, 129]. Therefore, the introduction of new molecules with immunosuppressive features without causing significant hypercalcemia has

been strongly encouraged since a while [130]. Actually, drug development efforts should keep on designing vitamin D analogues retaining further distinct separation between immunomodulatory and hypercalcemic potency. Thus, the use of molecules as BXL-01-0029 or elocalcitol, with less or none hypercalcemic activity—and, therefore, without systemic toxicity—seems suitable for inclusion in immunosuppressive regimens, since they own the potentiality to lower the doses of current immunosuppressants and, thus, to reduce the side-effects associated with immunosuppression.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Can High Altitude Influence Cytokines and Sleep?

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The number of persons who relocate to regions of high altitude for work, pleasure, sport, or residence increases every year. It is known that the reduced supply of oxygen (O_2) induced by acute or chronic increases in altitude stimulates the body to adapt to new metabolic challenges imposed by hypoxia. Sleep can suffer partial fragmentation because of the exposure to high altitudes, and these changes have been described as one of the responsible factors for the many consequences at high altitudes. We conducted a review of the literature during the period from 1987 to 2012. This work explored the relationships among inflammation, hypoxia and sleep in the period of adaptation and examined a novel mechanism that might explain the harmful effects of altitude on sleep, involving increased Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) production from several tissues and cells, such as leukocytes and cells from skeletal muscle and brain.

1. Background

In recent years, the interest in activities carried out at high altitudes has grown. Millions of people travel to regions of high altitudes (i.e., above 2500 m) for tourism, sport, work, or permanent residence. However, living in high altitudes can lead to hypoxia. The effects of exposure to hypobaric hypoxia, which is present at high altitude, are dependent on the length of time spent at high altitude and the altitude reached.rt Because O_2 is required for the maintenance of vital functions, blood oxygenation can affect several physiological functions. Exposure to hypobaric hypoxia can result in extreme conditions, such as acute mountain sickness (AMS), high altitude pulmonary edema (HAPE), and high altitude cerebral edema (HACE), as well as other conditions, such as headache, nausea, vomiting, and gastrointestinal alterations [1–5].

Alterations in cardiovascular and respiratory functions promoted by altitude have been previously described. More recently, attention has focused on neurobiological functions, including sleep, cognition, and humor [6, 7]. Thus, this review discusses the effects of hypoxia stimulated by high altitude on sleep, with an emphasis on neuroimmunoendocrine interactions.

2. Methods

For this study, we conducted a systematic and integrative review of the literature, using source articles indexed by the ISI database, PubMED and MEDLINE by searching for books that addressed specific aspects related to altitude/hypoxia, cytokines, and sleep during the period from 1987 to 2012.

The keywords searched were "*cytokines and hypoxia,*" "*cytokines and altitude,*" "*inflammation and hypoxia,*" "*inflammation and altitude,*" "*sleep and hypoxia,*" "*sleep and altitude,*" "*sleep and cytokines,*" and "*sleep and inflammation*." These descriptors were used in a Boolean-specific basis to obtain various arrangements thought to maximize both the coverage and quality of the search. No restrictions were made regarding age or gender.

3. Altitude

The principal characteristic of exposure to high altitudes is the fact that there is an inverse correlation between altitude and the partial pressure of O_2. Therefore, at high altitudes, the body tries to adapt by generating many responses, including changes in skeletal muscle and in the endocrine and nervous systems [8].

Although the barometric pressure decreases with increasing altitude, the gas composition does not change until above the 1200 m level. Although the percentage of ambient oxygen is maintained at 20.93%, the increase in altitude decreases the O_2 partial pressure in expired air. This decrease promotes a partial impairment in the support of O_2, resulting in less oxygen transported by hemoglobin and consequently less O_2 available for tissues. In fact, all tissues that need O_2 for energy production are affected by hypoxia, and each tissue response depends on several factors, including the O_2 demand by the tissue, the time of exposure, and the individual's characteristics [9].

The classical response induced by high altitude includes respiratory and cardiovascular changes that are initiated within minutes after the person reaches the altitude [10]. In fact, there is an inverse correlation between increases in altitude and hemoglobin saturation. In addition, the number of hemoglobin molecules begins to increase, even at altitudes as low as 500 m. At the same time, alterations in hyperventilation occur at rest and during acute physical exercise. The heart rate increases in a manner similar to the increase seen in cardiac output, which attempts to compensate by decreasing the partial pressure of carbon dioxide in the arterial blood ($PaCO_2$); however, these alterations are not sufficient to affect the oxygen consumption (VO_2) decrease and aerobic energy production. As a result, remaining at high altitudes might result in fatigue and a significant decrease in the capacity to work and physically perform, especially aerobic and endurance exercise. In addition, it is possible to have an increase in blood pressure due to an increase of norepinephrine levels because of the impact of stimulated activities of rest and exercise [11, 12].

High altitude (above 3000 m) is a powerful stressor. Being at these altitudes can modify metabolic and physiological functions, and the body then tries to reestablish the homeostasis that was altered by hypoxia [13]. Several studies have shown that acute or chronic exposure to altitudes between 2500 and 5000 m results in sympathoadrenal responses that are exacerbated by metabolic alterations to other systems [13], including the immune system [12, 14].

Under these conditions, it is possible to produce a rapid adrenaline hormonal response and a transient increase in plasma cortisol concentrations [15, 16].

Altitude-induced hypoxia can also stimulate the release of other hormones involved in the recovery of homeostasis. One of those hormones is erythropoietin (EPO). Humans exhibit increased EPO concentrations two hours after exposure to high altitudes [17]. EPO is fundamentally important to the organization of the physiological response to altitude and can modulate the expression of many proteins. Increases in EPO and hemoglobin are essential for acclimatization and the maintenance of the O_2 supply to tissues.

It has been demonstrated that acute exposure to elevated altitudes can result in changes to several immunological parameters [12, 18]. Hypoxia for even a few hours is sufficient to induce significant changes in neutrophil and lymphocyte numbers, which are mainly characterized by reductions in cluster of differentiation (CD), cell numbers, and cellular proliferation [19]. Several studies have shown that acute hypoxia results in an increase in natural killer cells (NK cells) numbers and activity [20].

Studies have shown that lymphocytes and phagocytes present some signs of adaptation if the hypobaric stimulus is chronic, due to alterations in the production and release of substances such as cytokines and antibodies [21]. Other studies have shown that immunity mediated by T lymphocytes can be stopped by exposure to elevated altitudes [12, 22]. Facco et al. [21] confirmed that exposure to elevated altitudes can alter the number and cellular function and suggested that new studies be carried out to evaluate the expression of cytokines by T lymphocytes, particularly to determine the maintenance of the T helper cells (Th1/Th2) response.

It was suggested that remaining at an altitude of 4000 m above sea level was associated with increased plasma concentrations of IL-6 and Interleukin-1 receptor antagonist (IL-1ra). Furthermore, C-reactive protein (CRP) increases are associated with the development of pulmonary edema [23]. Numerous stressful events are associated with increases in cytokine release and disturbances in the pro/anti-inflammatory cytokine ratio [24]. Hypoxia alone seems to have a decisive role; however, the mechanisms responsible for the induction of cytokines under hypoxic conditions are not clear. Exposure to elevated attitudes can cause cellular damage due to increased oxidative stress and altered cytokine release; in turn, these cytokines participate in the recovery from cellular damage [25, 26].

4. Altitude and Inflammation

The exposure to hypoxia promotes several transcription factors, including nuclear factor-κB (NF-κB), which plays a central role in stimulating the proinflammatory cytokines TNF-α and IL-6 [27]. Similarly, several studies with rodents and humans have shown that effects-induced hypoxia can cause inflammation, including increase in transvascular leakage and oxidative stress with increased NF-κB expression in lungs followed by significant increase in proinflammatory cytokines IL-1, IL–6, and TNF-α [28–30].

A decrease in plasma cytokine concentration or the treatment with appropriate antagonists promotes partial reversion of the symptoms and illnesses, including cardiovascular disease, obesity, insulin resistance, and depression [31, 32]. Therefore, we suggest that sleep disturbances due to high altitudes could also be caused by increases in proinflammatory cytokines from several cells, such as skeletal muscle and immune cells, in association with the capillary leakage or repeated wakening aspects of AMS, which usually occur concurrently with the hyperopic phase of periodic breathing.

Hojman et al. [33] observed that augments the acute inflammatory effect in humans. In this study, the authors demonstrated that when EPO was given prior to a bolus injection of endotoxin, the levels of TNF-α and IL-6 were enhanced by 5- and 40-fold, respectively, whereas the endotoxin-induced increase in Interleukin-10 (IL-10) was not influenced by EPO. This interaction between EPO and inflammation may corroborate with sleep disruptions found at high altitudes.

However, Hojman et al. [34] used animal experiments to show that when EPO was expressed at supraphysiological levels, there were substantial metabolic effects, including protection against diet-induced obesity and normalization of glucose sensitivity, associated with a shift towards increased fat metabolism in the muscles.

Unfortunately, only limited information from well-controlled laboratory and field studies is available on this topic. Relatively, little is known about the influence of altitude on the interaction of cytokines and sleep. The significant effects (pro- and anti-inflammatory) of EPO in acute and chronic high altitudes should be investigated further. Thus, the sleep complains due to high altitudes could also be caused by increases in proinflammatory cytokines from several cells, such as skeletal muscle and immune cells, in association with the capillary leakage or repeated wakening aspects of AMS, which usually occur concurrently with the hyperopic phase of periodic breathing. This interaction between EPO and inflammation may corroborate with sleep disruptions found at high altitudes.

5. Cytokines

Cytokines are proteins produced and released by different cells, for example: leukocytes, muscle cells, and neurons. These proteins can act in a pleiotropic way or in synergy with other substances and can modulate the production of other cytokines [35]. Cytokines function in the regulation of metabolism by influencing hormone secretion, regulating the TH1/TH2 immune responses, and inducing inflammatory responses; in the nervous system, they influence complex neuronal actions and modulate thermoregulation, food intake, and neurobiological patterns [35, 36] during sleep.

Interleukin-1 (IL-1) increased in plasma concentration may cause fever, sickness behavior, increased heart rate, increased blood flow in many vascular beds, and increased sympathetic tone; changes in carbohydrate, fat, and protein metabolism also occur [24, 35]. The effects of IL-1 can be reversed by treatment with IL-1ra, an antagonist of IL-1, which functions to prevent IL-1 binding to its specific receptors [35].

The TNF-α is mainly produced by macrophages and neutrophils, but other cells, such as lymphocytes, NK cells, endothelial cells and neural cells, might also have the capacity to produce it [24]. TNF-α production occurs in response to a wide variety of stimuli, including infections and stimulation by other cytokines or mitogens [37]. TNF-α is a potent pleiotropic cytokine due to its ability to activate multiple signal transduction pathways and induce or suppress the expression of a number of genes. In addition, it has potent endogenous pyrogenic properties and may promote changes in the body's physiological temperature [38]. Moreover, tissues that present marked cachexia show high TNF-α activity, as observed in catabolic conditions, such as cancer and systemic inflammatory diseases [24].

The Interleukin-6 (IL-6) plays a significant role in regulating the pro-inflammatory response [24]. However, due to its capacity to stimulate the hypothalamus-pituitary-adrenal axis to produce cortisol and anti-inflammatory cytokines, such as interleukin-4 (IL-4) and it also has anti-inflammatory properties [24].

The Interleukin-10 (IL-10) has multiple biological activities and affects many different cell types. These include monocytes/macrophages, T cells, B cells, NK cells, neutrophils, endothelial cells, and peripheral blood mononuclear cells (PBMCs). IL-10 also acts in the regulation of inflammation because it is produced by adipose and muscle tissues, which are important to the pro/anti-inflammatory ratio in conditions such as physical exercise, obesity, and inflammatory diseases [39, 40].

Cytokines can penetrate the blood-brain barrier (BBB) and act indirectly on the brain by stimulating the production of chemical second messengers that carry information to targets such as NF-κB and adenosine [41, 42] as shown in Figure 1. The hypothesis that cytokines could influence the functions of the nervous system (NS) is based on observations that treatment with cytokines, such as Interferon-γ (INF-γ), promotes neuroendocrine alterations, and other studies show that there are receptors for these cytokines in many areas of the brain [38, 43, 44]. Additional studies have shown that an increase in proinflammatory cytokine concentrations promotes a decrease in the transendothelial electrical resistance and an increase in the permeability of the BBB [45]. Finally, it is possible that cytokines can be produced within the brain itself in response to neuronal activity [35].

More recently, several studies have shown the existence of an afferent neural pathway by which inflammation in the peritoneal cavity might influence the brain [46]. Subdiaphragmatic transection of the vagus produces reduction of fever, poor sleep, nocturnal excretion of norepinephrine, and hypothalamic production of IL-1 induced by lipopolysaccharides (LPS) in the peritoneal cavity [47], thereby validating this hypothesis. These alterations are not due to a reduction in the circulating levels of cytokines or to the attenuation of the inflammatory response induced by lipopolysaccharide (LPS) but rather to a defective translation of cytokines in the brain [48].

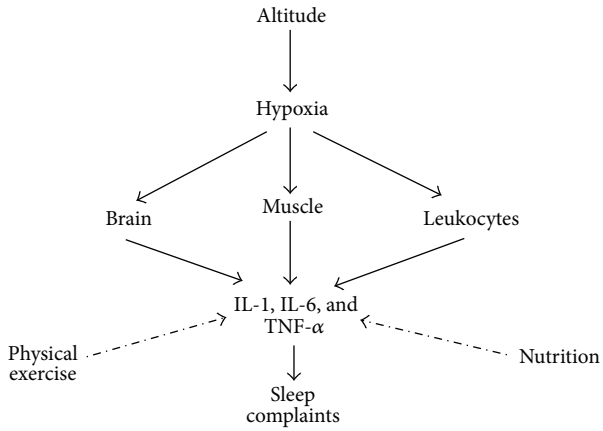

Figure 1: Solid line indicates stimulation; dotted line indicates inhibition.

High altitudes are potent stressors known to alter physiological and metabolic functions in the search for mechanisms to try to restore homeostasis by hypoxia disbalance. The acute or chronic exposure to altitudes between 2500 and 5000 m stimulates in a response sympathoadrenal leading to numerous other metabolic changes that, in turn, could affect several physiological systems including the production of cytokines and worsen the quality of sleep [49–51].

Currently, a strong relationship between sleep and immune process has been shown. The proinflammatory cytokines, including IL-1, IL-6, and TNF-α, are known as sleep-regulatory cytokines. However, sleep-promoting properties are also possessed by several other immune and proinflammatory cellular classes. Many studies reporting these relationships are focused on the perspective of low-grade inflammation associated with significant sleep alterations and on the perspective of immune dysregulation associated with several primary sleep disorders [52].

6. Altitude and Sleep

Sleep is a functional state that includes a complex combination of physiological and behavioral processes. It has some characteristic manifestations, such as a cyclic pattern, relative immobility, and an increase in the response threshold to external stimuli [53]. Sleep is very important, as it is evident from studies of acute or chronic sleep deprivation and sleep disorders; these impairments promote several alterations, including a marked increase in the production of stress hormones, including catecholamines and cortisol, a reduction in cognitive capacity, and a reduction in the state of alertness, among others [54].

Sleep can be divided into two phases: the nonrapid eye movement (nREM) phase, in which the electroencephalogram (EEG) records a synchronized tracing, and the rapid eye movement (REM) sleep phase, in which the electroencephalogram records signals similar to those in the wake period that are associated with the rapid eye movements [55, 56].

Two hypotheses attempt to explain the mechanisms involved in sleep regulation, and it is possible that these hypotheses are not mutually exclusive and could happen simultaneously. One hypothesis describes the role of circadian rhythms, while the other is related to the homeostatic effects of sleep [55].

The biochemical mechanisms that control sleep are very complex because sleep modulation is dependent on several factors, including carbon dioxide (CO_2) concentrations, as well as potassium, free radical, nitric oxide, hormone, and adenosine levels [57]. Proinflammatory cytokines play an important role in sleep regulation [58]. Some cytokines have an antisomnogenic action by decreasing prosomnogenic cytokine production, while others cytokines have the opposite effect [59].

Most of the existing studies on sleep and altitude were carried out in the field. There have also been studies carried out in normobaric hypoxic rooms that simulate conditions of high altitude [60]. High altitude has frequently been associated with sensations of suffocation when awakening from sleep. In fact, several studies showed that hypoxia directly acts on the architecture and quality of sleep in humans and rodents; these effects include increases in Stage I, decreases in REM sleep, lesions in cerebral regions that control sleep, and increases in the sensations of sleep deprivation and sleep fragmentation [61–63].

In fact, around 60% of persons subjected to altitudes of 3500 m or higher experience various sleep complaints. Recurring wakefulness is the most common characteristic due to the decreased O_2 saturation, which leads to sleep fragmentation [45, 64, 65]. In addition, hypoxia can cause poor sleep quality due to slight reductions in delta sleep, relative reductions in REM sleep, and agitation during the night [63]; however, overall total sleep time (TST) is not reduced. Therefore, the reduced subjective sleep quality is due to a higher arousal frequency. Despite previous studies suggesting that the impairment of sleep persists even after a season of acclimatization [64, 65], partial recovery of the damage during sleep can occur after spending some days at high altitude [26]. This finding has been shown in animal studies in which several days were spent in hypoxic conditions but not after a sudden ascent.

7. Altitude, Sleep, and Cytokines

To date, the effects of altitude on the architecture and quality of sleep are not well known [66]. Studies in rodents and humans suggest that prolonged exposure to hypoxia can alter circadian rhythms by reducing the amplitude of circadian oscillations and by possibly leading to changes in several variables, such as activity, hunger, metabolic rate, and the dark and light cycle [60, 67, 68]. The modification of melatonin and neurotransmitter release, metabolism in peripheral tissues, and modulation of several hormones and cytokines that participate in sleep regulation and gene expression responsible for the functions of the biological clock are also affected [69–71]. In part, this alteration on the sleep leads

to upregulation of proinflammatory cytokines in response at high altitude.

The relationship between sleep and cytokines was first established through observations that sleep deprivation increases INF-γ production. To date, the roles of several growth factors, including epidermal growth factor, fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, granulocyte-macrophage colony-stimulating factor and insulin-like growth factor-1 (IGF-1), have also been investigated for their roles in sleep modulation [59]. However, this review focuses on the pro- and anti-inflammatory cytokines IL-1β, IL-6, IL-10, and TNF-α.

8. Hypoxia, Physical Exercise, Cytokines and Sleep

In relation to physical exercise in hypoxia, few and contradictory studies evaluated the effect of exercise on condition of hypoxia on the production of cytokines [72]. The exercise performed under hypoxic conditions/high altitude represents an additional stress condition in relation to the exercise performed at sea level [73]. Even when the exercise intensity is relative, that is, taking into account that the maximum VO_2 and performance decreases as the altitude increases [73, 74]. So many factors should be taken into account when discussing the interaction hypoxia and cytokines. The increase in altitude or the extent of hypoxia is a primary factor that influences the level of variation in physiological and biochemical parameters which can modulate the immune response mediated by exercise [12]. Collectively, analyzing the results of the previously published works, one can speculate that there is a threshold elevation that should be followed to achieve the benefits associated with living or training at altitude with the least possible damage [12].

The concentration of cytokines, notably IL-6 and inflammatory markers such as the acute phase proteins CRP has its increased concentrations in response to a session with moderate exercise intensity of 50% $VO_{2\,Max}$ at an altitude of 4300 m over the same exercise at sea level [75]. However, in this study, the authors evaluated the effects of varying intensities of exercise in normoxic and hypoxic environments at equivalently 3100 m on immune regulation and metabolic responses and showed that during prolonged physical exercise at 40 and 60% of $VO_{2\,Max}$ this doesnot seem to dramatically alter the response of the selected immune system including IL-1 or TNF-α and metabolic markers. Exercise training that uses acute hypoxic environments does not adversely affect immune regulation system status and may be beneficial for those individuals looking to increase endurance performance [76].

One way to partially reverse the effects of hypoxia on sleep patterns can be by performing moderate exercise, taking into account that in normoxic condition physical exercise beside improving sleep also modulates the memory, attention, and mood state [77].

Physical exercise has been considered as the best strategy to prevent and treat chronic inflammatory diseases of low grade [78, 79], such as those generated by sleep disorders. Regular physical training is able to increase the production of anti-inflammatory cytokines and decrease the concentrations of circulating proinflammatory cytokines and can improve the quality of sleep.

9. Conclusions

The relationships among inflammation, hypoxia, and sleep are discussed in the present study; we conclude that hypoxia induced by elevated altitudes in the adaptation period results in a disturbance in the balance of homeostasis and affects several physiological systems. Consequently, severe changes in sleep architecture and sleep quality may occur. These changes might be mediated by increases in plasma concentrations of IL-1, IL-6, and TNF-α and possibly through the stimulation of EPO.

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Review Article

Inflammatory Signalings Involved in Airway and Pulmonary Diseases

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In respiratory diseases, there is an increased expression of multiple inflammatory proteins in the respiratory tract, including cytokines, chemokines, and adhesion molecules. Chemokines have been shown to regulate inflammation and immune cell differentiation. Moreover, many of the known inflammatory target proteins, such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2), and cytosolic phospholipase A_2 ($cPLA_2$), are associated with airway and lung inflammation in response to various stimuli. Injuriously environmental stimuli can access the lung through either the airways or the pulmonary and systemic circulations. The time course and intensity of responses by resident and circulating cells may be regulated by various inflammatory signalings, including Src family kinases (SFKs), protein kinase C (PKC), growth factor tyrosine kinase receptors, nicotinamide adenine dinucleotide phosphate (NADPH)/reactive oxygen species (ROS), PI3K/Akt, MAPKs, nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and other signaling molecules. These signaling molecules regulate both key inflammatory signaling transduction pathways and target proteins involved in airway and lung inflammation. Here, we discuss the mechanisms involved in the expression of inflammatory target proteins associated with the respiratory diseases. Knowledge of the mechanisms of inflammation regulation could lead to the pharmacological manipulation of anti-inflammatory drugs in the respiratory diseases.

1. Introduction

Inflammation is a protective response to cellular and tissue damage/injury. The purpose of this process is to destroy and remove the injurious agents and injured tissues, thereby promoting tissue repair. When this beneficial response occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results in chronic inflammation and destruction of normal tissue [1]. Moreover, inflammatory airway and lung diseases, such as asthma or chronic obstructive pulmonary disease (COPD), are characterized by chronic inflammation. Many of the known inflammatory target proteins, such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2), and cytosolic phospholipase A_2 ($cPLA_2$), are associated with inflammatory signaling pathways induced by various stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), adenosine-5′-triphosphate (ATP), cigarette smoke extract (CSE), lipoteichoic acid (LTA), or lipopolysaccharide (LPS) [2–6]. Airway smooth muscle is considered as an end-response effector regulating regional differences in ventilation by contracting in response to various proinflammatory mediators and exogenous substances released under homeostatic or pathologic conditions, such as asthma [7]. Lung cells, in particular alveolar epithelial type II cells, are susceptible to the injurious effects of oxidants. It has been shown that lung cells release inflammatory mediators and cytokines/chemokines, such as IL-1β, IL-6, IL-8, and TNF-α in response to oxidative stress [8]. Moreover, the

SFKs, PKC, growth factor tyrosine kinase receptors, NADPH oxidase/ROS, PI3K/Akt, and MAPKs are components of signaling cascades that respond to extracellular stimuli by targeting transcription factors, such as NF-κB and AP-1, resulting in the modulation of inflammatory gene expression [8]. Thus, this review will focus on some general aspects of inflammatory signaling regulation and summarize current knowledge regarding the presence and functional roles of these inflammatory signal molecules within the respiratory system, and their proposed involvement in the expression inflammatory target proteins in response to proinflammatory mediators during airway and lung inflammation. The pharmacological interventions protect against inflammation-induced airway and lung diseases will be discussed.

2. Inflammatory Target Proteins and Respiratory Diseases

2.1. Adhesion Molecules. Cell adhesion molecules play an important role in inflammatory responses. Leukocytes continuously circulate throughout the body in order to come in contact with antigens sequestered within tissues. To enter tissues, circulating leukocytes migrate from the blood, between vascular endothelial cells and into the tissue [9]. During this migration, leukocytes initially bind to endothelial cells via low affinity adhesion molecules. The low affinity adhesion in combination with the force of the blood flow results in rolling of leukocytes on endothelial cells. Subsequently, adhesion molecule affinity is upregulated and leukocytes firmly adhere to the endothelium [9]. Finally, bound leukocytes migrate between the endothelial cells and into the tissue. VCAM-1 is one of the inducible cell transmembrane glycoproteins of the immunoglobulin supergene family expressed on several cell types and plays an important role in a number of inflammatory and immune responses [10]. It was first identified as an adhesion molecule induced on endothelial cells by proinflammatory cytokines or LPS [11, 12]. In normal processes, VCAM-1 is important during development since a VCAM-1 knockout is an embryonic lethal. In pathogenesis, VCAM-1 expression is induced on endothelial cells during inflammatory bowel disease, atherosclerosis, infection, and asthmatic responses [13–15]. Upregulation of VCAM-1 expression on cytokine-triggered vascular endothelial cells enhances the targeted transmigration of PMNs into extravascular space of inflammation [10]. In airways, to reach the submucosa and airway lumen, circulating PMNs must first be recruited across the vascular endothelium [16] and then migrate through the interstitial matrix before interacting with airway epithelium. The pathogenesis of asthma, eosinophil migration into the lung is VCAM-1 dependent [17]. Accumulation of inflammatory cells within the airways can be influenced by expression of adhesion molecules on airway epithelium. Thus, similar processes that govern PMNs adhesion to lung airway resident cells may occur and contribute to the damage to these cells seen in inflammatory responses of asthma [18]. This event is crucial in the development of allergic inflammation and is mediated by adhesion molecules and cytokines [19]. ICAM-1 is an endothelial- and leukocyte-associated transmembrane protein long known for its importance in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration [20]. More recently, ICAM-1 has been characterized as a site for the cellular entry of human rhinovirus [21]. Because of these associations with immune responses, many researchers have hypothesized that ICAM-1 could function in signal transduction. Earlier studies showed that ICAM-1 gene is highly expressed in pulmonary fibroblasts of COPD patients [22]. In addition, blocking pulmonary ICAM-1 expression ameliorates lung injury in established diet-induced pancreatitis [23]. Thus, adhesion molecules play a key role in regulating inflammation in respiratory disorders (Figure 1).

2.2. Cytosolic Phospholipase A_2. There are three forms of PLA_2 in mammalian cells [24]. The first class of PLA_2 is secretary PLA_2 ($sPLA_2$) that is expressed in a variety of cell types [25] and it has no preference for AA at *sn*-2 position, requires millimolar amounts of Ca^{2+} for activity and is sensitive to sulfhydryl reducing agents, such as dithiothreitol (DTT), and is resistant to heat or acid conditions [25]. The second class of PLA_2 is calcium-independent PLA_2 ($iPLA_2$) that does not require Ca^{2+} for catalytic activity. $iPLA_2$ prefers plasmalogen substrates and does not appear to have a preference for the type of fatty acid at the *sn*-2 position [26]. The third class is the novel, high molecular weight (85 kDa) cytosolic PLA_2 ($cPLA_2$). $cPLA_2$ enzymes catalyze the hydrolysis of the *sn*-2 position of membrane glycerophospholipids, leading to production of free fatty acids and lysophospholipids. This reaction is of particular importance if the esterified fatty acid is arachidonic acid (AA) [24], which is converted by downstream metabolic enzymes to various bioactive lipophilic compounds called eicosanoids, including prostaglandins (PGs) and leukotrienes (LTs) [24]. PLA_2 could be the initial and rate-limiting enzyme in this conversion. The increase in $cPLA_2$ activation and expression following external stimuli, including proinflammatory cytokines, growth factors, and microbial toxin, is often observed in several systems [27]. The implication of $cPLA_2$ in inflammatory diseases has been confirmed by that the airway anaphylactic response in the $cPLA_2$ knockout mice is markedly reduced compared with that in the wild-type mice [28]. Moreover, $cPLA_2$-deficient mice have provided the most definitive evidence for the central role of $cPLA_2$ in eicosanoid [29] as well as in the pathogenesis of several inflammatory diseases, such as acute respiratory distress syndrome (ARDS) due to bacterial sepsis [30, 31]. These studies have demonstrated that there was a reduction in the bronchial lumen and alveolar thickening in the control mice that was remarkably absent in the $cPLA_2$ knockout mice. This outcome also appeared in 5-lipoxygenase (LO)-knockout mice and mice with PGD_2 receptor deficiency [32]. Thus, $cPLA_2$ seems to function as a crucial upstream regulator of the production of eicosanoids for airway resistance during allergic inflammation and is correlated to the process of asthma (Figure 1). The inhibition of $cPLA_2$-mediated pathways may also provide a therapeutic approach to airway and lung injury.

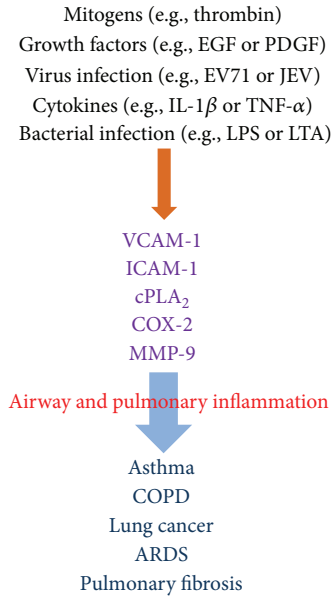

Figure 1: General overview of the inflammatory target proteins in respiratory diseases. Many of the known inflammatory target proteins, such as MMP-9, ICAM-1, VCAM-1, COX-2, and $cPLA_2$, are induced by various stimuli, such as mitogens, growth factors, virus infection, cytokines, and bacterial infection. EGF, epidermal growth factor; PDGF, platelet derived growth factor; EV71, enterovirus 71; JEV, Japanese encephalitis virus; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; LTA, lipoteichoic acid.

2.3. Cyclooxygenase-2. COX metabolites have diverse effects in the lung and are known to modify airway tone as well as inflammatory responses [3]. Three isoforms of COX have been identified [24]. COX-1 is constitutively expressed in most tissues and considered to be the "housekeeping" isoform that produces PGs which are required for maintenance of normal cell and organ function. In contrast, COX-2 is primarily an inducible isoform whose expression can be upregulated in many cell types by cytokines, mitogens, and endotoxin [3, 4]. It is highly expressed in inflamed tissues and believed to produce PGs involved in inflammatory processes [33]. COX-2 has multiple transcriptional regulatory sequences in its promoter region, including a TATA box, an NF-IL6 motif, two AP-2 sites, three Sp1 sites, two NF-κB sites, a CRE motif, and an E-box [34]. COX-2 gene expression can be induced by multiple cytokines and growth factors, via activation of transcriptional regulatory proteins that act on these promoter sites [35]. Thus, COX-2 appears to be the primary COX controlling PGE_2 synthesis in response to inflammation (Figure 1). COX effects are widespread and extremely complex; however, studies in knockout mice for COX-1 versus COX-2 reveal sometimes overlapping, not altogether predictable roles for these two enzymes [24]. The levels of prostanoids in bronchoalveolar lavage fluid are increased in asthma, and several studies have found enhanced expression of both COX-1 and COX-2 in the airways of asthmatics [36, 37]. A recent research has renewed interest in the role of prostanoids in allergic airway disease. Moreover, the expression of COX-2 protein induced by *Lactobacillus rhamnosus* GG (LGG), endotoxin, and lipoteichoic acid (LTA) in T84 epithelial cells [38]. The presence of COX-3 mRNA transcript, with a size of approximately 5.2 kb, was subsequently confirmed in human cells; COX-3 was in highest concentrations in the cerebral cortex and heart tissue [24]. The regulation of COX-3 transcription appears to be identical to that of COX-1. COX-3 is similar to COX-1 and COX-2 in terms of structure and enzymatic function. However, the retention of intron 1 in COX-3 seems to slow its enzymatic activity in comparison to COX-1 and COX-2. Thus, the inhibition of COX-2-mediated inflammatory pathway may provide a therapeutic approach to respiratory diseases.

2.4. Matrix Metalloproteinase-9. MMPs are proteolytic enzymes that are able to degrade extracellular matrix (ECM) components and, thus, play a role in cell migration and tissue remodeling. Moreover, they can splice and (in)activate cytokines and chemokines, thereby influencing the recruitment and function of inflammatory cells [39]. To date, 24 MMPs have been identified in mammals; cellular sources include inflammatory, stromal, and epithelial cells. Some MMPs are anchored to the cell surface, whereas others are secreted into the extracellular space. They are released as inactive proenzymes and are activated by proteolytic cleavage of the N-terminal domain. Most MMPs are constitutively secreted once they become translated [40]. In gelatinase subfamily of MMPs (MMP-2 and MMP-9), the catalytic domain that includes the Zn^{2+} binding site also contains repeats of fibronectin motifs allowing the ability to bind gelatin, their major substrate. Patients with asthma have an increased gelatinolytic activity linked to MMP-2 and MMP-9 and higher levels of tissue inhibitor of metalloproteinase-1 (TIMP-1; a natural inhibitor of MMPs) in their sputum [41]. The activated form of MMP-9 (85 kDa) was found in the sputum from 60% of asthmatics, but was absent from that of control subjects. Although less frequently detectable than pro-MMP-9 (pro-MMPs are catalytically inactive and are activated into the active MMP after cleaving of the prodomain), pro-MMP-2 (72 kDa) was also found more frequently in asthmatics (50%) than in control subjects (5%). In addition, patients with COPD have an increased gelatinolytic activity in sputum linked to MMP-2 and MMP-9 [41]. In smokers with emphysema, MMP-8 and MMP-9 levels in bronchoalveolar lavage (BAL) fluid were significantly higher than in smokers without emphysema [42]. *In vitro* cultured human airway smooth cells and A549 cells, TNF-α and IL-1β induce MMP-9 expression and cell migration [2, 43, 44] via various signaling pathways, such as PKC, MAPKs, NF-κB, and AP-1. Thus, MMPs and their inhibitors (TIMPs) play multiple functions in physiological processes and interact with many other mediators regulating inflammatory processes, cell behavior, and angiogenesis. These mediators are implicated in many intricate loops of reciprocal interactions rendering the understanding of the role of MMPs in regulatory processes difficult. In many respiratory diseases, MMPs are

overexpressed or oversecreted leading to both deregulation of physiological homeostatic processes and ECM degradation and disorganization (Figure 1).

3. Inflammatory Signalings Involved in the Respiratory Diseases

3.1. Protein Kinase C. PKCs are important in many cellular responses in the lung, including permeability, contraction, migration, hypertrophy, proliferation, apoptosis, and secretion [45]. PKC is a family of serine/threonine kinases characterized by at least eleven different isotypes. PKC isotypes are differentially regulated by calcium (Ca^{2+}), diacylglycerol, and phospholipids and differ in structure, expression, intracellular localization, substrate utilization, and mechanisms of activation [45]. The PKC isotypes are subdivided into three groups: the classical, novel, and atypical. This subdivision is based on the structural and functional differences in the conserved domains C1–C4. The classical PKCα, PKCβI/II, and PKCγ isotypes are Ca^{2+} and diacylglycerol dependent [46]. The novel PKCε, PKCδ, PKCη, PKCθ, and PKCμ isotypes contain C2 domains that lack Ca^{2+}-binding ability but still retain functional C1A and C1B domains that can bind the endogenous diacylglycerol and exogenous phorbol esters [47]. The atypical PKCι, PKCλ, and PKCζ isotypes lack a functional C2 domain and contain a single C1 domain that lacks the ability to bind diacylglycerol and phorbol esters. Therefore, the mechanism of activation of the atypical PKC isotypes is both Ca^{2+} and diacylglycerol independent. PKCζ and PKCλ have been implicated in signaling through lipid metabolites including phosphatidylinositol 3-phosphates [47]. Moreover, PKCs are important signaling intermediates in chronic airway diseases like asthma and COPD. PKCs have been implicated in airway inflammation, bronchospasm, and mucous production [46]. Resident airway epithelial cells produce proinflammatory mediators under the regulation of PKCδ [48]. Increased PKCδ activity increases NF-κB-dependent proinflammatory cytokine generation in human airway epithelial cells, while expression of a dominant negative PKCδ mutant has inhibiting effects. In human airway smooth muscle cells, PKCα, βI, δ, ε, μ, γ, and ζ are found in the cytosol and βII in the membrane under basal conditions [49]. The proinflammatory neuropeptide bradykinin (BK) causes activation of PKCα, βI, δ, and ε when applied to airway smooth muscle cells. BK also induces COX-2 protein expression and PGE_2 accumulation in human airway smooth muscle cells via a PKCε-dependent signaling. PKCα is increased in the lungs of patients with COPD and is thought to be important in the hypertrophy and proliferation of airway smooth muscle cells [46]. PKCζ activity is also increased in proliferating human airway smooth muscle cells. On the other hand, PKC is important in mediating the effects of proinflammatory cytokines by phosphorylating $cPLA_2$ leading to the release of AA from phospholipids with subsequent production of bioactive eicosanoids in activated cells [50]. PKC is a key regulator of fibrosis in human pulmonary interstitial fibroblasts. At least three PKCs are expressed in interstitial fibroblasts, including PKCα, δ, and ε [46]. Activation of PKCα causes decreased collagen expression via the extracellular signal-regulated kinase kinase (MEK)/ERK signaling cascade, a response that is opposed by PKCε [51]. Selected PKCs are activated by LPS, leading to the production of the proinflammatory cytokines, such as TNF-α, IL-β, and IL-6 [52]. In addition, thrombin causes an increase in cytosolic [Ca^{2+}] and activation of selected PKCs [53]. TNF-α has been shown to induce MMP-9 expression via a PKCα-dependent pathway in A549 cells [2]. Taken together, these studies indicated that PKCs play a critical role in mediating inflammation and respiratory diseases (Figure 2). Because multiple signaling pathways contribute to the key cellular responses important in lung biology, therapeutic strategies targeting PKCs may be more effective if combined with inhibitors of other pathways for additive or synergistic effect. Mechanisms that regulate PKC activity, including phosphorylation and interaction with isozyme-specific binding proteins, are also potential therapeutic targets in the respiratory diseases.

3.2. NADPH Oxidase/ROS. ROS are products of normal cellular metabolism and are known to act as second messengers. Under physiological conditions, ROS participate in maintenance of cellular "redox homeostasis" to protect cells against oxidative stress. In addition, regulation of redox state is important for cell activation, viability, proliferation, and organ function. However, overproduction of ROS, most frequently due to excessive stimulation of either reduced NADPH by proinflammatory cytokines or the mitochondrial electron transport chain and xanthine oxidase, results in oxidative stress. Oxidative stress is a deleterious process that leads to airway and lung damage and consequently to several respiratory inflammatory diseases/injuries [8]. ROS are intracellularly generated from several sources, including mitochondrial respiration, cytochrome P450, the NADPH oxidase system, and xanthine/xanthine oxidase [54]. However, the major ROS generating enzyme is NADPH oxidase, a membrane-bound multicomponent enzyme complex that is present in phagocytes as well as nonphagocytic cells [55]. ROS produced by NADPH oxidase have two major roles. First, superoxide produced by NADPH oxidase 2 is required for respiratory burst that occurs in phagocytes, leading to microbial killing. The second role of NADPH oxidase is associated with the regulation of cell signaling [55]. ROS derived from NADPH oxidase can specifically and reversibly react with proteins, altering their activity, localization, and half-life [8]. Activated phagocytic cells generate ROS via assembly and activation of the NADPH oxidase complex, which comprises membrane-associated flavocytochrome b_{558} ($gp91^{phox}$) and $p22^{phox}$ and various cytosolic cofactors ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, and the GTPase, Rac1) and mediates transmembrane electron transfer from the major cellular electron donor, NADPH, to reduce molecular O_2 to superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) [54]. A number of homologs of the main business end of NADPH oxidase, $gp91^{phox}$, have been discovered, and mammalian systems are now known to contain seven NADPH oxidase homologs, comprising NADPH oxidase 1–5 (NADPH oxidase 2 being the new

name for gp91^{phox}) and two larger dual oxidases, DUOX1 and DUOX2, which are widely expressed in many cell types to mediate a variety of biological functions, such as cell mitosis, differentiation, migration, and immune regulation [56]. In *in vitro* studies, using macrophages, alveolar and bronchial epithelial cells, ROS have been shown to induce gene expression of inflammatory mediators, such as IL-1 and TNF-α [57, 58]. Patients with asthma demonstrate increased generation of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Increased production of ROS has been demonstrated by many cell types within the lung in asthma, including macrophages, antigen-presenting cells (APCs), neutrophils, and eosinophils [59]. Excessive production of ROS correlates with the degree of airway hyperresponsiveness, as quantified by methacholine challenge. In addition, oxidative stress also contributes to a proteinase-antiproteinase imbalance, both by inactivating antiproteinases, such as α1-antitrypsin and secretory leukocyte proteinase inhibitor, and by activating proteinases, such as MMPs [8]. On the other hand, oxidants also promote inflammation by activating NF-κB or AP-1, which orchestrates the expression of multiple inflammatory genes recognized to be important in COPD, such as TNF-α. Recently, phagocytic NADPH oxidase-ROS signaling has been shown to play a critical role in promoting TNF-α-induced, NF-κB-dependent acute inflammatory responses, and tissue injury specifically in the lungs, which is effected by preferential leukocyte infiltration [8]. Thus, oxidative stress plays a critical role in inflammatory responses in airway and lung diseases via the upregulation of redox-sensitive transcription factors (such as AP-1 or NF-κB) and thereby proinflammatory genes (such as MMP-9, VCAM-1, ICAM-1, COX-2, or cPLA$_2$) expression. Inflammation itself results in oxidative stress in the airways and lungs. Taken together, NADPH oxidase/ROS play a critical role during development of airway and lung diseases (Figure 2).

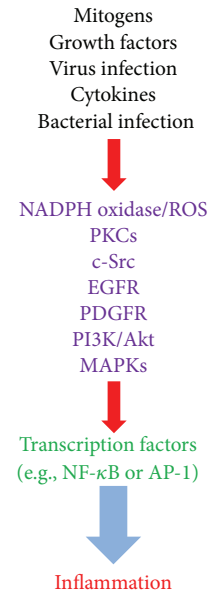

Figure 2: The signaling pathways in airway and pulmonary inflammation. Various stimuli, such as mitogens, growth factors, virus infection, cytokines, and bacterial infection, can induce inflammation via the NADPH oxidase/ROS, PKCs, c-Src, EGFR, PDGFR, PI3K/Akt, and MAPKs pathways and the transcription factors, such as NF-κB and AP-1 in lung resident cells.

3.3. PI3K/Akt. The PI3K family are central signaling elements in a diverse array of cellular functions, including growth, proliferation, migration, and survival. It is, therefore, understandable that dysregulation of PI3K has been implicated in the induction and/or progression of a variety of disease states, including those of the respiratory tract, ranging from asthma to cancer [60]. PI3Ks have been divided into three classes according to theirstructure and lipid substrate specificity [61]. The most extensively investigated are class I PI3Ks. Type I PI3Ks are activated by cell surface receptors, such as growth factors, insulin, and G-protein-coupled receptors (GPCRs). Class II PI3Ks comprised α, β, and γ isoforms, which are characterized by the presence of a C2 domain at the C terminus. They predominantly use phosphatidylinositol and phosphatidylinositol 4-phosphate [PI(4)P] as substrates. The class III PI3Ks only use phosphatidylinositol as a substrate. Class I PI3Ks are further divided into class IA and class IBPI3Ks. Structurally, PI3Ks IA exist as heterodimeric complexes in which a catalytic p110 subunit (designated as α, β, or γ) is in association with a particular regulatory subunit (designated as p85, p55, and p50) [61]. Importantly, PI3K IA signals downstream of receptor tyrosine kinase and Ras. The single class PI3K IB consists of the p110γ catalytic subunit complexed to the p101 regulatory subunit and signals downstream of GPCRs and Ras, which is activated by $\beta\gamma$ subunits from GPCRs, such as the receptors for chemokines [61]. Despite limitations in selectivity, the two commercially available PI3K inhibitors, wortmannin and LY294002, have contributed greatly to our understanding of the biological role of PI3K in lung inflammation [62]. Moreover, previous study indicated that intratracheal administration of LY294002 significantly reduced ovalbumin-(OVA-) induced increases in total cell counts, eosinophil counts, and IL-5, IL-13, and CCL11 (eotaxin) levels in BAL fluid and dramatically inhibited OVA-induced tissue eosinophilia and airway mucus production [63]. This study confirmed that LY294002 markedly attenuated OVA-induced serine phosphorylation of Akt, a direct downstream substrate of PI3K. In addition, other studies also showed that LY294002 and wortmannin attenuated eosinophilic airway inflammation and airway hyperresponsiveness in a murine asthma model [64]. Thus, PI3K inhibition was indicated to have therapeutic potential for the treatment of asthmatic airway inflammation. On the other hand, ROS induction is accompanied by activation of PI3K. LY294002 was shown to reduce chemokine-induced ROS generation in phagocytes, which was further confirmed by studies using PI3K knockout mice [62]. It was also reported that serum withdrawal (SW) killed human U937 blood cells by elevating cellular ROS levels, which occurred through PI3K activation [65]. Thus, PI3K family plays a prominent role in various airway and lung inflammation

(Figure 2). Moreover, inhibitors of PI3K/Akt may prove to be useful novel therapies in the treatment of respiratory diseases.

3.4. Src Family Kinases. SFKs are signaling enzymes that have long been recognized to regulate critical cellular processes, such as proliferation, survival, migration, and metastasis [8]. Src protein tyrosine kinase (PTK) family is categorized into nonreceptor tyrosine kinases and consists of nine members. Src, Fyn, Yes, and Yrk are ubiquitously expressed, whereas Blk, Fgr, Hck, Lck, and Lyn are expressed in more restricted patterns [66]. So far, Yrk has been detected only in chicken [66]. Src PTK family members are activated in response to the stimulation of a variety of cell surface receptors, such as tyrosine kinase receptors, integrin receptors, and G protein-coupledreceptors, and by cellular stress [66]. Src PTKs can also regulate the functional activity of these receptors. Moreover, we reported that TNF-α or IL-1β induces VCAM-1 and ICAM-1 expression via a c-Src-dependent pathway in human airway smooth muscle cells [8]. In addition, c-Src has been shown to regulate COX-2/PGE_2/IL-6-dependent airway inflammation via NADPH oxidase/ROS [67]. In human lung epithelial cells, in addition to activating NF-κB-inducing kinase (NIK) via TRAF2, TNF-α could activate c-Src through PKC. Systemic inhibition of these kinases using specific small molecule inhibitors for Src PTKs (either PP2 or SU-6656) significantly attenuated LPS-induced lung injury and capillary permeability and reduced LPS-dependent cytokine and chemokine levels in the lung and the serum [68]. Thus, the role of Src family PTKs in inflammatory responses is a rising area of research (Figure 2). However, application of small chemical inhibitors to effectively and specifically block Src PTKs could have a great clinical implication for airway and lung diseases with inflammatory responses as underlying mechanisms.

3.5. Growth Factor Tyrosine Kinase Receptors. Cell-surface tyrosine kinases receptors play pivotal roles in development, tissue repair, and normal cellular homeostasis. Aberrant expression or signaling patterns of these receptors have also been linked to the progression of a diversity of diseases, including asthma. Two major families of tyrosine kinases receptors, the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) families, have received a great deal of attention as potentially therapeutic targets for respiratory diseases, as these receptors have been shown to play key roles in chronic tissue remodeling in asthma, bronchitis, and pulmonary fibrosis. The EGFR system on epithelial cells and underlying mesenchymal cells (fibroblasts, myofibroblasts, and smooth muscle cells) drives numerous phenotypic changes during the progression of these pulmonary diseases, including mesenchymal cell hyperplasia, differentiation, and ECM production. The PDGFR system, located primarily on mesenchymal cells, transduces signals for cell survival, growth, and chemotaxis. The variety of EGFR and PDGFR ligands produced by the airway epithelium or adjacent mesenchymal cells allows for intimate epithelial-mesenchymal cell communication. In humans, the airway epithelium expresses EGFR ligands constitutively, including EGF, TGF-α, HB-EGF, amphiregulin, heregulin, and betacellulin. Expression of several EGFR ligands has also been investigated in diseases, such as COPD and asthma. Kohri et al. showed that *P. aeruginosa* bacterial supernatant induces mucin production in human airway epithelial cells (NCI-H292) via EGFR activation [69]. Multiple studies have also reported that stimulation of airway epithelial cells by LPS induces the secretion of IL-8 via a cellular cascade involving a TLR4/myeloid differentiation primary response gene (MyD)88/NF-κB-dependent pathway [70]. In addition, ROS have been shown to stimulate PDGFRα activation via c-Src family kinases [8]. There is accumulating evidence that PKC-dependent phosphorylation of $p47^{phox}$ is essential for PDGF-stimulated ROS generation, which is important for PDGF-induced MAPKs activation [8]. Taken together, these studies suggest that growth factor tyrosine kinase receptors may also play a key role in mediating expression of inflammatory genes (Figure 2).

3.6. Mitogen-Activated Protein Kinases. MAPKs are important components of signaling modules activated by neurotransmitters, cytokines, and growth factors, as well as chemical and mechanical stressors. In the airway, these external signals produce acute responses that modify smooth muscle contraction and may also induce chronic responses that modify airway structure [71]. Both acute and chronic events in airway remodeling result from altered expression of multiple genes encoding protein mediators of cell-cell signaling, ECM remodeling, cell cycle control, and intracellular signaling pathways [72]. In mammals, three groups of MAPKs have been identified: the extracellular signal-regulated protein kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 MAPK. ERK is activated by diverse stimuli, including growth factors and cytokines [73]. The p38 MAPK is activated by cellular stresses, including UV radiation, LPS, growth factors, and cytokines. The JNK is activated by many of the same stimuli that activate p38 MAPK, such as cellular stresses and numerous cytokines. Thus, the inhibition of MAPKs activity via pharmacological or genetic approaches blocks allergic inflammation of airways. Moreover, asthmatic patients demonstrated increased immunostaining for phospho (p)-ERK, p-p38 MAPK, and p-JNK [72]. p-ERK staining was observed especially in airway epithelium and smooth muscle cells. The phosphorylation of p38 MAPK was primarily observed in the basal layer of the columnar epithelium. It is likely that p38 MAPK drives basal metabolic processes for this particular cell type. There was significant correlation between clinical severity of asthma and intensity of immunostaining for p-ERK and p-p38 MAPK and between p-ERK and the number of tissue eosinophils and neutrophils in the airways. p-JNK primarily stained airway smooth muscle cells. Early studies of p38 MAPK demonstrated that IL-1β and TNF-α activate the p38 MAPK in monocytes [74]. Furthermore, inhibition of the p38 MAPK pathway was shown to exert anti-inflammatory effects through inhibition of IL-1β, IL-6, and TNF-α expression [75]. In airway smooth muscle cells, there is solid evidence that both ERK and p38 MAPK pathways contribute to IL-1β-induced COX-2 expression and PGE_2 synthesis [76, 77].

In a mouse model of chronic lung inflammation (allergic inflammation), significant inhibition of TNF-α, IL-4, IL-13, and RANTES (regulated on activation, normal T-cell expressed and secreted) in lung homogenates was observed with JNK inhibitor, SP600125 [78]. In addition, we also found that LTA or IL-1β could induce $cPLA_2$, COX-2, or MMP-9 in human airway smooth muscle cells or A549 cells [3, 44]. Therefore, MAPKs play an important role in mediating airway and lung inflammation (Figure 2).

3.7. NF-κB. NF-κB is viewed as a master regulator of inflammatory responses because it plays an essential role in the evolution as well as the resolution phase of inflammation. NF-κB controls a wide spectrum of biological effects ranging from immune and stress-induced responses to cell fate decisions such as proliferation, differentiation, tumorigenesis, apoptosis, and tissue remodeling [8]. NF-κB usually exists as a heterodimeric complex of p50 and p65/RelA subunits. In unstimulated cells, NF-κB is found in the cytoplasm as an inactive non-DNA-binding form, associated with an inhibitor protein called inhibitory κB (IκB) which masks the nuclear translocation signal and so prevents NF-κB from entering the nucleus. Upon cell stimulation with various NF-κB inducers, IκBα is rapidly phosphorylated on two serine residues, which targets the inhibitor protein for ubiquitination by the E3 ubiquitin-ligases (E3RSIκB) and subsequent degradation by the 26S proteasome [79]. The released NF-κB dimer can then be translocated into the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. NF-κB is activated by numerous extracellular stimuli, including cytokines such as TNF-α and IL-1β, viruses and environmental particulates (PM10s), and oxidative stress [8]. Exogenous H_2O_2 also activated NF-κB in a murine model of ROS-induced acute lung injury. Administration of OTC (L-2-oxothiazolidine-4-carboxylate) resulted in significant reduction of NF-κB translocation into the nucleus and expression of adhesion molecules, chemokines, and cytokines [80]. Previous study demonstrated that NF-κB activation occurred rapidly in the ovalbumin (OVA) model of allergic airways disease and that NF-κB activation predominantly occurred in the epithelial cells of the conducting airways, in association with enhanced mRNA expression of NF-κB-regulated chemokine genes, including MIP-2 and eotaxin [81]. A novel cyclin-dependent kinase inhibitor (BAI) has been shown to downregulate TNF-α-induced expression of cell adhesion molecules by inhibition of NF-κB activation in human pulmonary epithelial cells [82]. Recently, we also demonstrated that overexpression of HO-1 protects against TNF-α-mediated airway inflammation by downregulation of TNFR1-dependent oxidative stress and NF-κB activation [83]. Taken together, these results show that NF-κB plays a key role in mediating the expression of inflammatory proteins in airway and lung inflammation and injury (Figure 2).

3.8. AP-1. AP-1 transcription factor typically consists of combinations of Jun (c-Jun, Jun B, Jun D) and Fos proteins (c-Fos, Fos B, Fra-1, Fra-2), which bind to the promoters of target genes. It was found to be responsible for the transcriptional activation of various genes that were activated by phorbol esters (such as PMA) via activation of PKC [84]. AP-1 may be activated via PKC and by various cytokines, including TNF-α and IL-1β, via several types of PTK and MAPKs, which themselves activate a cascade of intracellular kinases [85]. Certain signals rapidly increase the transcription of the fos gene, resulting in increased synthesis of Fos protein. Other signals lead to activation of kinases that phosphorylate c-Jun, resulting in increased activation. Specific Jun and Fos kinases are now recognized and may play a key role in the regulation of cellular responsiveness to cytokine signals. Recent studies showed that sirtuin 1 (SIRT1) directly interacted with c-Jun and repressed the transcriptional activity of AP-1, thus decreasing MMP-9 expression [86]. More recently, it was reported that SIRT1 decreased c-Fos/c-Jun acetylation induced by p300 and inhibited the transcriptional activity of AP-1 and subsequent COX-2 expression and PGE_2 generation [87]. Thus, AP-1 may play a critical role in mediating expression of various inflammatory proteins. There is evidence for increased expression of c-Fos in epithelial cells in asthmatic airways [88], and many of the stimuli relevant to asthma that activate NF-κB will also activate AP-1. Thus, AP-1 is also a key factor in respiratory diseases (Figure 2).

4. Therapeutic Implications

Kinase pathways have become recognized as key cellular signal transducers, and several protein kinase inhibitors are in development for the treatment of respiratory diseases. The pyridinylimidazole compounds, exemplified by SB203580, were originally prepared as inflammatory cytokine synthesis inhibitors that subsequently were found to be selective inhibitors of p38α and β MAPK [89]. SB203580 inhibits the catalytic activity of p38 MAPK by competitive binding inthe ATP pocket. These drugs inhibit many inflammatory cytokines, chemokines, and inflammatory enzymes [89]. SB203580 was shown to attenuate BAL TNF-α production in an ovalbumin challenged rat model of asthma [90] and SB2439063 reduced neutrophilia and mediator expression in rat COPD models [91]. In addition, a recent study also indicated that in acute and chronic animal models of asthma, SP600125 (a JNK inhibitor) reduces BAL accumulation of eosinophils and lymphocytes, cytokine release, serum IgE production, and smooth muscle proliferation after repeated allergen exposure [92]. Intratracheal administration of LY294002 reduced OVA-induced increases in total cell counts, eosinophil counts, and IL-5, IL-13, and CCL11 (eotaxin) levels in BAL fluid and dramatically inhibited OVA-induced tissue eosinophilia and airway mucus production [63]. Inhibition of SFKs using specific inhibitors for Src PTKs (either PP2 or SU-6656) attenuated LPS-induced lung injury and capillary permeability and reduced LPS-dependent cytokine and chemokine levels in the lung and the serum [68].

RNAi is the process of sequence-specific, post-transcriptional/transcriptional gene silencing through small interfering RNA (siRNA). RNAi is a popular method of

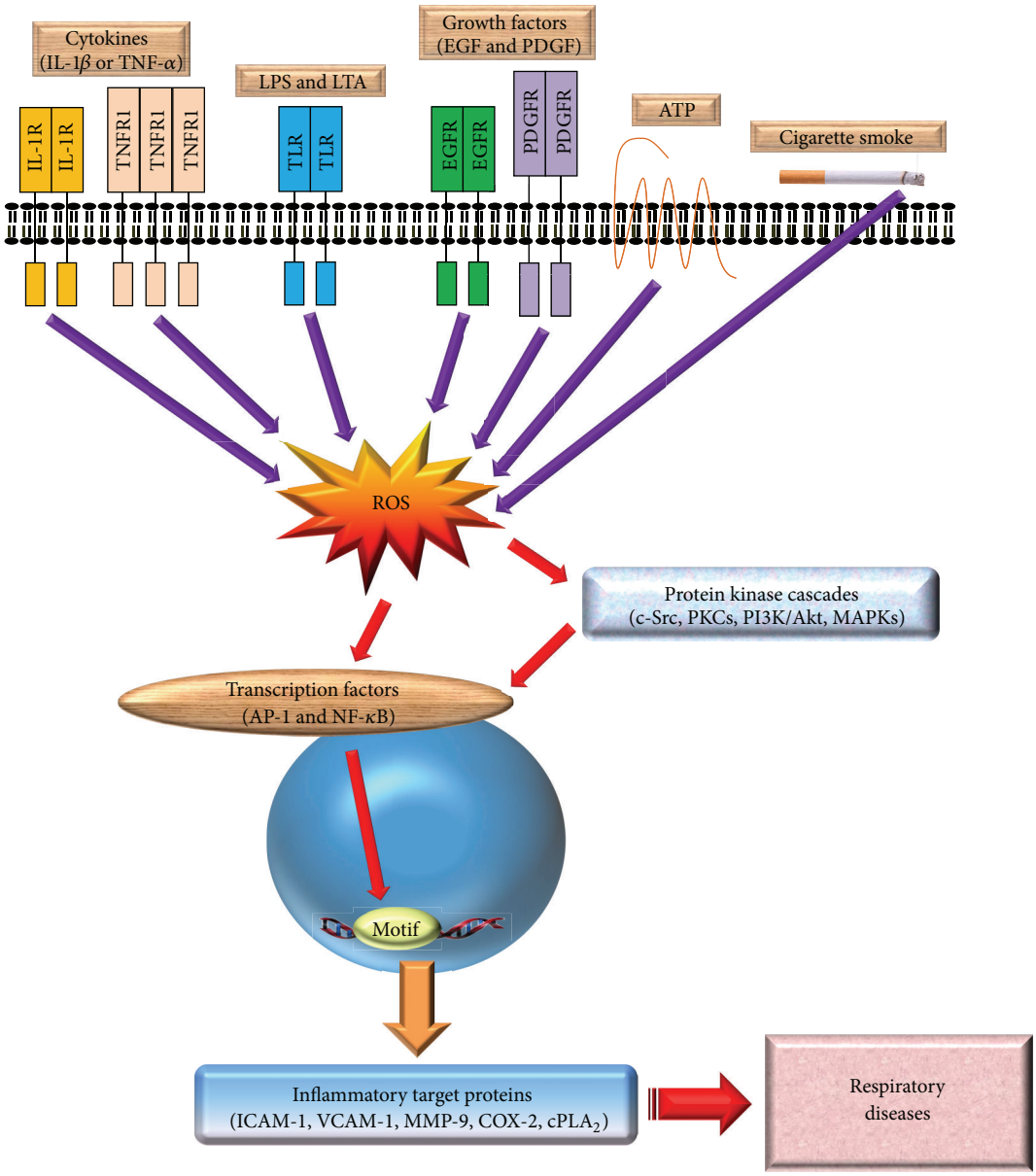

Figure 3: General overview of the inflammatory signaling pathways in respiratory diseases. Many of the known inflammatory target proteins, such as MMP-9, ICAM-1, VCAM-1, COX-2, and $cPLA_2$, are associated with inflammatory signaling pathways induced by various stimuli, including TNF-α, IL-1β, ATP, cigarette smoke, LTA, or LPS. Moreover, the SFKs, PKCs, growth factor tyrosine kinase receptors, NADPH oxidase/ROS, PI3K/Akt, and MAPKs are components of signaling cascades that respond to extracellular stimuli by targeting transcription factors, such as NF-κB and AP-1, resulting in the modulation of inflammatory gene expression associated with pulmonary diseases.

controlling gene expression and has a potential in the development of drugs for several diseases, such as various types of cancer and viral infections. Gene therapy for asthma has already been developed and has demonstrated promising results in animal models [93]. Recent progress in delivering siRNA to the respiratory system has also improved the therapeutic feasibility of RNAi for asthma. IL-5 has been suggested to be involved in the development of airway hyperresponsiveness. Huang et al. indicated that siRNA against IL-5 decreases airway eosinophilia and hyperresponsiveness [93]. In the context of allergic immune responses, activation of STAT6 is pivotal for Th2-mediated IgE production and development of airway inflammation and hyperreactivity [94]. Moreover, STAT6 siRNA has been shown to inhibit allergic airway inflammation and hyperreactivity in mice [94].

Inflammatory airway and lung diseases are characterized by chronic inflammation and oxidant/antioxidant imbalance, a major cause of cell damage/injury. Numerous studies have shown the effectiveness of polyphenols in limiting the progression of chronic diseases. This is likely to occur, at least in part, because of the antioxidant capacity of these molecules,

which extends from the availability of hydroxyl groups and the presence of conjugated double bonds. Resveratrol has been reported to increase antioxidant capacity and reduce various markers of oxidative stress [8]. Recently, Lee et al. indicated that resveratrol inhibits the activation of NF-κB (p65) by TNF-α or PMA and reduces ATP-induced mucin secretion from cultured primary rat tracheal surface epithelial (RTSE) cells [95]. On the other hand, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a novel radical scavenger protects neurons by reducing endothelial injury and by ameliorating neuronal damage caused by brain ischemia. Treatment of edaravone decreases interstitial edema and inflammatory cell infiltration as well as prevents the process of pulmonary fibrosis [8]. In addition, reports of clinical benefit in airway and lung diseases for increased vitamins C and E and other dietary antioxidants have been varied. Morita et al. indicated that vitamin E treatment prior to injury largely prevents the increase in pulmonary permeability index and moderates the increase in lung lymph flow, increases the PaO_2/FiO_2 ratio, ameliorates both peak and pause airway pressure increases, and decreases plasma conjugated dienes and nitrotyrosine [96]. Erdosteine is a thiol antioxidant having mucoactive properties and the ability to reduce bacterial adhesiveness. This compound was introduced as a mucolytic agent for the treatment of chronic airway and pulmonary diseases. Erdosteine breaks the disulfide bonds of mucus glycoproteins, affecting the physical properties of the mucus, thus leading to increased cough clearance [97]. In addition, erdosteine has been reported to have antioxidant, anti-inflammatory, and antibacterial activity [97]. Negro et al. showed that erdosteine at a dose of 600 mg/day proved effective in significantly reducing ROS levels in peripheral blood of stable COPD patients who are current smokers, together with reduction in levels of some chemotactic proinflammatory cytokines (IL-6 and IL-8) in their bronchial secretions [98].

Recently, Greene and Gaughan represent new therapeutic targets and medicines that target specific microRNAs (miRNAs) and may have potential in the treatment of asthma [99]. miRNAs are regulatory RNAs that affect protein synthesis [99]. There have been a number of studies in the field of miRNA that implicate specific miRNAs in the pathophysiology of asthma. For example, studies using mouse models have identified miRNAs that are altered in response to allergen challenge. Certain miRNAs that are involved in the regulation of IL-13 and the TH2 response, key components of the asthmatic response, have been shown to be amenable to modulation by pre-miRs and anti-miRs. Other studies have identified miRNAs that are implicated in bronchial smooth muscle hyperresponsiveness and proliferation. Thus, developing miRNA-based medicines to treat the pulmonary manifestations of asthma could yield therapeutics with new properties that have the potential to treat both the inflammation and hyperresponsiveness associated with this disease.

5. Conclusions

There is an increasing evidence that inflammatory proteins, such as VCAM-1, ICAM-1, $cPLA_2$, COX-2, and MMP-9 are involved in the pathogenesis of respiratory diseases, such as asthma and COPD (Figure 3). Moreover, various inflammatory signaling pathways, including PKCs, NADPH oxidase/ROS, EGFR, PDGFR, c-Src, PI3K/Akt, MAPKs, AP-1, and NF-κB, are involved in the regulation of these inflammatory proteins (Figure 3). Further exploration of the role of VCAM-1, ICAM-1, $cPLA_2$, COX-2, and MMP-9 in these highly prevalent diseases is crucial to identify which are possible therapeutic targets. The development of new inhibitors that are highly specific but have no major adverse effects is essential. As targeted delivery of inflammatory proteins inhibitors directly to the airway and lung might result in fewer side effects, this option should also be explored. Although the use of inhibitors of inflammatory signaling pathways in the treatment of respiratory diseases seems very attractive, further studies are needed to identify the exact role of inflammatory signaling molecules in these diseases and to develop highly specific inhibitors.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Anti-Inflammatory Dimethylfumarate: A Potential New Therapy for Asthma?

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Asthma is a chronic inflammatory disease of the airways, which results from the deregulated interaction of inflammatory cells and tissue forming cells. Beside the derangement of the epithelial cell layer, the most prominent tissue pathology of the asthmatic lung is the hypertrophy and hyperplasia of the airway smooth muscle cell (ASMC) bundles, which actively contributes to airway inflammation and remodeling. ASMCs of asthma patients secrete proinflammatory chemokines CXCL10, CCL11, and RANTES which attract immune cells into the airways and may thereby initiate inflammation. None of the available asthma drugs cures the disease—only symptoms are controlled. Dimethylfumarate (DMF) is used as an anti-inflammatory drug in psoriasis and showed promising results in phase III clinical studies in multiple sclerosis patients. In regard to asthma therapy, DMF has been anecdotally reported to reduce asthma symptoms in patients with psoriasis and asthma. Here we discuss the potential use of DMF as a novel therapy in asthma on the basis of *in vitro* studies of its inhibitory effect on ASMC proliferation and cytokine secretion in ASMCs.

1. Introduction

Asthma is a disease of the airways characterized by chronic inflammation associated with airway hyperresponsiveness (AHR) and airway wall remodeling. In the past decades, numerous immunological studies of lung fluids and animal studies suggested that asthma is a disease caused by the deregulation of the immune response to inhaled or eaten allergens that leads to structural changes of the airway tissue which increase with the duration of the disease [1–3]. New clinical studies, especially in childhood asthma, suggest that inflammation and remodeling occur independent of each other in parallel or even that airway wall remodeling especially of the airway smooth muscle occurs before any signs of inflammation can be found [4–7]. Therefore the question if the pathophysiology of the airway smooth muscle cell is crucial for the pathogenesis of asthma was reactivated [8].

The increased mass of cells within airway smooth muscle (ASM) bundles is one of the most striking pathological features in the asthmatic airway and inversely correlates with lung function in asthma [9]. The role of the airway epithelium as a master regulator of airway wall forming cells has recently been demonstrated; however, the mechanism(s) by which a deranged epithelium affects the underlying cell types has to be studied in more detail [10]. In Figure 1, we provide two examples of the airway wall obtained from nonasthmatic adults and from two moderate asthmatics. Both tissue sections of the asthmatic airways demonstrate the well-known loss of epithelium integrity, the significant increase of the basement membrane thickness, and the increased number of ASM bundles. In contrast, there is no clear increase of the thickness and structure of the subepithelial fibroblast/myofibroblast cell layer (Figure 1).

Recent studies support the hypothesis that the increase of the ASM bundles in the airway wall of asthma patients is an early event developing independently in parallel to inflammation [4–7]. Comparing the airway wall structure in biopsy material of 53 school children with treatment-resistant asthma to that of 16 healthy age-matched controls provided evidence that remodeling, especially the increase of ASM bundle size, was independent of proinflammatory

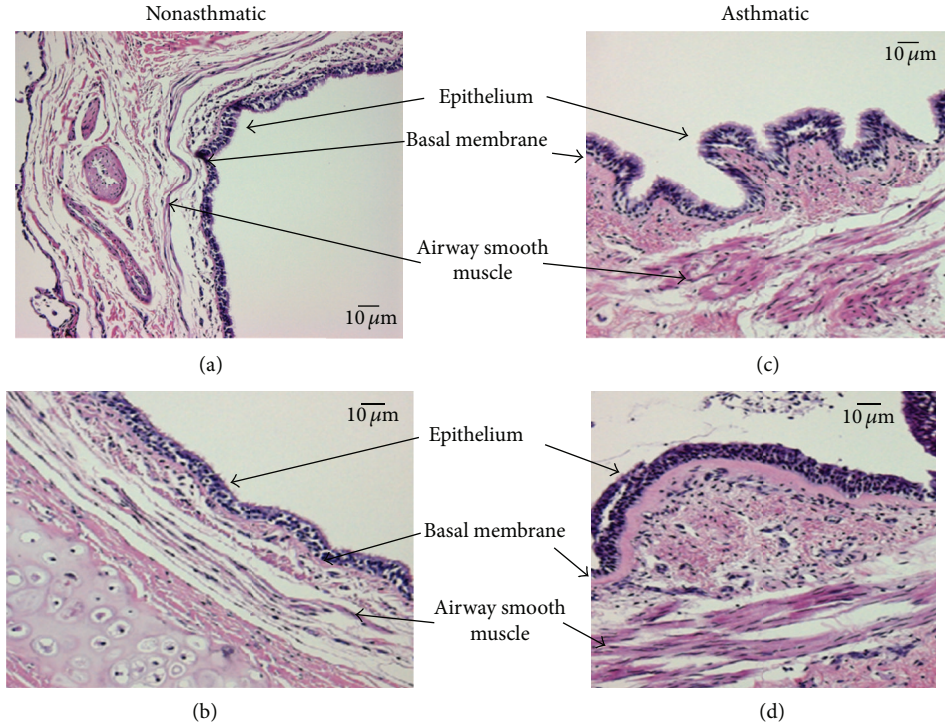

Figure 1: Histological tissue sections of two patients with moderate asthma and two nonasthma controls. Arrows indicate the loss of epithelium integrity, the increase of the basement membrane thickness, and the increased number of ASM bundles in the asthmatic airways (c, d), compared to nonasthmatic airways (a, b).

Th2-cell derived cytokines (IL-4, IL-5, and IL-13), while eosinophil counts varied over a wide range [4]. Assessing endobronchial biopsy specimen of ASM obtained from 47 wheezing preschool children and 21 nonwheezing controls, it was documented that an increased mass of ASM occurred in the majority of wheezing children [7]. In a nonhuman primate model of asthma and COPD, a striking rearrangement of the smooth muscle cell bundles from a nonstructured into a spiral-like formation surrounding the airway was described [11, 12]. These findings suggested that allergic as well as nonallergic asthma triggers induce a pathological reorganization of ASM bundles by an unknown mechanism. Furthermore, it was reported that inhalation of either methacholine or house dust mite allergens by volunteering patients with mild asthma leads to airway wall remodeling within only eight days, which was prevented by inhalation of a long-acting β2-agonist [5]. In addition, removal of ASM cells by thermoplasty significantly reduced asthma symptom of several years and is today regarded as a therapeutic option for severe asthma [13–15]. Several *in vitro* and *in vivo* studies have shown that ASM cells (ASMCs) secrete a variety of mediators, which enable them to interact with immune cells and to modulate the inflammatory response and remodeling in asthma [9, 16, 17]. Together these observations strongly support the central role of ASMC in the pathogenesis of asthma and therefore they are interesting targets for asthma therapy [18, 19].

Inhaled long-acting β2-agonists (LABAs) combined with glucocorticoids (GCs) remain the most effective therapy for asthma. However, a considerable number of asthma patients do not respond to inhaled GCs [20]. In addition, the current therapy only controls disease symptoms, but none of the existing asthma medications cures the disease. This emphasizes a need for new therapeutic options to treat asthma more efficiently [21–23].

Dimethylfumarate (DMF) is a potent anti-inflammatory medication for psoriasis and it has also been shown to suppress inflammation in other chronic inflammatory diseases, especially MS [24]. Interestingly, DMF has been anecdotally reported to reduce asthma symptoms in patients suffering from asthma and psoriasis. In experimental studies, DMF inhibited proliferation and proinflammatory transcription factors as well as the secretion of asthma-relevant cytokines in primary human lung cells [25–28]. In this paper we describe how ASMC-derived CXCL10, CCL11, or RANTES may contribute to airway inflammation in asthma and how these chemokines can be controlled by the anti-inflammatory action of DMF.

2. Airway-Smooth-Muscle-Cell-Derived Chemokines Contribute to Airway Inflammation

ASMC hyperplasia and hypertrophy in asthmatic airways had already been described in 1922 and was considered as the main cause of AHR [8]. Interestingly, in recent years it became evident that ASMCs hypertrophy may precede inflammation and that ASMC are an important source of inflammatory mediators and therefore actively contribute

to airway inflammation [4–6]. Proinflammatory cytokines activate the ASMC to secrete further chemokines that subsequently attract immune cells such as mast cells [16] or T lymphocytes [9] into the ASM bundle. These immune cells then interact with ASMC and alter their contractile function, enhance proliferation, and further amplify the secretion of proinflammatory factors. For instance, it has been reported that mast-cell-derived tryptase enhanced ASMC contractility [29], induced ASMC proliferation [30], and increased TGF-β1 secretion [31]. Similarly, T lymphocytes infiltrated the asthmatic ASM bundle and induced ASMC proliferation [9, 32]. In the following, we will focus on ASMC-derived chemokines CXCL10, CCL11 (eotaxin), and RANTES, which are crucially involved in the trafficking of immune cells into the airway in asthma.

3. CXCL10 (IP-10)

ASMC-derived CXCL10 is a potent chemoattractant for human lung mast cells [33]. In a disease-specific pattern, ASM bundles are infiltrated by activated mast cells in asthma, as this pathology was neither observed in patients with eosinophilic bronchitis nor in nonasthmatic controls [16, 34]. Consequently, it was hypothesized that the ASMC itself attracts mast cells by secreting chemokines such as CXCL10. This assumption was supported by studies in ASMC of asthma patients, which expressed higher levels of CXCL10 than ASMC derived from nonasthmatic controls. In addition, CXCL10 has been detected in the ASM bundles of asthmatic airway biopsies only and all mast cells within the ASM bundles expressed CXCR3, which is the receptor for CXCL10 [33]. A wide range of asthma relevant stimuli have been reported to increase signaling pathways that may lead to incerased CXCL10 secretion [35–38].

CXCL10 is secreted by ASMC after stimulation with proinflammatory cytokines such as TNF-α, IFN-γ, or IL-1β, which activated MAPK JNK, NF-κB, STAT 1, and the transcriptional coactivator CREB-binding protein [25, 39–41]. In addition, CXCL10 secretion by ASMC is sensitive to changes in cellular glutathione (GSH) levels [27], suggesting a link of this signaling pathway to the asthma-associated upregulation of mitochondria, which control the cellular redox system [42]. Interestingly, the thiazolidinedione ciglitazone strongly inhibited cytokine-induced CXCL10 protein without affecting CXCL10 mRNA level, suggesting that CXCL10 is regulated on the posttranslational level in ASMC [41]. In this context, it would be of interest if the posttranscriptional regulation of CXCL10 in asthma occurs through the recently described modified translation control [43].

4. CCL11 (Eotaxin)

CCL11 is a potent eosinophil chemoattractant and eosinophilia is a prominent pathology of the asthmatic airway [44–46]. *In vivo*, the asthmatic ASM bundle showed strong signals of CCL11 immunoreactivity and CCL11 mRNA [47]. In addition, in bronchial biopsies of asthmatic patients, CCL11 expression correlated with asthma severity [44, 48]. *In vitro*, ASMC secreted CCL11 [26, 35] and it has been shown that ASMCs derived from asthmatic patients produce higher levels of CCL11 mRNA and protein than those derived from nonasthmatic controls [49, 50]. CCL11 secretion from ASMCs can be induced by Th1 and Th2 cytokines including TNF-α, IL-1β, IL-13, or IL-4 [26, 35, 51, 52] and critically involves the activation of NF-κB [26, 35]. In addition to its chemoattractant function, CCL11 has been proposed to stimulate ASMC hyperplasia, as ASMCs express the CCL11 receptor CCR3, which, upon activation, induces ASMC migration but in this study did not induce ASMC proliferation [49]. However, in a different study CCL11 increased [^{3}H]-thymidine incorporation and DNA synthesis and decreased the rate of apoptosis in ASMC [53].

5. RANTES

RANTES is a chemoattractant for eosinophils, T cells, and monocytes and thus has been linked to asthma pathology [54, 55]. In asthma, the ASM bundles are infiltrated by T lymphocytes [9] and ASMCs produce elevated levels of RANTES mRNA [56]. ASMC-derived RANTES was therefore proposed to participate in the chemotaxis of T lymphocytes to the ASM bundle. IFN-γ and TNF-α stimulated RANTES secretion from ASMC *in vitro* [26, 35, 51] and this was dependent on the activation of NF-κB [26, 35], MAPK JNK [57], and AP-1 [58]. Like CCL11, RANTES participated in airway remodeling by inducing ASMC proliferation and migration [53, 59]. Interestingly, recombinant RANTES is degraded by mast cell tryptase and thereby reduced RANTES-activated chemotaxis of eosinophils [55]. Furthermore, mast-cell-derived histamin has been shown to reduce RANTES secretion by ASMC *in vitro* [60]. The inactivation of RANTES by mast-cell-derived mediators might explain the phenomenon that the asthmatic ASM bundle is infiltrated by a much higher number of mast cells than of T lymphocytes [16].

6. Dimethylfumarate (DMF)

DMF is the ester of the unsaturated dicarboxylic fumaric acid (Figure 2). The German chemist Walter Schweckendieck described the anti-inflammatory properties of DMF in 1959 [61]. Schweckendieck postulated that psoriasis is caused by a dysfunctional citric acid cycle and hypothesized that DMF is metabolized to fumaric acid, which enters the citric acid cycle and thereby inhibits the inflammatory processes. Schweckendieck tested several forms of fumarates in self-experiments and his psoriasis improved [61]. Based on his findings, the physician Günther Schäfer developed a psoriasis therapy with a mixture of fumaric acid esters. In 1989 a controlled clinical study proved the efficacy of DMF in psoriasis [62] and soon thereafter a mixture of DMF with calcium, magnesium, and zinc salts of ethyl hydrogen fumarate was registered in Germany as Fumaderm for the systemic treatment of psoriasis. Fumaderm is widely used for the treatment of moderate to severe psoriasis vulgaris in Northern Europe [24, 63–65]. In 2003 a second-generation

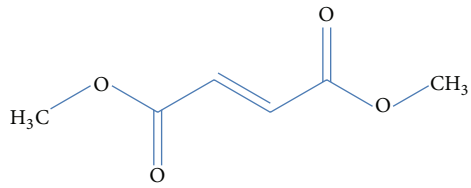

Figure 2: Chemical structure of dimethylfumarate.

fumaric acid derivate, BG-12, which only contains DMF in enteric-coated microtablets, has been developed (no authors listed BG 12). BG-12 has been successfully tested in phase II and III clinical studies for the oral treatment of multiples sclerosis [66–68].

7. Clinical Use and Pharmacokinetic of DMF

Fumaderm is administered orally in slowly increasing doses till a clinical effect is observed. The initial dose is 30 mg DMF per day, which can be increased to a maximum daily dose of 720 mg DMF [64, 65, 69]. In several clinical studies, Fumaderm showed an excellent antipsoriatic effect causing an improvement of about 75% of the baseline PASI (psoriasis area and severity index) in up to 70% of the patients tested [64, 69–71]. Even though side effects such as gastrointestinal complaints or flushing occur, Fumaderm has been shown to be very safe as long-term treatment for psoriasis with no long-term toxicity, higher risk for infections or malignancies [72].

Although Fumaderm has been used for many years, its pharmacokinetic is still poorly understood. DMF is the main ingredient of Fumaderm and is clinically most efficacious [73]. However, after oral administration of Fumaderm only, monomethylfumarate (MMF) with serum peak concentrations at around 20 μM, but not DMF, was detectable in blood plasma [68, 74]. It was therefore hypothesized that DMF's hydrolysis-product MMF is the actual active compound. However, this notion has been questioned by many *in vitro* studies showing that MMF is pharmacologically less effective when compared to DMF. For instance, in human endothelial cells, DMF reduced the expression of VCAM-1, ICAM-1, and E-selectin with IC_{50} values of approximately 50 μM, whereas monoethylfumarate at concentrations of 10–100 μM showed no inhibitory effect [75]. Similarly, in human keratinocytes, DMF at concentrations of 7–140 μM inhibited IL-1β-induced phosphorylation of MSK-1, whereas MMF at 140 μM had no effect on MSK-1 activation [76]. DMF has been shown to rapidly react with glutathione (GSH) under physiological conditions *in vitro* [77]. It was therefore proposed that DMF is released into the bloodstream where it is absorbed by cells and conjugated to GSH, explaining why DMF is not detectable in plasma after oral intake. This assumption was supported by a study, showing that DMF-GSH-conjugate metabolites are secreted in the urine of DMF-treated psoriasis patients [78]. However, the complex pharmacokinetic of DMF makes it difficult to relate concentrations of DMF used in cell culture models to DMF concentrations in target tissue or plasma levels *in vivo*.

8. The Anti-Inflammatory Action of DMF in Psoriasis

Several *in vitro* and *in vivo* studies have proven the potent anti-inflammatory effects of DMF and its favorable safety profile in the treatment of psoriasis [79]. DMF reduced the proinflammatory contribution of several cell types including T lymphocytes, mononuclear blood cells, dendritic cells (DCs), endothelial cells, and keratinocytes, which are all crucially involved in the inflammatory process in psoriasis, as will be discussed in the following.

In purified human T lymphocytes, DMF inhibited the proinflammatory transcription factor NF-κB and induced apoptosis [80, 81]. *In vivo* studies in psoriasis patients showed that DMF reduced the total number of peripheral blood T lymphocytes and the number of T lymphocytes in psoriatic lesions [82, 83]. Regarding its anti-inflammatory action, DMF inhibited the maturation of DC by reducing the expression of interleukin- (IL-) 12, IL-6, major histocompatibility complex (MHC) class II, cluster of differentiation (CD)80, and CD86, mainly through the inhibition of NF-κB, and mitogen- and stress-activated protein kinase- (MSK-) 1. These immature DCs were shown to generate fewer IFN-γ- and IL-17-producing T lymphocytes [84].

In human keratinocytes and mononuclear blood cells, DMF inhibited mRNA and protein expression of CXCL8, CXCL9, and CXCL10 [85]. Furthermore, the expression of CXCL8 and IL-20 mRNA in human keratinocytes was inhibited by DMF, which was mediated by reduced MSK-1, NF-κB, and cAMP response element-binding protein (CREB) activation [76, 86]. In human peripheral blood mononuclear cells, DMF reduced GSH level and upregulated heme oxygenase- (HO-) 1 expression, which resulted in the inhibition of TNF-α, IL-12, and IFN-γ secretion [87]. In addition, DMF inhibited macrophage migration inhibitory factor- (MIF-) induced human keratinocytes proliferation by reducing MSK-1, 90 kDa ribosomal S6 kinase (RSK), CREB, and JunB phosphorylation [86].

In endothelial cells, DMF inhibited the nuclear entry of NF-κB, resulting in a reduced expression of TNF-induced tissue factor [88]. Furthermore, the expression of the adhesion molecules intercellular adhesion molecule- (ICAM-) 1, vascular cell adhesion molecule- (VCAM-) 1, and E-selectin on endothelial cells was inhibited by DMF, resulting in impaired lymphocyte rolling and adhesion [89]. DMF also had antiangiogenic properties by the inhibition of vascular endothelial growth factor receptor (VEGFR)2 expression on human endothelial cells [90].

9. DMF as Potential Treatment for Multiple Sclerosis

Multiple sclerosis (MS) is a chronic disease of the central nervous system, which is characterized by inflammation, demyelination, axonal loss, and glial proliferation. Currently therapy for MS is parenteral administered and is only partially effective, as patients do not remain relapse-free after treatment [91]. Clinical phase II and III studies investigating

a potential use of DMF for oral treatment of relapsing-remitting MS (RRMS) as well as a number of *in vitro* studies on MS-relevant cells have shown very promising results.

10. Clinical Studies

Schimrigk et al. [92] performed the first open-label, baseline controlled clinical study with Fumaderm in patients with RRMS. They showed that Fumaderm treatment significantly decreased the number and volume of gadolinium-enhancing lesions. In addition, they reported elevated levels of the cytokine IL-10 and decreased expression of the proinflammatory cytokine IFN-γ. Furthermore, they found that apoptosis was increased in lymphocytes [92]. In 2008, Kappos et al. [66] published the results of a multicentre, randomized, double-blind, placebo-controlled phase IIb study testing the efficacy and safety of BG-12 (contains DMF only) in RRMS patients. Patients treated with BG-12 showed a dose-dependent reduction of MS lesions compared to the placebo group and there was a trend of a lower annualized relapse rate. BG-12 was generally well tolerated and showed a favorable safety profile [66]. This study was followed by a placebo-controlled phase III clinical study, confirming that BG-12 treatment resulted in a significant reduction of MS lesions compared to placebo. Furthermore, the study showed that the proportion of patients who had a relapse as well as the annualized relapse rate was reduced and a decrease of the disability progression rate was observed in BG-12-treated MS patients [67, 93].

11. Mode of Action of DMF in MS

DMF has been shown to initially reduce cellular reduced glutathione (GSH) in different cell types, including neuronal cells [94] and astrocytes [95], which resulted in the activation of the Nrf2 (nuclear factor erythroid-derived-2- (E2) related factor)/Keap-1 pathway in astrocytes, neuronal cells, and primary central nervous system cells [94–96]. A reduction of GSH and an activation of Nrf2 induced the expression of antioxidant enzymes such as NAD(P)H quinone oxidoreductase- (NQO-) 1, glutamate-cysteine ligase catalytic subunit (GCLC), or HO-1, resulting in reduced oxidative stress, proinflammatory cytokine secretion, and proliferation in these cells [87, 94, 95]. Therefore, the activation of the antioxidant Nrf-2 pathway is regarded to mediate DMF's beneficial effects in MS.

12. DMF as a Potential Asthma Therapy

In primary human lung mesenchymal cells, DMF inhibited the activation of the proinflammatory transcription factor NF-κB [26, 28, 35]. NF-κB activity was reported to be increased in the airways of asthmatic patients [97]. In addition, proinflammatory cytokines such as TNF-α activated NF-κB in ASMC *in vitro*, which resulted in the secretion of a variety of proinflammatory factors including RANTES, CXCL10, or CCL11 [26, 27, 35]. *In vitro,* inhibition of NF-κB downregulated the release of a range of proinflammatory mediators by ASMC [98]. Furthermore, the inhibition of NF-κB reduced airway inflammation in a mouse model of asthma [99].

In resting cells, NF-κB is retained in the cytosol in a complex formed with IκB (inhibitor of NF-κB). Upon stimulation, IκB is degraded, which allows free and activated NF-κB to enter the nucleus where it binds to specific NF-κB-sensitive DNA sequences which are located within the promoter regions of many proinflammatory genes [100]. NF-κB activity is regulated by posttranslational modifications such as phosphorylation, glutathionylation, or modification of histones that wind up NF-κB target genes. Protein glutathionylation is a redox-regulated process, whereby a cysteine-thiol of a protein forms a disulfide bond with the cysteine-thiol of GSH [101]. Interestingly, IκBα contains cysteine thiols, which are susceptible to glutathionylation [102].

In human cultured ASMC, DMF inhibited the nuclear entry of NF-κB and the binding of NF-κB to the corresponding DNA sequence [26, 35]. In a subsequent study, we provided evidence that the inhibitory effect of DMF on NF-κB nuclear entry is mediated by glutathionylation of IκBα, which inhibited its degradation [35]. Furthermore, DMF reduced NF-κB phosphorylation and altered the chromatin environment by inhibiting MSK-1-induced histone H3 phosphorylation in ASMC and keratinocytes [26, 35, 86]. Consequently, DMF inhibited the secretion of NF-κB-dependent cytokines such as interleukin (IL)-6, GM-CSF, eotaxin, RANTES, and CXCL10 when stimulated with TNF-α in ASMC and lung fibroblasts [25, 26, 28, 38]. An interesting study by Van Ly et al. has shown that DMF increased rhinovirus (RV) replication and failed to reduce RV-induced IL-6 and IL-8 by human lung fibroblasts [37]. In another study on HIV-infected monocyte-derived macrophages, DMF reduced HIV replication and neurotoxin release [103]. This suggests a virus-specific effect of DMF leaving it an open question whether DMF may help to control virus-induced asthma exacerbations.

In addition to NF-κB inhibition, DMF downregulated the secretion of PDGF-BB-induced IL-6 by airway smooth muscle cells and lung fibroblasts, which was most likely mediated by DMF's inhibitory effect on AP-1 and CREB in these cells [26, 28]. Besides inhibition of proinflammatory cytokine secretion, DMF was shown to reduce PDGF-BB-stimulated ASMC and lung fibroblast proliferation, suggesting a beneficial effect on airway remodeling in asthma [28, 36].

The airways of asthma patients are exposed to increased oxidative stress [104], which may alter ASMC proliferation and proinflammatory cytokine secretion through redox-sensitive signaling pathways [105, 106]. As described earlier, DMF activated the Nrf2 antioxidant response pathway, by reducing cellular GSH levels [94–96]. Once activated, Nrf2 binds to the antioxidant response elements (AREs) within the promoter of antioxidant genes such as HO-1 initiating their transcription. DMF reduced intracellular-reduced GSH level and upregulated HO-1 in ASMC [27, 35]. HO-1 is an inducible enzyme, which protects the lungs from increased oxidative stress [107]. In addition, HO-1 protected the lungs against hyperoxic injury and attenuated allergen-induced airway inflammation and hyperreactivity in animal models

Table 1: DMF effects on primary human lung cells.

Factors	DMF effect	After stimulation	Cell type	Reference
CXCL10	Inhibition at 10–100 μM DMF	TNF-α and/or IFN-γ and/or IL-1β	ASMC	[25, 27]
G-CSF	No effect Inhibition at 10 μM DMF	IFN-γ TNF-α	ASMC	[27]
Eotaxin	Inhibition at 10–100 μM DMF	TNF-α	ASMC	[26, 35]
RANTES	Inhibition at 10–100 μM DMF	TNF-α	ASMC	[26, 35]
IL-8	No effect at 0.01–1 μM DMF	Rhinovirus	Lung fibroblasts	[37]
GM-CSF	Inhibition at 100 μM DMF	TNF-α and IL-1β prior to stimulation with human serum (10%)	ASMC	[38]
IL-6	Inhibition at 10–100 μM DMF	TNF-α or PDGF-BB	ASMC and lung fibroblasts	[26, 28]
	No effect at 0.01–1 μM DMF	Rhinovirus	Lung fibroblasts	[37]
Cell proliferation	Inhibition at 10–100 μM DMF	PDGF-BB	ASMC and lung fibroblasts	[28, 36]

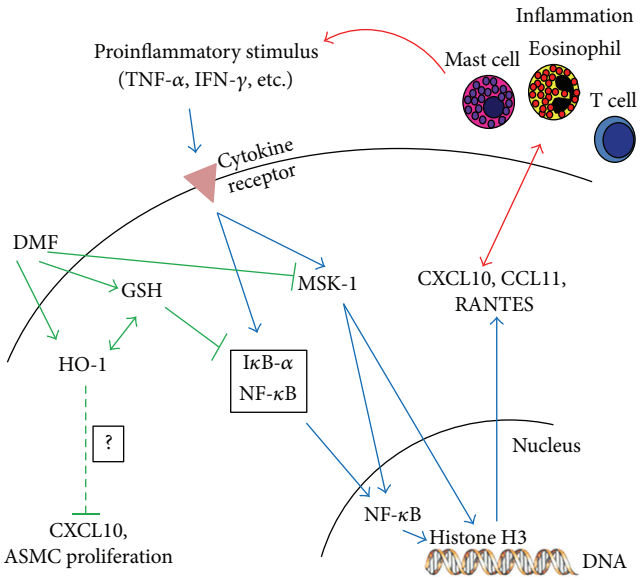

Figure 3: Secretion of chemokines such as CXCL10, CCL11, or RANTES by ASMC attracts immune cells (eosinophils, T cells, or mast cells) into the asthmatic airway. In turn, these immune cells secrete proinflammatory cytokines (TNF-α, IFN-γ, etc.), which stimulate proinflammatory signaling molecules such as NF-κB or MSK-1 in ASMC, enhancing its proinflammatory function. DMF reduces intracellular reduced glutathione (GSH) level and thereby induces HO-1 expression and IκB-glutathionylation. DMF-induced HO-1 decreased CXCL10 and ASMC proliferation by an unknown mechanism. IκB-glutathionylation inhibited IκB degradation and subsequent NF-κB nuclear entry. Furthermore, DMF inhibited MSK-1-mediated histone H3 and NF-κB phosphorylation, leading to reduced secretion of CXCL10, CCL11, and RANTES. DMF reduces ASMC chemokine secretion and may therefore inhibit the crosstalk between ASMC and immune cells, leading to airway inflammation.

of asthma [108, 109]. We demonstrated that DMF induced HO-1 and thereby inhibited PDGF-BB-induced ASMC proliferation and CXCL10 secretion [27, 36]. Importantly, DMF inhibited CXCL10 by ASMCs more efficiently when combined with the GC fluticasone, suggesting a GC sparing effect of DMF [27]. Furthermore, others have shown that activation of Nrf2 and induction of HO-1 inhibit TGF-β-induced ASMC proliferation and secretion of IL-6 [110]. In this context, it is of importance that in ASMC-derived from patients with severe asthma the binding of Nrf2 to the antioxidant response elements as well as the expression of HO-1 was reduced when compared to ASMC of nonasthmatic controls [110] suggesting that activation of Nrf2 and upregulation of HO-1 by DMF may compensate this pathology. Together these results emphasize a potential beneficial effect of Nrf2-mediated HO-1 induction in asthma. Regarding other anti-inflammatory actions of DMF, it reduced CXCL10 expression in cells stimulated with either a cytomix (IL-1β, TNF-α, and IFN-γ in combination) or IFN-γ alone, which has been shown to be insensitive to GC therapy [25, 27, 41]. Thus DMF may act as an anti-inflammatory drug in steroid-resistant asthma and significantly reduce healthcare costs. A summary of DMF effects on primary lung cells is summarized in Table 1 and the postulated beneficial action of DMF is provided in Figure 3.

13. Conclusion

Current asthma therapy is not sufficient to control symptoms in all asthma patients and does not cure the disease. Thus, it is important to find new therapeutic options to treat asthma. CXCL10, CCL11, and RANTES derived from ASMC are believed to be crucially involved in chemotaxis of immune cells into the asthmatic airways and are therefore actively involved in the development of airway inflammation in asthma. DMF reduced the secretion of CXCL10, CCL11, and RANTES as well as ASMC proliferation by inhibiting the proinflammatory transcription factor NF-κB and by upregulation of HO-1. Furthermore, DMF overcame GC resistance and had a GC-sparing effect in a cell culture model of asthma. Taken together, DMF's strong anti-inflammatory and antiproliferative effects in cultured human ASMC suggest that it may be beneficial in asthma therapy.

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Research Article

3,5,4′-tri-O-acetylresveratrol Ameliorates Seawater Exposure-Induced Lung Injury by Upregulating Connexin 43 Expression in Lung

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The aim of the present study was to examine the effects of 3,5,4′-tri-O-acetylresveratrol on connexin 43 (Cx43) in acute lung injury (ALI) in rats induced by tracheal instillation of artificial seawater. Different doses (50, 150, and 450 mg/kg) of 3,5,4′-tri-O-acetylresveratrol were administered orally for 7 days before modeling. Four hours after seawater inhalation, histological changes, contents of TNF-α, IL-1β and IL-10, and the expression of Cx43 in lungs were detected. Besides, the gap junction communication in A549 cells and human umbilical vein endothelial cells (HUVECs) challenged by seawater was also evaluated. Histological changes, increased contents of inflammatory factors, upregulation in gene level, and deregulation in protein level of Cx43 in lungs stimulated by seawater were observed. On the other hand, pretreatment with 3,5,4′-tri-O-acetylresveratrol significantly inhibited infiltration of inflammation, development of pulmonary edema, and contents of inflammatory mediators in lungs. Above all, 3,5,4′-tri-O-acetylresveratrol upregulated the expression of Cx43 in both gene and protein levels, and its intermediate metabolite, resveratrol, also enhanced the gap junction communication in the two cell lines. The results of the present study suggested that administration of 3,5,4′-tri-O-acetylresveratrol may be beneficial for treatment of inflammatorycells in lung.

1. Introduction

Drowning is the second accidental death causes in the world [1]. It is estimated that more than 500,000 people died from drowning each year. Basically, there are two different outcomes of drowning, death on the spot of drowning, and survival from the initial apnoea. However, with the lower respiratory tract challenged by water, the survivor may suffer acute lung injury (ALI), which is characterized by developing pulmonary inflammation and edema [2]. It was reported that inflammation factor secretion, pulmonary edema, and inflammatory spreading to entire lung or even both lungs were closely related to the alteration of communication between cells [3].

Gap junction channels (GJCs), connecting the cytoplasm between adjacent cells, are cell membrane channels, which provide a pathway for rapid exchange of ions, metabolites, and small intracellular signal molecules, such as Ca^{2+}, cyclic AMP, and so on. The critical contribution of GJCs to disease etiology has been intensively researched in recent years [4], and connexin 43 (Cx43), as the main mode of connection between alveolar epithelial cells, participates in a variety of acute/chronic lung disease occurrence and development [5]. Evidence proven that Cx43 may regulate Ca^{2+} signal path way, and this would play a pivotal role in acute lung injury [6].

3,5,4′-tri-O-acetylresveratrol (Figure 1), with three hydroxyls replaced by acetyls, is an analog of resveratrol. Several studies demonstrated that it exerted anti-γ-irradiation

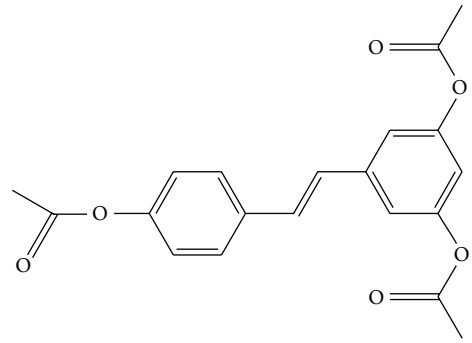

Figure 1: The structure of 3,5,4′-tri-O-acetylresveratrol.

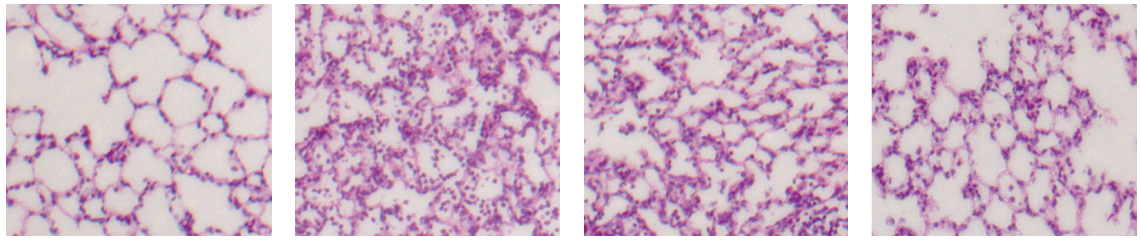

Figure 2: Microscopic findings of lung tissues stained with hematoxylin-eosin (×200). (a) control group; (b) seawater drowning group: edema, hemorrhage, thickened alveolar septum, and infiltration of inflammatory cells were observed in lung samples; (c) 3,5,4′-tri-O-acetylresveratrol (50 mg/kg) group; (e) 3,5,4′-tri-O-acetylresveratrol (450 mg/kg) group. Lung injuries were significantly alleviated by 3,5,4′-tri-O-acetylresveratrol, especially high dose of 3,5,4′-tri-O-acetylresveratrol.

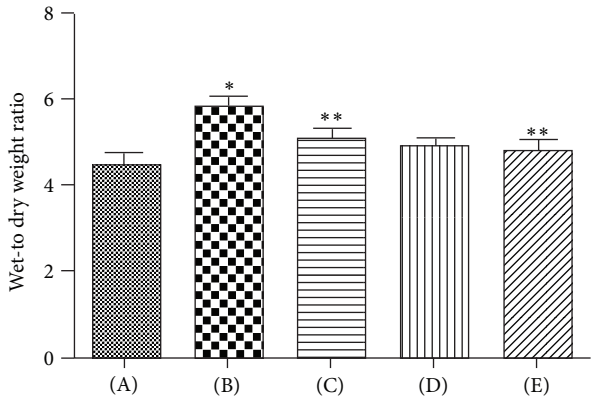

Figure 3: Effects of 3,5,4′-tri-O-acetylresveratrol on lung edema of rats following seawater aspiration. Data are mean ± SD, $n = 8$. (A) control group; (B) seawater drowning group; (C), (D), and (E) different doses of 3,5,4′-tri-O-acetylresveratrol groups. $^{*}P < 0.01$ versus control group; $^{*}P < 0.05$ versus $^{**}P$.

effects by inhibiting the expression of reactive oxygen species (ROS) [7] and acted as inhibitors of PAF-induced washed rabbit platelet aggregation [8]. Besides, our previous results showed that 3,5,4′-tri-O-acetylresveratrol might trigger the accumulation and concentration of resveratrol in lungs [9].

Based on the previous evidence, we put forward and proved in the present research the hypothesis that Cx43 participated in the inflammation induced by seawater instillation via affecting intracellular communication. While 3,5,4′-tri-O-acetylresveratrol could protect lungs by enhancing the expression of Cx43, suppressing inflammatory reaction and reconstructing intercellular communication.

2. Materials

2.1. Animal Preparation. Male Sprague-Dawley rats, weighing 180–220 g each, were obtained from the Animal Center (Fourth Military Medical University, Xi'an, China). The rats were housed in air-filtered, temperature-controlled units with 12-hour light-dark cycles and had free access to food and water. All experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Reagents. Seawater (osmolality 1300 mmol/L, pH 8.2, SW 1.05, NaCl 6.518 g/L, $MgSO_4$ 3.305 g/L, $MgCl_2$ 2.447 g/L, $CaCl_2$ 1.141 g/L, KCl 0.725 g/L, $NaHCO_3$ 0.202 g/L, NaBr 0.083 g/L) was prepared according to the major composition of the East China Sea provided by Chinese Ocean Bureau. 3,5,4′-tri-O-acetylresveratrol was obtained from the Pharmacy Department of Medicinal Chemistry with HPLC purity >99%. Resveratrol was purchased from Xi'an Grass Plant Technology Corporation (Xi'an, China), purity > 98%. Lucifer Yellow CH dilithium salt was purchased from Sigma

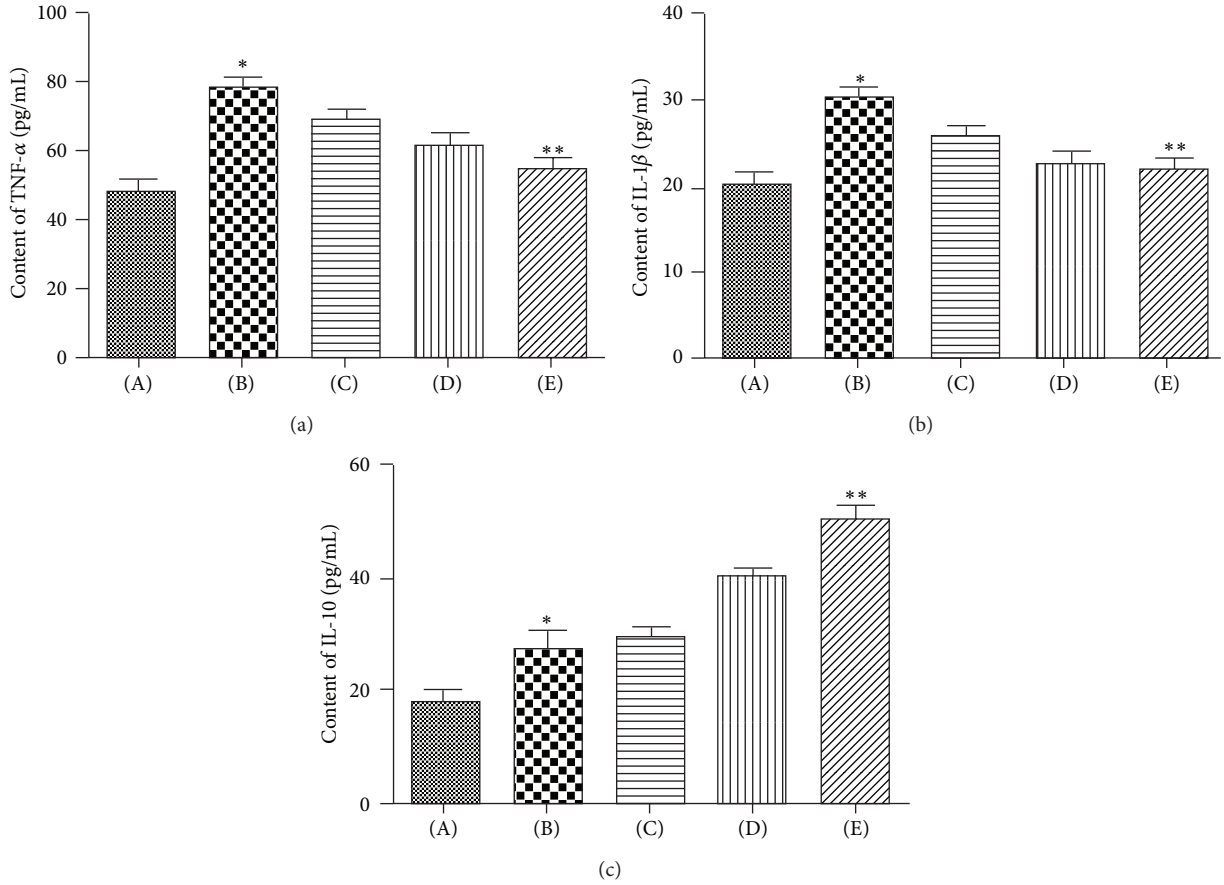

Figure 4: Effects of 3,5,4′-tri-O-acetylresveratrol on TNF-α (a), IL-1β (b), and IL-10 (c) of lung tissue. (A) control group; (B) seawater drowning group; (C), (D), and (E) different doses of 3,5,4′-tri-O-acetylresveratrol groups. The values of TNF-α, IL-1β, and IL-10 were increased by seawater instillation. 3,5,4′-Tri-O-acetylresveratrol pretreatment downregulated the level of TNF-α and IL-1β and upregulated the level of IL-10. $^{*}P < 0.05$ versus control group; $^{*}P < 0.01$ versus $^{**}P$.

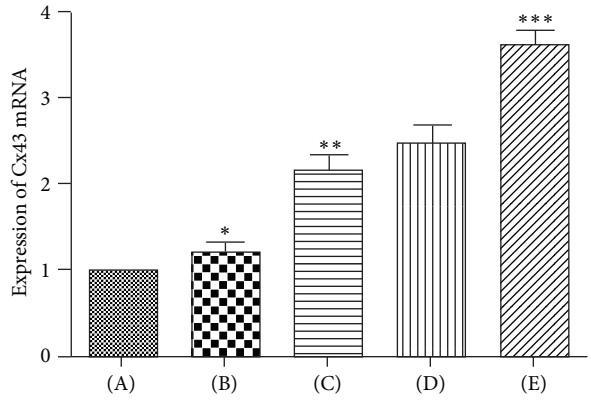

Figure 5: Effects of 3,5,4′-tri-O-acetylresveratrol on Cx43 mRNA expression. The expression level of Cx43 mRNA was taken as "1" in normal lung, and its expression in other groups was compared with the normal expression. (A) control group; (B) seawater drowning group; (C), (D), and (E) different doses of 3,5,4′-tri-O-acetylresveratrol groups. The expression of Cx43 mRNA was increased by seawater, 3,5,4′-tri-O-acetylresveratrol pretreatment upregulated Cx43 mRNA expression. $^{*}P < 0.05$ versus control group; $^{*}P < 0.01$ versus $^{**}P$; $^{**}P < 0.01$ versus $^{***}P$.

Chemical Company (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits of TNF-α and IL-1β were purchased from R&D Corporation (R&D Systems Inc., Minneapolis, MN, USA). ELISA kit of IL-10 was purchased from SenXiong Science and Technology Industrial Corporation (Shanghai, China). Anti-connexins 43 and anti-β-actin monoclonal antibodies were obtained from Anbo Biotechnology Company (Changzhou, China). Real Time PCR related reagents were provided by Takara Biotechnology (Dalian) Co., Ltd. The purity of all chemical reagents was at least at analytical grade.

2.3. Modeling and Grouping. SD rats were randomly assigned into 5 groups ($N = 8$).

(A) Control group: rats without any intervention.

(B) Seawater drowning group: the rats were anesthetized with pentobarbital sodium (100 mg/kg of body wt, administered i.p.). A heparin-filled blunt-ended polyethylene catheter was inserted into the left carotid artery to monitor the mean arterial pressure and obtain blood samples. After exposure of the

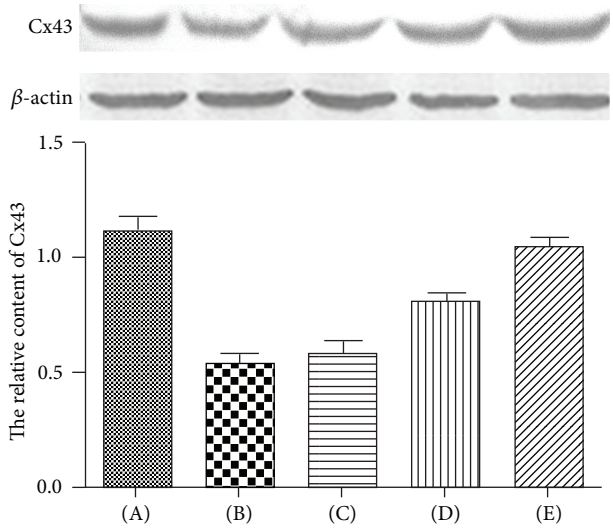

Figure 6: Effects of 3,5,4′-tri-O-acetylresveratrol on Cx43 in seawater stimulated lungs. (A) Control group; (B) seawater drowning group; (C), (D), and (E) different doses of 3,5,4′-tri-O-acetylresveratrol groups. After protein quantitation, western blot was performed to investigate the Cx43 content at 4 h after seawater administration. Ratios of Cx43 protein versus β-actin in three independent experiments were obtained by density scanning using an image analysis system. $^{*}P < 0.01$ versus control group; $^{*}P < 0.01$ versus $^{**}P$; $^{**}P < 0.01$ versus $^{***}P$.

trachea, a 20 min stable baseline period was followed, then a syringe (1 mL) was inserted into the trachea and seawater (4 mL/kg) was instilled at a steady speed within 4 min into both lungs. All rats were sacrificed at 4 h after seawater instillation.

(C) 3,5,4′-tri-O-acetylresveratrol (50 mg/kg) + Seawater drowning group: 3,5,4′-tri-O-acetylresveratrol was administered daily orally for 7 days before modeling.

(D) 3,5,4′-tri-O-acetylresveratrol (150 mg/kg) + Seawater drowning group: 3,5,4′-tri-O-acetylresveratrol was administered daily orally for 7 days before modeling.

(E) 3,5,4′-tri-O-acetylresveratrol (450 mg/kg) + Seawater drowning group: 3,5,4′-tri-O-acetylresveratrol was administered daily orally for 7 days before modeling.

The doses of 3,5,4′-tri-O-acetylresveratrol (50, 150, and 450 mg/kg) used here were based on previous dose-response and time-course studies carried out in our laboratory. All rats were anesthetized and exsanguinated through aortic transection 4 hours after modeling. The lungs were moved out rapidly from thoraxes and processed in the manners described below.

2.4. Histology. At the end of the experiments, lung tissues of the same lobe from every rat were fixed with 10% formalin for 24 h, and then embedded in paraffin. After deparaffinization and dehydration, the lungs were cut into 5 μm-thick sections with a microtome and stained with haematoxylin and eosin.

2.5. Lung Wet-to-Dry Weight Ratio. Lung wet-to-dry ratio (W/D) was used to quantify the magnitude of pulmonary edema. The lung tissues, obtained 4 h after modeling, were weighed immediately, and then dried to constant weight at 70°C for 72 h and weighed again. The wet-to-dry ratio was calculated through dividing the wet weight by the dry weight.

2.6. Measurement of Cytokines. Levels of TNF-α, IL-1β, and IL-10 in the lung tissues were determined by using commercially available ELISA kits. Briefly, lung tissues were homogenized in cool phosphate-buffered saline (lung tissue to PBS 1 : 5). The assay was carried out according to the manufacturer's instructions.

2.7. Western Blot Analysis for Cx43. The lungs were perfused with pH 7.4 PBS to remove the blood cells from the pulmonary circulation, and then, the tissue samples from each group were collected and the total proteins were extracted. Protein concentrations were determined by BCA protein assay kit. The protein samples were boiled, separated on a 12% SDS-polyacrylamide gel, electrotransferred to nitrocellulose membranes, blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20, and incubated overnight at 4°C with monoclonal antibodies against Cx43 (1 : 200) and β-actin (1 : 5000). After repeated washing, the secondary antibody (anti-rabbit IgG peroxidase conjugated, 1 : 10000) was incubated, and bands were visualized by using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The results were expressed as the ratio to β-actin level in the same protein samples.

2.8. Determination of Cx43 mRNAs in Lung Tissues. The total RNA of the lung samples was extracted with TRIZOL reagent (Takara). RNA concentration was tested by spectrometric analysis. Cx43 and β-actin were examined by Real Time PCR following the manufacturer's instructions (Takara Perfect Real Time). The mRNA of Cx43 gene was normalized to the level of β-actin. Genes and primers are listed as follows: Cx43, (forward) 5′-GGAAATCGAACGGCTGGGCGT-3′, (reverse) 5′-TCGCGTGAAGGGAAGAAGCGAT-3′; β-actin (forward) 5′-GCACTGTGTTGGCATAGAGGTC-3′, (reverse) 5′-ACGGTCAGGTCATCACTATCGG-3′. Amplification and detection were carried out by using Bio-Rad My iQ detection system (Edinburgh Biological Science and Technology Development co., LTD, Shanghai, China).

2.9. Cell Culture and Treatment. The human lung epithelial cell line, A549 (obtained from ATCC, Rockville, MD, USA), was maintained in 1640 medium supplemented with 10% fetal calf serum. The human umbilical vein endothelial cell (HUVEC) line was maintained in ham's F12medium supplemented with 10% fetal calf serum. Both of the two cell lines were treated with 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. After incubated in the presence or absence of resveratrol (200 μmol/L), seawater (0.25 mL per

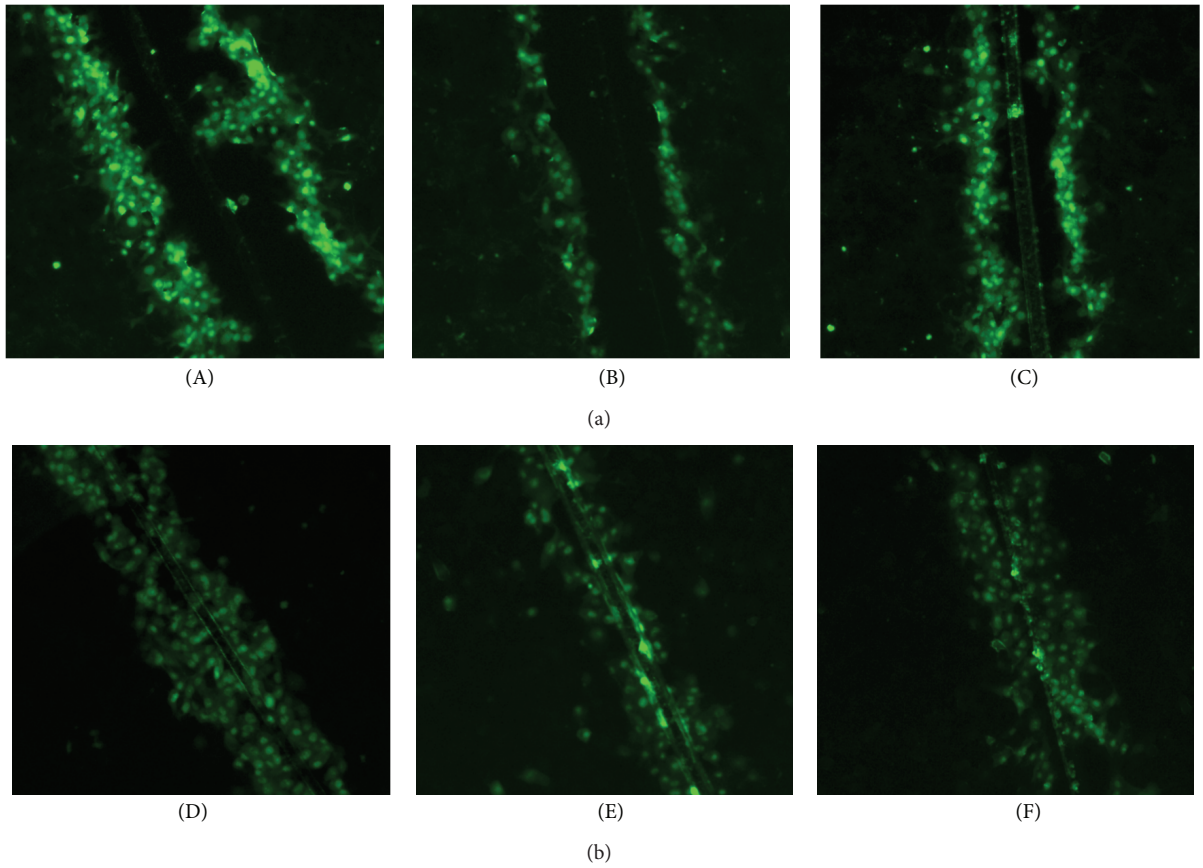

Figure 7: Dye transfer in A549 cells (a) and HUVEC (b) on slides. The panels show a region of A549 cells and HUVEC scrape-loaded with dye ((A) and (D) control; (B) and (E) seawater; (C) and (F) resveratrol). Green indicates Lucifer yellow, including cell initially loaded with dye and recipient cells linked to the donors by gap junctions.

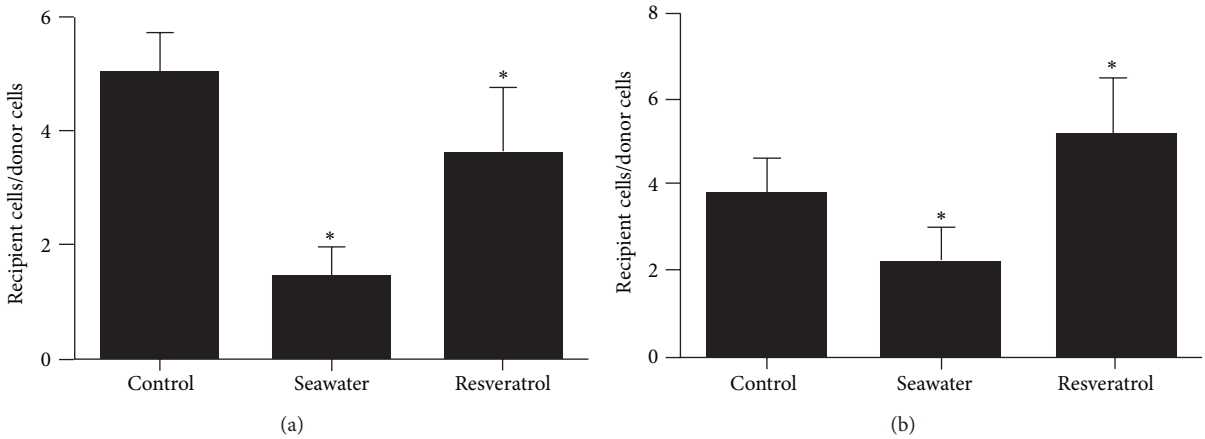

Figure 8: Quantification of dye transfer in G control, G seawater, and G resveratrol ($^{*}P < 0.01$), (a) A549 cells; (b) HUVEC.

1 mL total volume) was added to A549 and HUVEC cells and the cells were stimulated for the 4 h.

2.10. Scrape-Loading and Dye Transfer Technique. A dye transfer assay was used to assess gap junction communication. A549 cells were rinsed with 2 mL PBS after medium was removed from the plates. 2 milliliters of 0.075% Lucifer yellow CH(LY), a fluorescent membrane-impermeable cell marker dye dissolved in PBS, were added to the cells, and two scrape lines (parallel, equidistant scrapes per well) were made by gently passing a diamond-tipped pen (tip diameter, 0.25 mm) across the cultures. The plates were placed for 5 minutes at 37°C in a humidified 5% CO_2 incubator. The dye

solution was then discarded, and the dishes were rinsed twice with PBS to remove background fluorescence. After that, cells were examined with an inverted confocal microscope (FV1000 IX81, Olympus) at emission/excitation wavelengths of 528/425 nm. The images were quantified by counting the number of donor and recipient cells and calculating a cell coupling index with the ratio of recipient to donor cells. The value of dye transfer, defined as the number of secondary recipient cells visualized by Lucifer yellow CH, was recorded for only one side of the scrape (Figure 8).

2.11. Statistical Analysis. Statistical analysis was performed with SPSS 13.0 for Windows. Numeric variables are expressed as means ± SD. Statistically significant differences between experimental conditions were performed by one-way analysis of variance (ANOVA) followed by Dunnett's test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of 3,5,4′-tri-O-acetylresveratrol on Histopathological Changes. The results showed that 4 hours after seawater inhalation induced pulmonary edema, alveolar damage, and infiltration of inflammatory cells in the lung tissues and alveoli (Figure 2(b)), but pretreatment with different doses of 3,5,4′-tri-O-acetylresveratrol could significantly improve the lung injury (Figures 2(c) and 2(d)).

3.2. Effects of 3,5,4′-tri-O-acetylresveratrol on the Lung Edema. To observe the lung edema, we observed the lung wet/dry weight ratios (Figure 3). The wet/dry ratios significantly increased in Seawater drowning group compared with the control ($n = 8$, $P < 0.05$). However, administration with 3,5,4′-tri-O-acetylresveratrol markedly reduced the lung edema.

3.3. Effects of 3,5,4′-tri-O-acetylresveratrol on TNF-α, IL-1β, and IL-10 Levels. We also examine the effects of 3,5,4′-tri-O-acetylresveratrol on the levels of TNF-α, IL-1β, and IL-10 in the lung tissues. As shown in Figure 4, four hours after seawater instillation, TNF-α and IL-1β contents significantly increased. Pretreatment with 3,5,4′-tri-O-acetylresveratrol markedly inhibited the expression of these inflammatory mediators. Meanwhile, the release of IL-10 was elevated by 3,5,4′-tri-O-acetylresveratrol.

3.4. Effects of 3,5,4′-tri-O-acetylresveratrol on Cx43 Expression. Cx43 mRNA expression was examined using Real Time PCR on the lung samples 4 h after seawater exposure (Figure 5). The results showed that seawater aspiration increased the Cx43 mRNA level ($P < 0.05$), while pretreatment with different doses of 3,5,4′-tri-O-acetylresveratrol obviously upregulated the level of Cx43 mRNA ($P < 0.05$).

We then performed western blot to study the changes of Cx43 in protein levels (Figure 6). When challenged with seawater, the reactive pattern of Cx43 protein was opposite to that of its mRNA. Namely, the protein levels of Cx43 in lungs decreased 4 h after seawater exposure ($P < 0.05$).

3.5. Effects of Resveratrol on Dye Transfer in A549 and HUVEC. Similar cell numbers were initially loaded with dye in all treatment groups (Figures 7(a) and 7(b)). However, compared with ratios of the control group, there were fewer labeled neighboring cells of seawater groups, and the treatment with resveratrol significantly increased the number of labeled neighboring cells.

4. Discussion

In the present study, we demonstrated that (i) intratracheal instillation of seawater (4 mL/kg) induced obvious histological changes, pulmonary edema, and inflammation in a dosage dependence manner; (ii) seawater stimulation could obviously suppress the cellular communication between cultured cells; (iii) pretreatment with 3,5,4′-tri-O-acetylresveratrol could effectively alleviate the seawater-induced lung injuries; (iv) resveratrol, intermediate metabolite of 3,5,4′-tri-O-acetylresveratrol, could rebuild or strengthen the seawater impaired communication between cells.

Although several promising pharmacological therapies have been studied for patients with ALI and ARDS, none of these pharmacological treatments obviously reduced mortality [10]. Resveratrol (3,5,4′-tri-O-acetylresveratrol) is a polyphenolic compound which is a phytoalexin synthesized by a wide variety of plant species. It has lots of pharmacological properties, such as antioxidation, anti-inflammatory, cardioprotection, cell cycle inhibition, and neuroprotection [11]. However, resveratrol has some shortages of its own. For example, it is not stable with short half-life and it has a low bioavailability [12]. While resveratrol's analog, 3,5,4′-tri-O-acetylresveratrol may overcome some of those disadvantages to some extent. Evidence showed that this analog was effective in inhibiting the expression of reactive oxygen species (ROS) [7] and PAF-induced washed rabbit platelet aggregation [8]. More importantly, it may lead to accumulation and concentration of resveratrol in lungs.

ALI/ARDS can be divided into two categories based on origin: direct (or pulmonary) ALI and indirect (extrapulmonary) ALI [13]. Seawater drowning-induced acute lung injury (SWD-ALI) belongs to direct ALI. Clinical studies indicated that white blood cell and neutrophils apparently increased in most acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) patients' plasma with bilateral diffuse or localized alveolar infiltrates on chest X-ray [14]. Our previous results also showed that infiltration of inflammatory cells [15] and permeability of alveolar wall to Evans blue [16] apparently increased in SWD-ALI rat model, which to some extent explained the possible mechanism of seawater drowning-induced acute lung injury.

Inflammation response is a series of complex pathological process, including release of cytokines, growth factors and chemokine, and migration of neutrophils, monocytes and lymphocytes to tissue spaces. All the above pathological processes need the coordination of communication between cells. Evidence showed that communication between flanking cells stimulated by Cx43 provided the foundation for the onset and development of inflammation in lung tissues [17].

We found, in the present study, that seawater exposure resulted in upregulation of Cx43 in gene level, deregulation of Cx43 protein, and secretion of inflammatory factors, such as TNF-α and IL-1β, while pretreatment with 3,5,4′-tri-O-acetylresveratrol reduced the contents of inflammation factor. Besides, IL-10, as a famous inhibitory inflammation factor, was increased by 3,5,4′-tri-O-acetylresveratrol. More importantly, 3,5,4′-tri-O-acetylresveratrol markedly upregulated the expression of Cx43 in gene and protein levels, and its intermediate metabolite contributed to the reconstruction of cellular communication in A549 and HUVEC.

Pulmonary edema is another critical issue of acute lung injury, which results from breathing membrane barrier (BBB) dysfunction, and capillary permeability increase [18, 19]. Accumulation of liquid rich in protein in alveolar space may increase the mortality of ALI/ARDS. It was demonstrated, in a gunshot lung injury rabbit model, that cellular communication played an important role in capillary permeability increase and leakage of liquid into alveolar space [20]. In addition, it was found that inhibition of Cx43 mRNA and protein expression led to the increase of single cell permeability [21]. We found, in the present study, that seawater inhalation led to lung edema and seawater stimulation also suppressed cellular communication of HUVEC. However, 3,5,4′-tri-O-acetylresveratrol could alleviate the lung edema in rats suffering seawater instillation, and its metabolic intermediates, resveratrol, recovered the cellular communication restrained by seawater exposure.

Connexin 43 (Cx43), which plays a key role in regulating of inflammation and microvascular permeability, increased in gene level while was inhibited in protein level upon seawater stimulation. This may mean that seawater stimulation would enhance the transcription but inhibit the translation of Cx43. We observed the facilitating effect of 3,5,4′-tri-O-acetylresveratrol on Cx43 in SWD-ALI and two kinds of cells stimulated by seawater. Our results suggested that 3,5,4′-tri-O-acetylresveratrol alleviated seawater instillation induced inflammation and pulmonary edema probably via activation of Cx43.

Authors' Contribution

L. Ma and Y. Li authors contributed to the paper equally.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Low-Grade Inflammation and Spinal Cord Injury: Exercise as Therapy?

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An increase in the prevalence of obesity in people with spinal cord injury can contribute to low-grade chronic inflammation and increase the risk of infection in this population. A decrease in sympathetic activity contributes to immunosuppression due to the lower activation of immune cells in the blood. The effects of physical exercise on inflammatory parameters in individuals with spinal cord injury have not been well described. We conducted a review of the literature published from 1974 to 2012. This review explored the relationships between low-grade inflammation, spinal cord injury, and exercise to discuss a novel mechanism that might explain the beneficial effects of exercise involving an increase in catecholamines and cytokines in people with spinal cord injury.

1. Background

Low-grade inflammation is an immune system response that occurs when the body detects injurious stimuli. There can be a neuroendocrine response, such as fever, a blood response, and a metabolic response. White blood cells and cytokines work together to fight against the injury. Interleukins and tumor necrosis factor-alpha (TNF-α) are important cytokines that play a role in a wide variety of inflammatory reactions. Wang et al. [1] demonstrated that spinal cord injury was associated with increased serum concentrations of C-reactive protein (CRP, a marker of inflammation in the acute phase), interleukin 6 (IL-6), and endothelin-1. These changes suggest that spinal cord injury, independent of obesity, is associated with a state of chronic inflammation, and this association may explain, at least in part, the increase in the atherogenic risk in these patients [1].

With increasing age and time since injury, many individuals with spinal cord injury have an inactive lifestyle, which is associated with deconditioning and secondary health conditions (e.g., osteoporosis, upper extremity pain, obesity, diabetes, and cardiovascular disease), resulting in reduced participation and quality of life [2]. An increase in the prevalence of obesity in people with spinal cord injury can further contribute to low-grade chronic inflammation and increase the risk of cardiovascular disease and type 2 diabetes in this population [3]. Obesity is characterized by an excessive increase in the amount of adipose tissue, which acts as an organ secreting a large variety of proteins, including the pro-inflammatory cytokines interleukin-6 (IL-6) and TNF-α [4].

However, other factors in addition to obesity may contribute to the development of low-grade chronic inflammation in this population. For example, spinal cord injury causes a decrease in sympathetic activity, leading to significant changes in the metabolism of carbohydrates and lipids, such as decreased HDL cholesterol and increased insulin resistance [5]. This decrease in sympathetic activity also contributes to immunosuppression due to the lower activation of immune cells in the blood.

Exercise is an important therapy for the rehabilitation of individuals with spinal cord injury [6, 7]. This is the first to simultaneously discuss the mechanisms related to low-grade inflammation and spinal cord injury, and we propose exercise as a therapy. Our hypothesis is that exercise increases the sympathetic system, causing an increase in catecholamines and leading to enhanced lipolysis and the activation of immune cells in the blood. These effects lead to a decrease in body fat, particularly visceral fat, and the decreased production of inflammatory cytokines by lymphocytes in blood, leading to a decrease in the chronic inflammation found during low-grade inflammation. Thus, the objective of this work was to explore the relationships between low-grade inflammation, spinal cord injury, and exercise to discuss a novel mechanism that might explain the beneficial effects of exercise, which may include an increase in catecholamines and cytokines in people with spinal cord injury.

2. Methods

In this paper, we conducted a systematic and integrative review of the literature using source articles indexed by the ISI database, PubMed, and Medline by searching for books that addressed specific aspects related to low-grade inflammation, spinal cord injury, and exercise therapy which were published between 1974 and 2012.

We used the following search terms: "*inflammation and spinal cord injury*" ($n = 1607$), "*inflammation and exercise*" ($n = 3566$), "*inflammation and immune system*" ($n = 78611$), "*spinal cord injury and cytokines*" ($n = 1087$), "*spinal cord injury and obesity*" ($n = 154$), and "*spinal cord injury and exercise*" ($n = 1787$), for a total of 86812 articles. From this total, we selected 72 articles that were specific to the present topic. All the descriptors were searched by Boolean "*or, and, not*" to obtain various arrangements to maximize the search and the quality of research. No restrictions were made regarding age, gender, or experimental design.

3. Spinal Cord Injury

Worldwide, the incidence of spinal cord injuries is 22 occurrences per million people [8]. In the United States, it is estimated that spinal cord injury occurs in approximately 20% of spinal fractures and that from 10 to 15% of patients presenting with severe neurological damage have considerable morbidity and a 5% mortality rate [7]. Because of the high incidence and cost involved in the diagnosis, treatment, and rehabilitation of these patients, spinal cord injury is a major socioeconomic problem [9].

The term spinal cord lesion refers to any lesion that occurs within the medullary canal of neural elements which results in sensory, motor, and autonomic deficits. The individual may become paraplegic (injury between the lower thoracic vertebrae and the lumbar spine) or quadriplegic (lesion in the high thoracic or cervical spine) depending on the region of the spinal injury. Paraplegic individuals have paralysis of the lower limbs and difficulty in staying seated. In turn, tetraplegia is characterized by paralysis of all four extremities, upper and lower, along the trunk musculature [10].

A spinal cord injury can be caused by trauma, viruses, tumors, and schistosomiasis. Spinal cord injury is one of the most debilitating injuries because it is characterized by limited or no neurological recovery [8]. Several situations can lead to spinal cord injury, including automobile accidents, falls from heights, and injury by firearms and sports injuries [8].

4. Spinal Cord Injury and Low-Grade Inflammation

Spinal cord injury is a classic inflammatory process because the pathophysiology of spinal cord injury is characterized by disruption of the axons and cell membranes, cell death, leukocyte migration, and degradation of the myelin sheath [11, 12]. Factors such as an increase in the proinflammatory activity of immune cells and toxic metabolites released from disrupted cells may induce additional tissue damage. Previous studies have demonstrated that alterations in the immune system in individuals with spinal cord injury may further contribute to a low-grade inflammatory process [13, 14]. Over time, individuals with spinal cord injury also exhibit chronic low-grade inflammation [1]. Wang et al. [1] showed that injury itself was associated with increased serum concentrations of C-reactive protein (CRP, a marker of inflammation in the acute phase), IL-6 and endothelin-1. These changes suggest that spinal cord injury per se is associated with a state of chronic inflammation, which may explain, at least in part, the increase in the atherogenic risk in these patients.

In addition to chronic low-grade inflammation, people with spinal cord injury have lower fitness levels, decreased total body lean mass, decreased energy expenditure, fat accumulation in the lower limbs, and abdominal characteristics that increase the risk of this population developing obesity [15, 16].

5. Obesity and Inflammation

Obesity is characterized by an excessive increase in the amount of adipose tissue [17], and it is a low-grade inflammatory disease. The adipose tissue acts as an organ secreting a large variety of proteins, including the proinflammatory cytokines interleukin-6 (IL-6) and TNF-α and the anti-inflammatory proteins adiponectin and IL-10 [18, 19]. An increase in the prevalence of obesity in spinal cord injury patients can further contribute to low-grade chronic inflammation, increasing the risk of cardiovascular disease in this population [3]. Obesity contributes to metabolic dysfunction,

including increases in circulating cytokines (IL-6, IL-1, and TNF) [20], a decrease in protective factors, such as adiponectin, and communication between cells in inflammatory and metabolic diseases [21].

Cytokines are proteins that are produced and released by different cells, including leukocytes, muscle cells, and neurons. These proteins can act in a pleiotropic way or in synergy with other substances and can modulate the production of other cytokines [22]. Cytokines function in the regulation of metabolism by influencing hormone secretion, regulating Th1/Th2 immune responses, and inducing inflammatory responses. In the nervous system, they regulate complex neuronal actions and modulate thermoregulation, food intake, and neurobiological patterns during sleep [23].

Interleukin-1 (IL-1) family members, such as IL-1α, IL-1β, IL-1ra, and IL-18, are produced by various cells, such as lymphocytes, in response to inflammation produced by infection and microbial endotoxins [24]. In addition, an increased IL-1 plasma concentration may cause fever, sickness, increased heart rate, increased blood flow in many vascular beds, and increased sympathetic tone; changes in carbohydrate, fat, and protein metabolism also occur [22–25].

TNF-α is mainly produced by macrophages and neutrophils, but other cells, such as lymphocytes, NK cells, endothelial cells, and neural cells, might also have the capacity to produce it [25]. TNF-α is produced in response to a wide variety of stimuli, including infections and stimulation by other cytokines or mitogens [24]. TNF-α is a potent pleiotropic cytokine due to its ability to activate multiple signal transduction pathways and induce or suppress the expression of a number of genes. In addition, it has potent endogenous pyrogenic properties and may promote changes in the body's physiological temperature [26]. Moreover, tissues that present marked cachexia show high TNF-α activity, as observed under catabolic conditions, such as cancer and systemic inflammatory diseases [25].

Cytokines can penetrate the blood-brain barrier (BBB) and act indirectly on the brain by stimulating the production of chemical second messengers that carry information to targets, such as NF-κB and adenosine [27–29]. The hypothesis that cytokines could influence the functions of the nervous system (NS) is based on observations that treatment with cytokines, such as interferon-γ (INF-γ), promotes neuroendocrine alterations. Other studies have identified receptors for these cytokines in many areas of the brain [26, 30, 31]. Additional studies have shown that an increase in proinflammatory cytokine concentrations promotes a decrease in the transendothelial electrical resistance and an increase in the permeability of the BBB [32]. Finally, it is possible that cytokines may be produced within the brain itself in response to neuronal activity [22].

More recently, several studies have shown the existence of an afferent neural pathway through which inflammation in the peritoneal cavity might influence the brain [11]. Subdiaphragmatic transection of the vagus leads to fever reduction, poor sleep, nocturnal norepinephrine secretion, and hypothalamic IL-1 production induced by lipopolysaccharides (LPS) in the peritoneal cavity [32], thereby validating this hypothesis. These alterations are not due to a reduction in the circulating levels of cytokines or the attenuation of the inflammatory response induced by lipopolysaccharide (LPS) but rather to a defective translation of cytokines in the brain [33].

6. Sympathetic Activity and Inflammation

Norepinephrine and epinephrine are key hormones that prepare the body for one of its most primeval reactions: the "fight or flight" response. Catecholamines increase the contractility and conduction velocity of cardiomyocytes, leading to increased cardiac output and an increase in blood pressure, which leads to increased vascular tone and resistance. This results in an increased "pre-load" in the right atrium, causing the heart rate to drop due to the Starling mechanism. Catecholamines also facilitate breathing (bronchi become dilated) and mobilize the body's metabolic reserves (lipolysis and glycogenolysis) to provide vital energy [34].

Spinal cord-injured individuals with a cervical or high thoracic lesion experience diminished sympathetic nerve traffic below the level of injury [35], including reduced leg sympathetic nerve activity recorded by microneurography [36], decreased whole-body noradrenaline spillover [37], and the impaired release of adrenaline from the adrenal medulla [38]. Based on these findings, it can be hypothesized that not only do these individuals experience a smaller increase in overall sympathoadrenergic activity during arm exercise but also the sympathetic activity in subcutaneous adipose tissue will be less affected in areas proximal (e.g., the clavicular region) to the injury than distal areas (e.g., the umbilical region). Lower plasma catecholamine responses to arm exercise have been found in individuals with spinal cord injury compared with healthy subjects [39]. In accordance with a differentiated autonomic response, hand heating elicits perspiration above but not below the segmental level of interrupted sympathetic output [40].

Catecholamines are the main regulators of lipolysis plasma hormones in humans. Catecholamines regulate lipolysis by stimulating α-adrenergic receptors and can therefore decrease or increase lipolysis, depending on their concentration and binding affinity of the receptor. During exercise, the increase in circulating catecholamines stimulates lipolysis by activating the α-adrenergic receptor [41, 42].

The brain and the immune system are some of the body's major adaptive systems [34], and they communicate with each other extensively in an attempt to regulate body homeostasis [43]. Key systems involved in this crosstalk are the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system, which consists of the adrenergic sympathetic nervous system, the vagus-mediated parasympathetic nervous system, and the enteric nervous system [34–44]. Over many decades, an increasing body of evidence has demonstrated that lymphocytes and phagocytes are capable of synthesizing and releasing not only neuropeptides but also neurotransmitters and hormones. Furthermore, these cells have adrenergic and cholinergic functions. Thus, by coexisting in the nervous and immune system, these mediators

become the universal language of the neuroendocrine-immune-modulating network [45], which enables the nervous, endocrine, and immune system to regulate and fine-tune their functional responses positively or negatively, thereby allowing the body to rapidly adapt to various changes in internal and external environments.

We are now beginning to understand that catecholamines are an integral part and potent modulators of these neuroendocrine-immune/inflammatory interactive networks. Through direct communication via sympathetic nerve fibers that innervate lymphoid organs [45], catecholamines can modulate mouse lymphocyte proliferation and differentiation [46] and the cytokine production of rodent. Th cells [47] and human peripheral blood mononuclear cells (PBMCs) [48]. These interactions are facilitated by adrenergic receptors expressed on murine lymphocytes [47], rat natural killer (NK) cells [49], rodent macrophages and neutrophils [50, 51], and human PBMCs [52]. Consequently, we must better understand the sources, distribution, and roles of catecholamines and their receptors in immunity and inflammation.

7. Exercise as an Anti-Inflammatory Therapy

A sedentary lifestyle is a risk factor for diseases, with several clinical studies illustrating a reduction of mortality and morbidity in physically active individuals compared to sedentary individuals [53, 54]. The effects of regular or chronic exercise on basal levels of inflammatory markers have been used to recommend exercise as an anti-inflammatory therapy. According to Kasapis and Thompson [55], a single session of exercise triggers an increase in proinflammatory cytokine release, which is associated with leukocytosis and an increased plasma concentration of CRP. This proinflammatory response to acute exercise is accompanied by a sudden increase in oxidative stress, followed by adaptive mechanisms against inflammation [56].

Moreover, a longitudinal study showed that regular training induces a reduction in C-reactive protein levels, suggesting anti-inflammatory action, under several conditions, including insulin resistance and other cardiovascular/cardiometabolic diseases. Regular exercise is also associated with decreased IL-6 and TNF-α and an increase in anti-inflammatory substances, such as IL-4 and IL-10 [57], reinforcing the anti-inflammatory nature of exercise [58, 59].

Cytokines are released from not only mononuclear cells but also muscle cells. Starkie et al. showed that physical exercise directly inhibits endotoxin-induced TNF-α production in humans, most likely through IL-6 release from exercising muscle [60]. Typically, IL-6 is the first cytokine present in circulation after exercise, followed by an increase in IL-1ra and IL-10 [61]. IL-6 release depends on the intensity and duration of exercise and is directly related to the concentration of catecholamines in the blood. The role of IL-6 and the hypothesis of exercise-induced anti-inflammatory IL-6 release has been recently reviewed [62, 63]. Therefore, IL-6, a multifactorial cytokine, regulates cellular and humoral responses and plays a pivotal role in inflammation, being associated with several pathological conditions as a marker of low-grade inflammation [62, 63]. However, what is even more interesting concerning IL-6, as Fisman and Tenenbaum [62] commented, are the putative beneficial effects it has as an anti-inflammatory factor, which is particularly evident in insulin sensitivity during exercise.

A marked increase in circulating levels of IL-6 after exercise without muscle damage has been a remarkably consistent finding. The magnitude by which plasma IL-6 increases is related to exercise duration, the intensity of effort, the muscle mass involved in the mechanical work, and endurance capacity [63] IL-6 has been indicated as the strongest candidate for a humoral factor released after exercise, working in a hormone-like fashion, in which it is released by the muscle, now viewed as an endocrine organ, to influence other organs [63]. Due to its capacity to stimulate the hypothalamus-pituitary-adrenal axis to produce adrenaline, cortisol and anti-inflammatory cytokines, such as interleukin-4 (IL-4) and interleukin-10 (IL-10), IL-6 also has anti-inflammatory properties [25].

IL-10 was described as a product of lymphocytes that might inhibit Th1 cytokine production. The IL-10 family consists of five other less-studied cytokines: interleukin-19 (IL-19), interleukin-20 (IL-20), interleukin-22 (IL-22), interleukin-24 (IL-24), and interleukin-26 (IL-26). Several tissues may function as sources of IL-10, including lymphocytes and adipose and skeletal muscle. Additionally, IL-10 has multiple biological activities and affects many different cell types, including monocytes/macrophages, T cells, B cells, NK cells, neutrophils, endothelial cells, and peripheral blood mononuclear cells (PBMCs). IL-10 also acts in the regulation of inflammation because it is produced by adipose and muscle tissues, which are important to the pro-/anti-inflammatory ratio under conditions such as physical exercise, obesity, and inflammatory diseases [64, 65].

8. Can Physical Exercise Improve Inflammation in Spinal Cord Injuries?

The protective effect of exercise against diseases associated with chronic inflammation may, to some extent, be ascribed to anti-inflammatory activity. Several studies have shown that markers of inflammation are reduced following longer-term behavioral changes involving reduced energy intake and increased physical activity [58]. The data presented in that study highlighted the idea that the beneficial effect of exercise seems to be related to its ability to decrease inflammatory cytokine levels and/or increase anti-inflammatory cytokines, which might also be true for pathological conditions and physical limitations, such as spinal cord injury.

In a recent review, Martin et al. [66] showed consistent evidence that exercise and physical training are effective in improving the physical fitness of people with spinal cord injury. These benefits were dependent on the time, intensity, and volume of exercise [66]. Regarding the effects of physical activity, some studies have shown that exercise can promote anti-inflammatory effects. Petersen and Pedersen [59] showed that, in healthy subjects, exercise induced the release of IL-6, which is primarily synthesized in muscle

fibers and can be released into the blood stream to stimulate the circulation of other anti-inflammatory cytokines, such as receptor antagonist IL-1 (IL-1RA) and IL-10, and inhibit the production of the proinflammatory cytokine TNF-α. Furthermore, IL-6 stimulates lipolysis and fat oxidation, and, after exercise, its concentration will depend mainly on the duration and intensity of exercise performed [67].

Considering the factors mentioned above, we believe that acute exercise causes an increase in sympathetic activity, and this response is intensity dependent, causing an increase in plasma catecholamines and other hormones, thereby modulating the neuro-immuno-endocrine axis in individuals with spinal cord injury. Thus, it is suggested that increasing catecholamines through physical exercise leads to enhanced lipolysis and the activation of immune cells in the blood. These effects lead to a decrease in body fat, particularly visceral fat, and a decreased production of inflammatory cytokines by lymphocytes in the blood, leading to a decrease in the low-grade inflammation found in this population [59, 68–70].

9. Conclusions

The relationships between low-grade inflammation, spinal cord injury, and exercise as therapy are discussed in the present review. Individuals with spinal cord lesions have a higher risk for obesity and, consequently, low-grade chronic inflammation due to the accumulation of visceral fat and the subsequent increased production of proinflammatory cytokines by adipose tissue. Additionally, the decrease in sympathetic activity observed in this population leads to an attenuation of lipolysis, dyslipidemia, and increased macrophage infiltration in adipose tissue, which contribute to chronic inflammation.

In contrast, acute exercise causes an intensity-dependent increase in sympathetic activity, leading to an increase in plasma catecholamines and a subsequent increase in IL-6 in the blood. This IL-6 release into the circulation after exercise is followed by an increase in IL-1ra and IL-10 anti-inflammatory cytokines. This increase in sympathetic response leads to enhanced lipolysis and the activation of immune cells in the blood. These effects lead to a decrease in body fat, particularly visceral fat, and the decreased production of inflammatory cytokines by lymphocytes in the blood. Given the evidence cited in the study, we conclude that exercise could be an interesting nonpharmacological therapy to decrease chronic low-grade inflammation.

In this sense, we believe that exercise could be considered a modulator of the neuro-immuno-endocrine axis in spinal cord injury patients, acting as a potential option to improve the quality of life in these individuals.

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Review Article

Mitochondrial Dysfunction: A Basic Mechanism in Inflammation-Related Non-Communicable Diseases and Therapeutic Opportunities

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Obesity is not necessarily a predisposing factor for disease. It is the handling of fat and/or excessive energy intake that encompasses the linkage of inflammation, oxidation, and metabolism to the deleterious effects associated with the continuous excess of food ingestion. The roles of cytokines and insulin resistance in excessive energy intake have been studied extensively. Tobacco use and obesity accompanied by an unhealthy diet and physical inactivity are the main factors that underlie noncommunicable diseases. The implication is that the management of energy or food intake, which is the main role of mitochondria, is involved in the most common diseases. In this study, we highlight the importance of mitochondrial dysfunction in the mutual relationships between causative conditions. Mitochondria are highly dynamic organelles that fuse and divide in response to environmental stimuli, developmental status, and energy requirements. These organelles act to supply the cell with ATP and to synthesise key molecules in the processes of inflammation, oxidation, and metabolism. Therefore, energy sensors and management effectors are determinants in the course and development of diseases. Regulating mitochondrial function may require a multifaceted approach that includes drugs and plant-derived phenolic compounds with antioxidant and anti-inflammatory activities that improve mitochondrial biogenesis and act to modulate the AMPK/mTOR pathway.

1. Background

The burden of noncommunicable diseases is increasing as such diseases are now responsible for more than three in five deaths worldwide. Atherosclerosis and cancer, in which tobacco use and excessive energy intake are determining factors, are the most frequently occurring of these diseases and are potentially preventable [1, 2]. Obesity and associated metabolic disturbances, which have been increasing worldwide in recent years, are the main factors that underlie noncommunicable diseases and are the consequences of unhealthy diets and physical inactivity [3]. Approximately 10–20% of patients with severe obesity, defined as a body mass index (BMI) > 40, present with no other metabolic complications. These patients are referred to by the oxymoronic designation of "metabolically healthy" obese [4–7]. Such a designation implies that most obese patients are not "metabolically healthy. " Hence, risk factors for the appearance of noncommunicable diseases have emerged. The reasons for these two phenotypes are unknown; the phenotypes might represent different transitions on a disease timeline, and different levels of either chronic inflammation or insulin resistance are likely contributors. Other contributors include gradual differences in glucose tolerance, inflammatory responses, adipose tissue distribution, patterns of adipokine secretion, and age.

Emerging obesogenic factors are likely to present with significant differences in the elderly, and consequently the

prevalence of obesity is expected to increase with increasing age. Therefore, it is likely not coincidental that most comorbidity associated with obesity and hence with noncommunicable diseases correlates with aging; the processes may share basic mechanisms, particularly mitochondrial age within an individual [7]. Of note, the prevalence of obesity is lower in people over 70 years of age, an effect attributed to the selective mortality of middle-aged people [8].

Current recommendations to decrease food intake and increase physical exercise do result in metabolic improvements, but such lifestyle changes are rarely sustained, despite strong motivation. However, several communities have undertaken initiatives to prevent noncommunicable diseases, and the lessons learned from the implementation of such initiatives should be examined further [9]. The active manipulation of energy sensors and effectors might be a possible alternative therapeutic procedure. Our aim is to provide a succinct review of the scarce and disseminated data that link mitochondrial dysfunction to the pathogenesis of energy-related complications and to discuss a possible multifaceted therapeutic approach.

2. Food Availability Links Mitochondrial Dysfunction and the Vicious Cycle of Oxidative Stress and Inflammation

Mitochondrial defects, systemic inflammation, and oxidative stress are at the root of most noncommunicable diseases such as cancer, atherosclerosis, Parkinson's disease, Alzheimer's disease, other neurodegenerative diseases, heart and lung disturbances, diabetes, obesity, and autoimmune diseases [10–16]. Obesity and obesity-related complications as well as impairment of mitochondrial function, which is required for normal metabolism and health (Figure 1), are universally associated with these conditions. The exact mechanisms that associate mitochondrial dysfunction, obesity, and aging with metabolic syndrome remain a topic of debate [17–22].

Body weight is controlled by molecular messengers that regulate energy status in a limited number of susceptible tissues, including the liver, adipose tissue, skeletal muscles, pancreas, and the hypothalamus [7, 23]. Mouse models of diet-induced obesity have revealed important morphological and molecular differences with respect to humans, particularly those related to the development of fatty liver (NAFLD: nonalcoholic fatty liver disease) or nonalcoholic steatohepatitis (NASH) [24–30] (Figure 2). High expectations for a human therapy after the generation of leptin-deficient animals (Ob/Ob) were countered by the determination that leptin is not a therapeutic option in humans [28].

Endoplasmic reticulum (ER) and mitochondrial stress, with the consequent oxidative stress, are immediate consequences of attempts to store excess food energy [23, 29]. Under normal weight conditions, adipose tissue-derived adipokines maintain the homeostasis of glucose and lipid metabolism; however, in obese conditions, the dysregulated production of adipokines favours the development of metabolic syndrome and related complications, particularly the accumulation of triglycerides in nonadipose organs that are not designed to store energy [19]. Other adipokines may cause inflammation and oxidative stress [31], but unknown factors are involved because interventions to ameliorate insulin resistance do not lead uniformly to clinical improvement [32]. It is of paramount importance to understand the mechanisms that disrupt ER homeostasis and lead to the activation of the unfolded protein response and mitochondrial defects in metabolic diseases in order to correctly manage noncommunicable diseases [33].

Incidentally, the role of genetics in low-energy expenditure and chronic food intake, although potentially significant, remains poorly understood [29, 30]. The genetic-selection hypothesis, which attempts to explain the high prevalence of obesity and diabetes in humans, remains controversial, since the recent abandonment of the "thrifty" gene hypothesis [34–38]. As a result, the roles of oxidative stress, inflammation, mitochondrial dysfunction, nutritional status, and metabolism might be reinforced in hypotheses regarding the pathogenesis of noncommunicable diseases (Figures 3 and 4).

Inflammation plays a vital role in host defence. Tissue damage, fibrosis, and losses of function occur under chronic inflammatory conditions. Growing evidence links a low-grade, chronic inflammatory state to obesity and its coexisting conditions as well as to noncommunicable diseases [10–16]. This low-grade inflammatory state is aggravated by the recruitment of inflammatory cells, mainly macrophages, to adipose tissue. Inflammatory cell recruitment is likely due to the combined effects of the complex regulatory network of cells and mediators that are designed to resolve inflammatory responses [7]. Anti-inflammatory drugs have shown to reverse insulin resistance and other related conditions that result from circulating cytokines that cause and maintain insulin resistance [19, 23, 39–42]. Therefore, it is likely that inflammation *per se* is a causal factor for noncommunicable diseases rather than an associated risk factor.

It is also important to highlight that adipose tissue is comprised of multiple types of cells that have intrinsic and important endocrine functions, particularly those mediated by leptin and adiponectin. Recruited and resident macrophages secrete the majority of inflammatory adipokines, specifically tumour necrosis factor α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), among others. The major roles of TNFα and other inflammatory cytokines in the progression of metabolic complications are likely related to oxidative stress [43, 44]. In adipose tissue macrophages, increased concentrations of saturated free fatty acids (FFAs) stimulate the synthesis of TNFα directly through the Toll-like receptor 4 (TLR4) or indirectly through cellular accumulation. Both macrophages and adipocytes possess TLR4 receptors that, upon lipid-dependent activation, induce NF-KB translocation to the nucleus and the subsequent synthesis of TNFα and IL-6 [7, 43, 44]. However, recruited macrophages have unique inflammatory properties that are not observed in resident tissue macrophages, and the recruitment of these cells is mainly modulated by MCP-1, the most important molecule of the CC chemokine family [7]. In this setting, the roles and polarisation of adipose tissue macrophages (ATMs) seem established [45]. M1 or "classically activated" ATMs

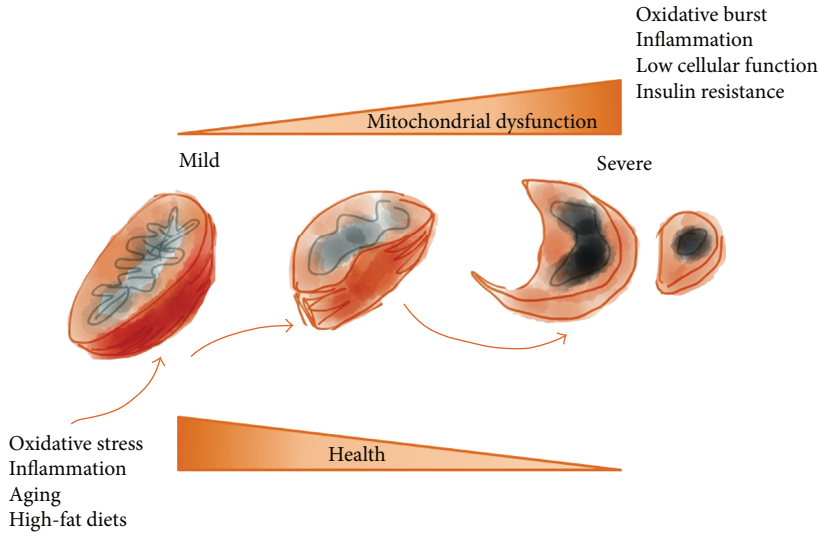
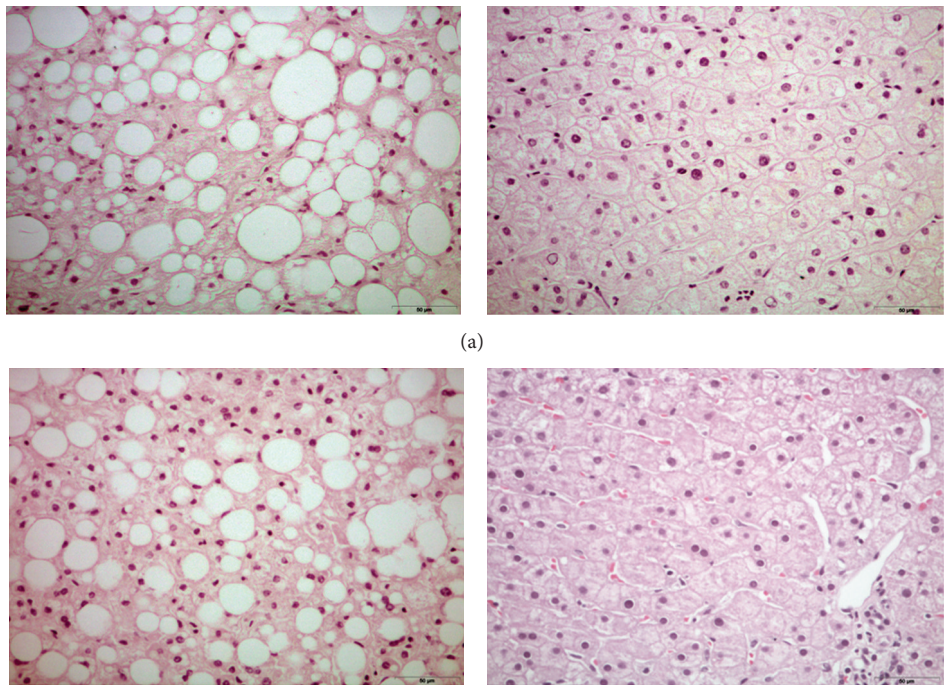


FIGURE 1: Mutations in mitochondrial DNA are accompanied by different disease-suggestive phenotypes (myopathies, neuropathies, diabetes, and signs of reduced lifespan and premature aging). Severe mitochondrial dysfunction triggers a high level of oxidative and inflammatory damage, impairs tissue function, and promotes age-related diseases.



(a)

(b)

FIGURE 2: Clinically, it is evident that, in severe obesity, (a) the presence of liver steatosis may vary from more than 80% to less than 5% of patients. Conversely, in most obese patients with some degree of liver steatosis (b), this condition disappeared in a relatively brief period of time after significant weight loss due to bariatric surgery.

are increased, and M2 or "alternatively activated" ATMs are decreased in the adipose tissues of both obese mice and obese humans, as discussed below [46, 47].

It is frequently assumed that, in contrast to hormones, chemokines influence cellular activities in an autocrine or paracrine fashion. However, chemokines may be relevant effectors in chronic systemic inflammation as the confinement of these molecules to well-defined environments is unlikely. Specifically, alterations in plasma MCP-1 concentrations in metabolic disease states, the presence of circulating chemokine reservoirs, recent evidence of novel mechanisms of action, and certain unexplained responses associated with

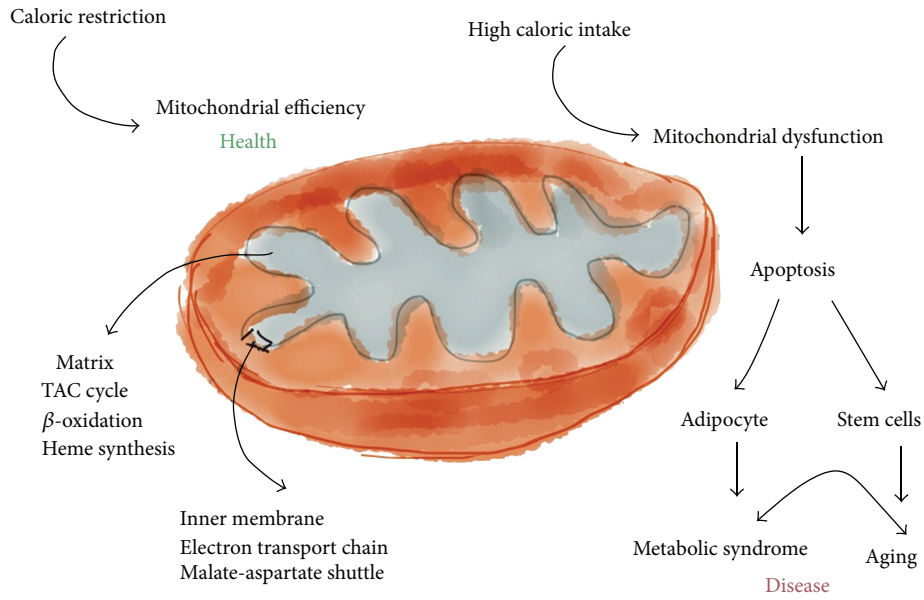


Figure 3: The mitochondrial matrix hosts the mitochondrial metabolic pathways (TAC cycle, β-oxidation, and haem synthesis), and the inner membrane contains the electron transport chain complexes and ATP synthase. Exchange carriers such as the malate-aspartate shuttle are also essential. Under caloric restriction, the mitochondrion achieves the highest efficiency, and high caloric intake produces dysfunction and a consequent increase in apoptosis, which promotes metabolic syndrome and age-related diseases.

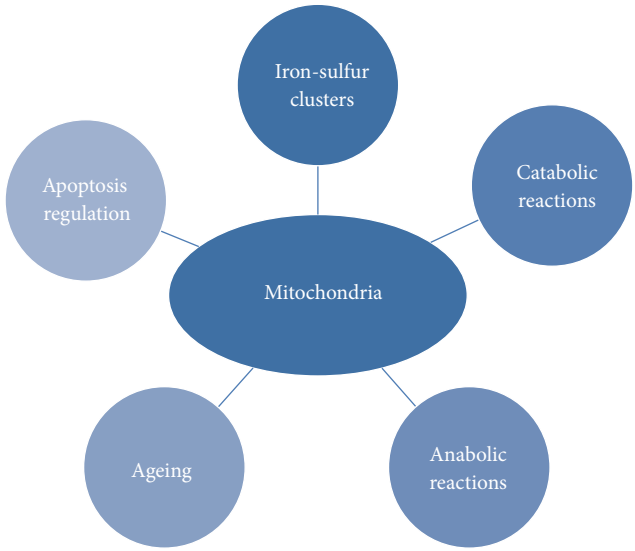


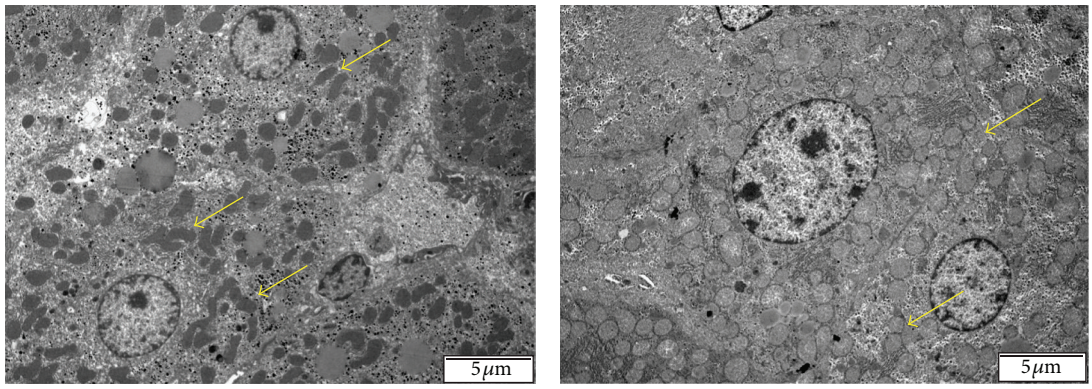
Figure 4: Schematic and abridged representation of the multiple roles of mitochondria in cellular processes that are associated with the pathogenesis of the more prevalent diseases.

metabolic disturbances suggest that MCP-1 might have a systemic role in metabolic regulation [48–50]. How and when obesity might initiate an inflammatory response remains controversial, but the underlying mechanism likely depends on the activation of the c-Jun N-terminal kinase (JNK) in insulin-sensitive tissues, as JNK is likely the principal mechanism through which inflammatory signals interfere with insulin activity [7].

ER stress responses and mitochondrial defects are also linked to the mTOR pathway, discussed below, which is essential for the regulation of numerous processes, including the cell cycle, energy metabolism, the immune response, and autophagy. Therefore, the specific cellular changes associated with metabolic alterations, particularly mitochondrial dysfunction, require further attention.

3. Mitochondria: Bioenergy Couples Metabolism, Oxidation, and Inflammation

Mitochondria are essential organelles that, among other functions, supply the cell with ATP through oxidative phosphorylation, synthesise key molecules, and buffer calcium gradients; however, they are also a source of free radicals (Figures 1, 3, and 4). It is not surprising that mitochondrial health is tightly regulated and associated with the homeostasis and aging of the organism. Within these processes, the antagonistic and balanced activities of the fusion and fission machineries constantly provide adequate responses to events caused by inflammation (Figure 5) [23, 50–54]. A shift towards fusion favours the generation of interconnected mitochondria, which contribute to the dissipation and rapid provision of energy. A shift towards fission results in numerous mitochondrial fragments. Apparently, the mixing of the matrix and the inner membrane allows the respiratory machinery components to cooperate most efficiently. Furthermore, fusion maximises ATP synthesis. In quiescent cells, mitochondria are frequently present as numerous morphologically and functionally distinct small spheres or short rods [51, 55, 56]. Upon the exposure of cells to stress, fusion optimises mitochondrial function and



(a) (b)

Figure 5: Mitochondrial fusion (a) and fission (b) processes in the liver (arrows). Mitochondrial morphology is basically controlled by metabolism and inflammation, and each change in morphology is mediated by large guanosine triphosphatases of the dynamin family, consistent with a model in which the capacity for oxidative phosphorylation is maximised under stressful conditions.

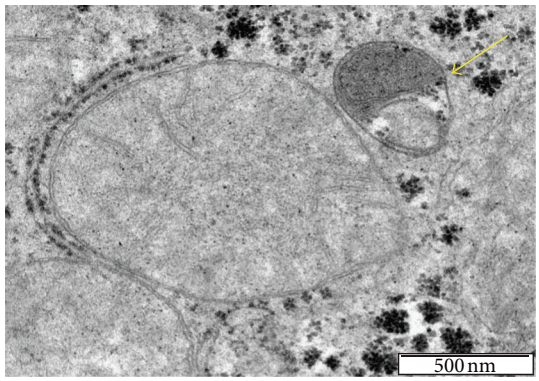


Figure 6: The complete elimination of mitochondria by autophagy (arrow) is a process linked to mitochondrial fission and fusion. Mitochondria also employ quality-control proteases to eliminate damaged molecules through the transcriptional induction of chaperones or the ubiquitin proteasome quality-control pathway.

plays a beneficial role in the maintenance of long-term bioenergetics capacities. In contrast, the mitochondrial fission machinery contributes to the elimination of irreversibly damaged mitochondria through autophagy [55–58]. This process, also called mitophagy, is extremely important under both physiological and pathological conditions (Figure 6). A detailed discussion of the importance of mitophagy is beyond the scope of this review; however, as an example of its importance, recall that amino acids are not stored in the body but are instead mobilised by proteolysis under conditions such as starvation, reduced physical activity, and disease [59]. Furthermore, intense exercise may modulate hepatic metabolism through similar mechanisms [60]. More recently, the mitochondrial E3 ubiquitin protein ligase 1 (Mul 1) was identified as a key protein that promotes mitophagy and skeletal muscle loss [61]. Mitochondrial fission *per se* triggers organelle dysfunction and muscle loss. The opposite is observed when mitochondrial fission is inhibited. The same authors [61] also demonstrated that the overexpression of Forkhead box O3 (FoxO3) induces mitochondrial disruption via mitophagy.

Therefore, it is not surprising that mitochondrial diseases often have an associated metabolic component, and consequently mitochondrial defects are expected in inflammation, aging, and other energy-dependent disturbances [58, 62]. In such disturbances, cellular oxidative damage caused by the generation of reactive oxygen species (ROS) that exceed the natural antioxidant activity is likely an initiating factor in inflammation and aging [63, 64]. Several potential therapeutic approaches are currently available to slow down age-related functional declines [65], including antioxidant treatments [66]; however, the effectiveness of existing antioxidants is likely suboptimal because these antioxidants are not selective for mitochondria [67]. However, recent experiments with a mitochondria-targeted antioxidant have been successful in animal models [67]. Similar assumptions can be made for endothelial cells, in which oxidation and the accompanying inflammation are recognised factors for atherosclerosis. Oxidative stress, which is mainly derived from mitochondrial dysfunction, decreases NO synthesis, contributes to hypertension, upregulates the secretion of adhesion molecules and inflammatory cytokines, and is responsible for the oxidation of low-density lipoproteins [68, 69].

Defective mitochondrial function in muscle tissues leads to reduced fatty acid oxidation and the inhibition of glucose transport, indicating that insulin-stimulated glucose transport is reduced. This is a hallmark of insulin resistance and type 2 diabetes. The chronic production of excess ROS and inflammation result in mitochondrial dysfunction potentially inducing lipid accumulation in these tissues and the endless vicious cycle of insulin resistance [70–74]. Mitochondrial ROS have also been related to the increased activity of uncoupling proteins (UCP), which uncouple ATP synthesis from electron transport. UCP activity leads to heat generation without ATP production, and long-term reductions in ATP

levels affect cellular insulin signalling. The roles of the UCPs and the metabolically relevant differences between brown and white adipose tissues were reviewed recently [75–77].

The mitochondria of obese individuals are different from those of lean individuals. Alterations in mitochondrial morphology, impaired mitochondrial bioenergetics, increased mitochondrial lipid peroxides, decreased ATP content, and mitochondrial dysfunction further increase the risks of developing metabolic complications [78, 79]. In comparison to those of lean individuals, mitochondria in obese individuals have lower energy-generating capacities, less clearly defined inner membranes, and reduced fatty acid oxidation. These differences might promote the development and progression of obesity and might also have therapeutic implications [80, 81]. Impaired mitochondrial function could account for the insulin resistance that is closely associated with increased lipid content in the muscles of patients with type 2 diabetes. Altered mitochondrial function is the major factor that leads to increased muscular lipid accumulation and decreased insulin sensitivity [80, 81]. More recently, a model was created in which the amount of mitochondrial activity in adipocytes and hepatocytes can be altered based on the properties of the mitochondrial protein mitoNEET, which is located at the outer membrane [70]. Despite the prevalence of obesity in this model, mitoNEET overexpression during periods of high caloric intake resulted in systemwide improvements in insulin sensitivity, thereby providing a model of a "metabolically healthy" obese state with minimal tissue lipotoxicity that is similar to the clinically observed condition [82]. Alterations in mitoNEET expression might modulate ROS concentrations and mitochondrial iron transport into the matrix [70, 82, 83]. The mitochondrial fusion protein mitofusin-2 (Mfn-2), another useful protein in studies of mitochondrial dysfunction, regulates cellular metabolism and controls mitochondrial metabolism. In cultured cells, mitochondrial metabolism was activated in Mfn-2 gain-of-function experiments, whereas Mfn-2 loss-of-function reduced glucose oxidation, mitochondrial membrane potential, oxygen consumption, and mitochondrial proton leakage [84]. It is defective in the muscles of obese and type 2 diabetes patients in which mitochondrial size is reduced [71].

Therefore, a detailed characterisation of the proteins involved in mitochondrial fusion and fission and studies of the mechanisms that regulate these two processes are relevant to human pathology and might have a great therapeutic potential to improve metabolism and to decrease the generation of oxidative stress and excessive inflammatory response [85].

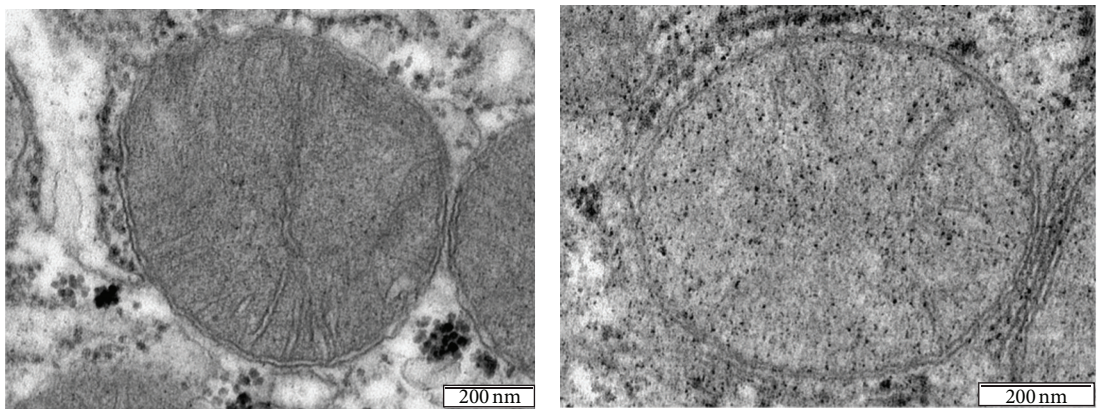
4. Is There a Link between Mitochondria and Nutrient Availability? The Possible Roles of Inflammation and Apoptosis

Apoptosis is another basic process to consider in metabolic diseases. Excess food intake leads to mitochondrial dysfunction and higher apoptotic susceptibility. Mitochondria specialise in energy production and cell killing. Only 13 proteins are encoded by the mitochondrial DNA, a circular molecule of 16 Kb. The remaining necessary proteins are encoded in the nuclear DNA [86]. Mitochondria are composed of outer and inner specialised membranes that define two separate components, the matrix and the intermembrane space [87]. Mitochondria regulate apoptosis in response to cellular stress signals and determine whether cells live or die [88]. Thus, it is conceivable that the availability or ingestion of nutrients could be a main candidate in the regulation of cell death and that mitochondria could have been selected as a nutrient sensor and effector. This could explain the influence of apoptosis-related proteins on mitochondrial respiration [89].

A common laboratory finding is that the morphology of the mitochondria changes when mice are supplied with a high-fat diet (Figure 7) and that optimal mitochondrial performance is achieved under conditions of calorie restriction. Excess food intake impairs respiratory capacities, likely through mTOR, and increases the susceptibility of the cell to apoptosis and additional stress [90, 91]. Of note, apoptotic protein levels are increased in the adipocytes of obese humans, and the depletion of proapoptotic proteins protects against liver steatosis and insulin resistance in mice fed a high-fat, high-cholesterol diet [92]. These conditions are relevant to the development of metabolic syndrome, as nutritional imbalances in Western diets lead to mitochondrial dysfunction and higher susceptibilities to inflammation, apoptosis, and aging [22].

5. AMP-Activated Protein Kinase (AMPK) Not Only Influences Metabolism in Adipocytes but Also Suppresses the Proinflammatory Environment

AMPK has anti-inflammatory actions that are independent of its effects on glucose and lipid metabolism [93]. The action of AMPK is not necessarily identical in all tissues. In adipose tissues, the role of AMPK is largely unknown because laboratory techniques to explore the action of this kinase in terminally differentiated adipocytes have not been fully established. Several agents have been used to activate AMPK experimentally, including AICAR (5′-aminoimidazole-4-carboxamide ribonucleoside), metformin, rosiglitazone, resveratrol and other polyphenols, statins, and several adipocytokines. In adipocytes, AMPK appears to increase the insulin-stimulated uptake of glucose, likely by increasing the expression of GLUT4, yet inhibits glucose metabolism [94]. Studies of the effects of AMPK on lipolysis in adipocytes have been controversial; some authors have reported an antilipolytic effect, while others have suggested that AMPK stimulates lipolysis [95, 96]. However, the activation of AMPK by metformin in human adipose tissues increases the phosphorylation of acetyl-CoA carboxylase (ACC) and decreases the expression of lipogenic genes, leading to reductions in malonyl-CoA, which is the precursor for fatty acid synthesis; malonyl-CoA also regulates fatty acid oxidation through the inhibition of carnitine palmitoyl-transferase 1, the rate-limiting enzyme for fatty acid entry into the mitochondria [97, 98]. Adipose tissue secretes adipocytokines, which influence metabolic and inflammatory pathways through the recruitment of



(a) (b)

Figure 7: The nutrient availability of food in "natural" conditions for mice is likely low and near the condition known as calorie restriction. In the laboratory, however, mice are usually fed *ad libitum*, and certain biases cannot be discarded. However, mitochondria from mice fed a chow diet (a) display rapid morphological changes when mice are fed with high-fat diets (b).

macrophages and the consequent transition from the M2 state to M1 [7, 41]. These actions contribute to the development of disease (Figure 8). Conversely, adiponectin has been reported to induce adipose macrophages to switch to the anti-inflammatory M2 state [99]. AMPK is anti-inflammatory, as it inhibits the synthesis of proinflammatory cytokines and promotes the expression of IL-10 in macrophages; adiponectin and leptin levels may also be regulated by AMPK [100] (Figure 8). Finally, brown adipocytes contain high numbers of mitochondria that express UCP1, which permit thermogenesis. Exposure to cold temperatures stimulates AMPK and may play a role in the differentiation of fatty oxidising brown adipose tissue, thus leading to greater energy expenditure [101]. Therefore, we hypothesise that the chronic manipulation of the AMPK/mechanistic target of rapamycin (mTOR) pathway might represent a therapeutic approach for preventing noncommunicable diseases (Figure 8). Metformin, along with salicylate, polyphenols, and rapamycin, has a long history of safe and effective use, but other modulators are currently under development and will likely permit the design of tissue-specific activators of this pathway.

6. Metformin and/or Rapamycin and Plant-Derived Polyphenols: An Apparent Treatment of Choice for Metabolic Syndrome and Obesity-Related Complications?

The first therapeutic approaches to metabolic disturbances are reduced caloric ingestion and increased physical activity. The effects are based mainly on weight reduction, but usefulness in other common complications remains incompletely explored [102]. Bariatric surgery is also effective, even in "metabolically healthy" patients [103, 104]. The effectiveness of surgery for the treatment of metabolic disturbances is surprisingly higher than expected, and mechanisms associated with surgical effects are not completely understood.

Insulin resistance and mitochondrial dysfunction appear to be the most significant alternative therapeutic targets. Metabolic abnormalities are associated with inflammation. Normally, glycolysis yields pyruvate, which is further oxidised in the mitochondria. When oxygen becomes limiting, mitochondrial oxidative metabolism is restricted. The induction of an inflammatory response is an energy-intensive process, and the involved cells rapidly switch from resting to highly active states. This is observed in diseases such as cancer, atherosclerosis, or autoimmune diseases, and mechanistic insights suggest the common involvement of the transcription factor hypoxia-inducible factor 1α, AMPK, and the mTOR pathway. In addition, the activation of sirtuins, which act as NAD+ sensors that connect nutrition and metabolism to chromatin structure, is anti-inflammatory [105] (Figure 8).

The use of metformin, an AMPK activator used extensively to treat type 2 diabetes, has been indicated for other metabolic conditions based on the rationale that insulin-sensitising agents might be effective [106], and the mode of action of metformin has guided our own experiments on cancer, aging, and viral infection [65, 107, 108]. We have shown that the beneficial effects of this biguanide class drug, which was initially obtained from *Galega officinalis*, are universal in patients with metabolic complications and negligible in patients without such complications. The primary effect is thought to be the suppression of hepatic glucose production and hepatic lipogenesis [109]. Metformin activates AMPK in hepatocytes, resulting in the phosphorylation and inactivation of ACA, a rate-limiting enzyme in lipogenesis [110], and theoretically might be useful and safe in the treatment of NAFLD [111]. Surprisingly, the beneficial clinical effects seem to be limited, despite the effects of metformin on insulin resistance, most likely because long-term treatment is an absolute requirement for the prevention of progressive disease. Our own current experiments in animal models suggest new insights into this phenomenon. Metformin activates AMPK, but AMPK deficiency does not abolish

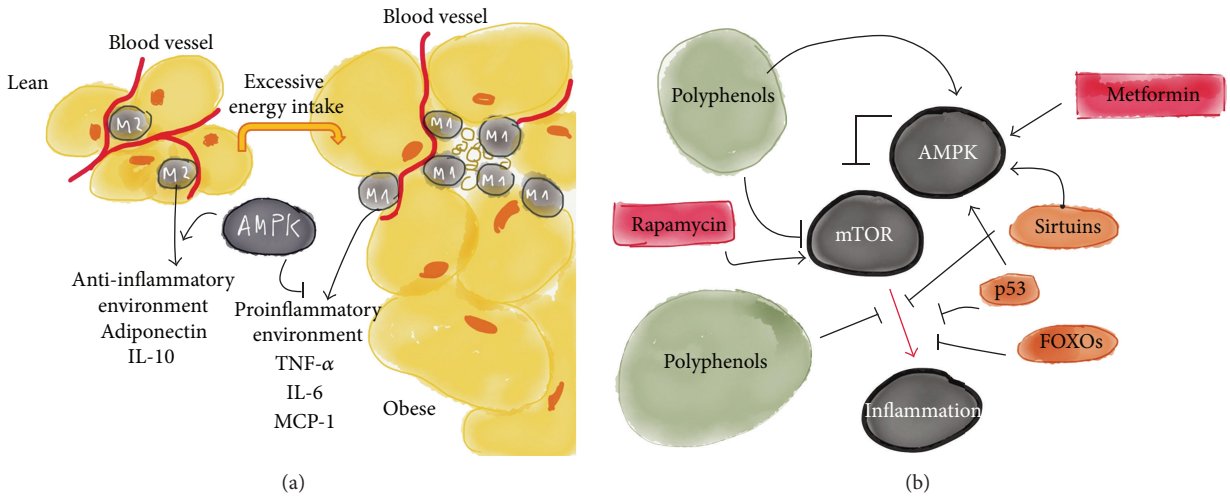


Figure 8: Activation of AMPK in macrophages promotes the switch from a proinflammatory to an anti-inflammatory phenotype by inducing a shift from glycolysis towards mitochondrial oxidative metabolism. In obesity, there may be a shift towards proinflammatory states, whereas in dietary restriction the balance may shift towards anti-inflammatory phenotypes through the activation of AMPK (a). The activation of AMPK implies the inhibition of mTOR, and several compounds are known to regulate this pathway (b). The inhibition of mTOR extends lifespan in model organisms and confers protection against a growing list of age-related pathologies. Several characterised inhibitors are already clinically approved, and others are under development.

the effects of metformin on hepatic glucose production, indicating that the role of AMPK is dispensable, as indicated previously [112]. This suggests that the overall effect of metformin is mediated through actions on mitochondrial function through decreases in the hepatic energy state and intracellular ATP content. Other studies suggest that metformin inhibits Complex I of the mitochondrial respiratory chain, but the exact mechanisms and pathways involved are unclear [113]. Sirtuin 3 (SIRT 3), a member of the family of nicotinamide adenine dinucleotide (NAD+) dependent deacetylase proteins, is a crucial regulator of mitochondrial function that controls the global acetylation of the organelle (all sirtuins regulate energy production and the cell cycle; Figure 8). SIRT3 induces the activity of Complex I and promotes oxidative phosphorylation. In SIRT3 knockout mice, mitochondrial proteins are hyperacetylated, and cellular ATP levels are reduced, effects that are aggravated by fasting [114]. As a complement, peroxisome proliferator-activated receptor gamma coactivator 1-alpha induces the expression of SIRT3 in the liver [115]. Therefore, mitochondrial function appears to be the key target of metformin; reductions in ATP production may mediate the hepatic and antihyperglycemic actions of the drug and downregulate SIRT3 expression [116]. However, metformin distinctively regulates the expression of different sirtuin family members [117, 118]. In summary, metformin acts against both insulin resistance and mitochondrial dysfunction and is currently an attractive candidate agent of choice in the management of metabolic disorders. We have recently reviewed this complex scenario and found the following: (1) the unique ability of metformin to activate AMPK while leading to the increased utilisation of energy occurs because metformin inhibits AMP deaminase; and (2) in metabolic tissues, metformin can inhibit cell growth by functionally mimicking the effects of a multitargeted antifolate [119].

Based on these and other findings, we have also demonstrated that plant-derived phenolic compounds interact with numerous targets and multiple deregulated signalling pathways that may be useful in the management of metabolic conditions [120–123]. The proposed mechanisms are direct antioxidant activity, attenuation of endoplasmic reticulum stress, blockade of proinflammatory cytokines, and blockade of transcription factors related to metabolic diseases [120]. Most polyphenols modulate oxidative stress and inflammatory responses through relevant actions in the process of macrophage recruitment. Interactions between the chemokine/cytokine network and bioenergetics, likely through the mTOR pathway, may also represent potential mechanisms for the prevention of metabolic disturbances [121]. Moreover, polyphenols attenuate the metabolic effects of high-fat, high-cholesterol diets when administered continuously at high doses, and we have described beneficial actions associated with the expression of selected microRNAs [122].

Inflammation lies at the heart of many diseases because the entire body is under metabolic stress, which induces symptoms and causes morbidity. Targeting altered metabolic pathways in inflammation may enhance our understanding of disease pathogenesis and point the way to new therapies. As mentioned, metformin, polyphenols, AICAR, salicylates, and corticoids all activate the AMPK/mTOR pathway. New compounds such as A-769662 are under scrutiny. Finally, rapamycin, which is also known as sirolimus and was first isolated from *Streptomyces hygroscopicus*, and several derivative compounds, including everolimus, temsirolimus, ridaforolimus, umirolimus, and zotarolimus, have been approved for a variety of uses, including posttransplantation

therapy, the prevention of restenosis following angioplasty, and as a treatment for certain forms of cancer. Drugs that inhibit the mTOR pathway could one day be used widely to slow aging and reduce age-related pathologies in humans [124]. The development of chemical inhibitors of mTOR, as well as drugs that target other components of the mTOR pathway, promises to aid research greatly while also providing drugs with potential therapeutic value.

7. Perspectives and Implications

Obesity, metabolic alterations, and age-related diseases are complex conditions that require a multifaceted approach that includes action on both the chemokine network and energy metabolism [123, 125]. The underlying mechanisms are far from being understood [126] although the association between obesity and insulin resistance seems to be well substantiated. However, obesity is not distributed normally throughout the population, and type 2 diabetes mellitus is not associated closely with increased body weight; also, the relationship with noncommunicable diseases is not straightforward. A working hypothesis is that adipose tissue has a limited maximum capacity to increase in mass. Once the adipose tissue has reached the expansion limit, fat is deposited in the liver and muscle tissues and causes insulin resistance. This process is also associated with the activation of macrophages, oxidative stress, and inflammation which produce cytokines that have negative effects on insulin sensitivity, induce the secretion of adipokines that cause insulin resistance, and suppress those that promote insulin sensitivity. However, a host of other mechanisms must be involved because metabolic responses are different among patients with maximum adipose tissue expansion. A more popular and recent hypothesis suggests a differential effect of lipophagy, which implies a tissue-selective autophagy with cellular consequences from the mobilisation of intracellular lipids. Defective lipophagy is linked to fatty liver tissues and obesity and might be the basis for age-related metabolic syndrome [127]. Increased adipose tissue autophagy may be responsible for more efficient storage. Autophagy also affects metabolism, oxidation, and proinflammatory cytokine production. Very recent evidence suggests that autophagy is increased in the adipose tissues of obese patients [128]. Inexpensive and well-tolerated molecules such as chloroquine, metformin, and polyphenols already exist and could be used to fine-tune the metabolic alterations derived from an excess of energy and, more specifically, to modulate autophagy in the liver. Whether these therapies will dampen the genetic expression of factors that affect the development of noncommunicable diseases remains to be ascertained.

Acknowledgments

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Research Article

Cigarette Smoke Suppresses the Surface Expression of *c-kit* and FcεRI on Mast Cells

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Chronic obstructive pulmonary disease (COPD) is a multicomponent disease characterized by emphysema and/or chronic bronchitis. COPD is mostly associated with cigarette smoking. Cigarette smoke contains over 4,700 chemical compounds, including free radicals and LPS (a Toll-Like Receptor 4 agonist) at concentrations which may contribute to the pathogenesis of diseases like COPD. We have previously shown that short-term exposure to cigarette smoke medium (CSM) can stimulate several inflammatory cells via TLR4 and that CSM reduces the degranulation of bone-marrow-derived mast cells (BMMCs). In the current study, the effect of CSM on mast cells maturation and function was investigated. Coculturing of BMMC with CSM during the development of bone marrow progenitor cells suppressed the granularity and the surface expression of *c-kit* and FcεRI receptors. Stimulation with IgE/antigen resulted in decreased degranulation and release of Th1 and Th2 cytokines. The effects of CSM exposure could not be mimicked by the addition of LPS to the culture medium. In conclusion, this study shows that CSM may affect mast cell development and subsequent response to allergic activation in a TLR4-independent manner.

1. Introduction

The incidence of chronic respiratory diseases like chronic obstructive pulmonary disease (COPD) and asthma is increasing dramatically and currently affect the lives of approximately 300 and 200 million people, respectively, worldwide [1, 2]. COPD is characterized by a complex interaction between inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators [3]. Cigarette smoke (CS) is the major player in the pathogenesis of COPD [3]. Exposure to CS activates an inflammatory cascade in the airways resulting in the production of a number of potent cytokines and chemokines with accompanying damage to the lung epithelium, increased permeability, and recruitment of macrophages and neutrophils [4]. CS contains high levels of reactive oxygen species [5] and LPS [6, 7]. LPS is a strong Toll-Like Receptor (TLR) 4 agonist [8]. TLRs are an evolutionarily conserved family of cell surface molecules which participate in innate immune response [9, 10]. The effects of smoking on inflammatory cell maturation and differentiation have not been well described. Upon encountering pathogens and/or proinflammatory mediators, cells undergo a transformation process termed "maturation," which, for example, enhances dendritic cell (DC) Ag-presenting capacity or ability to release inflammatory cytokines. The role of TLRs in maturation and development

of DCs [11] and B cells [12] has been extensively described. In this regard, it has been reported that CS exposure leads to (a) decreased sputum mature DCs in healthy smokers and patients with COPD [13], (b) impaired DC maturation and T-cell proliferation in thoracic lymph nodes of mice [14] and (c) suppressed generation of IL-12 and IL-23 from DCs mediated through ERK-dependent pathways [15].

Parental smoking during childhood and personal cigarette smoking in teenage and early adult life are associated with a lower risk of allergic sensitization in those with a family history of atopy. The underlying mechanisms for this association remain to be determined, but the findings are consistent with the hypothesis that the immune-suppressant effects of CS protect against atopy [16]. So far few studies have reported on the role of mast cells in human smokers and in animal models of emphysema. Mast cells normally reside close to epithelia, blood vessels, nerves, smooth muscle cells, and mucus-producing glands [17]. Mast cells play a crucial role in allergic reactions [18]. Interestingly, emerging evidence also suggests a role of mast cells in the pathogenesis of emphysema [19, 20]. Kalenderian et al. [19, 20] found that the levels of mast cell mediators, such as histamine and tryptase, are considerably elevated in BALF from smokers. The importance of mast cells is further supported by the fact that mast cell tryptase activity is correlated with the severity of COPD [21], and in COPD patients an accumulation of mast cells in the airways has been observed [22]. Mast cells located here could be exposed to inhaled environmental challenges, and mast cell activation results in the coordinated release of proinflammatory mediators into the surrounding tissue; activation of this cell type may result in pathology associated with chronic inflammatory stimuli [23, 24]. Mast cells play a crucial role in acute and allergic inflammation and have FcεRI on their surface [24]. Cross-linking of surface IgE molecules results in exocytosis of preformed mediators such as amines and proteases, as well as the release of newly generated mediators including leukotrienes, prostaglandins, and a variety of cytokines such as Th1 (TNF-α, IL-6) or Th2 cytokines (IL-13, IL-4, IL-5) [24].

Previously, we showed that CSM (without IgE/Ag activation) does not trigger degranulation of bone marrow-derived mast cells (BMMCs) but does induce the release of chemokines [2]. In addition, CSM exposure suppresses IgE-mediated mast cell degranulation and cytokine release but had no effect on leukotriene release. This suggests that exposure to CSM may lead to a reduced allergic activation of mast cells without affecting their response to other stimuli [25]. In contrast, CS exposure in vivo enhanced OVA-specific IgE levels, Penh values, and recruitment of inflammatory cells including mast cells in OVA-exposed allergic mice [26].

In the current study, we investigated the effect of CSM exposure on the mast cell development from bone marrow progenitor cells.

2. Materials and Methods

2.1. Reagents. Recombinant mouse IL-3 and SCF (stem cell factor) were purchased from PeproTech (tebu-bio, Heerhugowaard, The Netherlands). LPS (*Escherichia coli* 055.B5) was purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). RPMI 1640, Tyrode's buffer, fetal calf serum, nonessential amino acids were purchased from GIBCO BRL Life Technologies (GIBCO-BRL Invitrogen Corporation, Carlsbad, CA, USA). Penicillin, streptomycin, L-glutamine, sodium pyruvate, and 2-mercaptoethanol were obtained from Sigma-Aldrich.

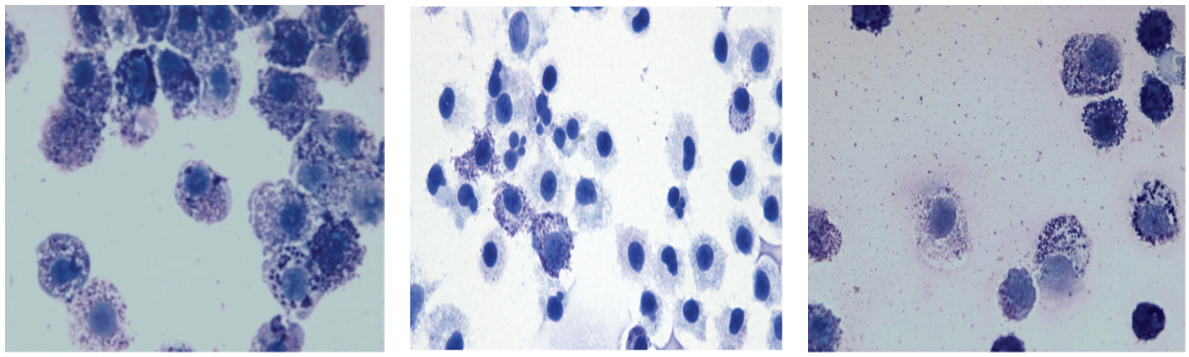
2.2. Production of Cigarette Smoke Medium (CSM). Cigarette smoke-conditioned medium (CSM) was produced as described previously [25]. CSM was generated by burning reference cigarettes 2R4F (University of Kentucky, Lexington, KY), using the TE-10z smoking machine (Teague Enterprises, Davis, CA, USA), which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35 mL puff volume drawn for 2 s, once per minute). Briefly, this machine was used to direct main- and side-stream smoke from one cigarette through a 5 mL culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically, and the media were standardized to the absorbance at 320 nm. The pH of the resultant extract was titrated to pH 7.4 and diluted with medium. This concentration (optical density [OD] = 4.0) was serially diluted with untreated media to 0.75%, 1.5%, and 3% OD and used in the indicated experiments. In preliminary experiments, a CSM concentration of 1.5% was found optimal in the culture experiments.

2.3. Mouse Bone Marrow-Derived Mast Cell (BMMC) Cultures and CSM Treatment. BMMCs were generated from bone marrow of male BALB/cBy mice as described previously [27]. Cells were cultured in RPMI medium supplemented with mitogen-stimulated spleen cell conditioned medium (see below) [28].

Cells were used for the experiments after 3 weeks when a mast cell purity >95% was achieved. Bone marrow cells were cocultured (1×10^6/mL) with either 1.5% CSM or LPS (100 ng/mL) during the third week of culture.

2.4. Pokeweed Mitogen-Stimulated Spleen Cell Conditioned Medium (PWM-SCM). Spleen cells from BALB/c mice (Charles River Breeding Laboratories) were cultured at a density of 2×10^6 cells/mL in RPMI 1640 medium containing 4 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM nonessential amino acids (complete RPMI 1640) containing lectin (8 μg/mL) and placed in 75 cm^2 tissue culture flasks. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. After 5–7 days, medium was collected, centrifuged for 15 min at 3200 ×g, filtered through a 0.22 μm Millipore filter, and used as PWM-SCM.

2.5. Toluidine Blue Staining. The granularity of the mast cells was determined by toluidine blue staining [29]. In brief, the cells were cytospun, fixed with Carnoy's fluid, and then stained by either 2 minutes with acid toluidine blue (pH = 2.7). Cells were examined by light microscopy.



(a) (b) (c)

Figure 1: Long-term culture in presence of CSM reduces the density of mast cell granules. BMMCs from BALB/c mice were cultured in presence with medium only (a), CSM (1.5%) (b), or LPS (c) (1 μg/mL) during the third week of culturing bone marrow cells as described in Section 2. Cells were stained with toluidine blue.

2.6. Mast Cell Degranulation Assay. The degranulation assay was performed as described before [28]. Briefly, approximately 2-3 $\times$ 10^6 cells from each group were resuspended in culture medium (enriched medium) and incubated with 1 μg/mL anti-DNP-IgE for 2 hr. After that, cells were washed and resuspended at a density of 0.6 $\times$ 10^6 cells/mL. Cells were aliquoted in 96 well plates (3 $\times$ 10^4 cells per well) and activated with indicated concentrations of DNP-conjugated ovalbumin (DNP-Ova) for 30 min. After incubation, supernatants were collected. Cells were subsequently lyzed using 0.1% NP-40 (Pierce) in order to quantify the total β-hexosaminidase activity present in these cells. Samples were incubated with 4-methylumbelliferyl glucosaminide (4-MUG) (Sigma) in 0.1 M citrate buffer (pH 4.5) for 1 h at 37°C. 4-MUG hydrolysis was determined by fluorimetric measurement (λex: 360 nm, λem: 452 nm) using a Millipore Cytofluor 2350 microplate reader. The percentage of β-hexosaminidase released was calculated by determining the ratio of fluorescence supernatant/fluorescence cell lysate corrected for the β-hexosaminidase activity present in the supernatant of nonchallenged cells.

2.7. Flow Cytometry Analysis. BMMCs were harvested, and after washing with cold PBS, the cell-surface Fc receptors were blocked with 2.4G2 (PharMingen, San Diego, CA, USA) before staining. We used a PE-conjugated anti-mouse *c-kit* (PharMingen) to stain *c-kit,* and mouse FcεRI was stained with an FITC-conjugated anti-mouse FcεRI antibody (PharMingen) and compared with matched isotype control antibodies. The cells were incubated with antibodies in 50 μL of PBS for 1 h at 4°C, washed with PBS, and analyzed on an FACSCantoII flow cytometer (Becton Dickinson, San Jose, CA, USA). Dead cells were gated out when performing the analysis.

2.8. Measurement of Cytokines. Briefly, approximately 1 $\times$ 10^6 cells for each experimental condition were resuspended in culture medium and incubated with 1 μg/mL anti-DNP IgE for 2 hr. After that, cells were washed and resuspended. Cells were aliquoted in 96 well plates (1 $\times$ 10^6 cells/mL) and activated with indicated concentrations of DNP-Ova for 16 h. IL-4, IL-5, IL-6, IL-13, and TNF-α concentrations in cell supernatants were quantitated using ELISA (Invitrogen and eBioscience), according to the manufacturer's instructions.

2.9. Statistical Analysis. Experimental results are expressed as mean ± S.E.M. Results were tested statistically by an unpaired two-tailed Student's t-test or one-way ANOVA, followed by a Newman-Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (version 5.04). Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. CSM Reduced the Granularity of Mast Cells during Culturing. Bone marrow cells were cultured with CSM or LPS during the third week of mast cell development. Cell granularity was analyzed by staining with toluidine blue (Figure 1(a)). Coculturing cells with CSM (1.5%) decreased the granularity of mast cells (Figure 1(b)). LPS (1 μg/mL) did not affect the granularity of cultured mast cells (Figure 1(c)).

3.2. CSM Decreased c-Kit (CD117) and FcεRI Expression on Mast Cells. Mast cell *c-kit* and FcεRI surface expression was determined after CSM exposure using flow cytometry. Coculture with CSM significantly suppressed the surface expression of *c-kit* and FcεRI on mast cells (Figure 2: upper and lower panels, control (a), and CSM (b)). In contrast, longterm culture with LPS did not change the surface expression of *c-kit* and FcεRI (Figure 2(c) upper and lower panels). FACS data of 3 representative experiments were quantified in (d) showing the mean fluorescence intensity (MFI) for each experimental group. Cell viability was not affected by either CSM or LPS treatment (data not shown).

3.3. Long-Term Exposure to CSM Modulates IgE/Ag-Mediated Degranulation and Cytokine Production by Mast Cells. Stimulation with IgE/Ag caused a dose-dependent degranulation of mast cells in the control group (Figure 3). Coculturing mast cells with CSM (1.5%) reduced IgE-mediated degranulation (Figure 3). In contrast, LPS, a TLR4 agonist, did not affect IgE/antigen-induced BMMC degranulation (Figure 3).

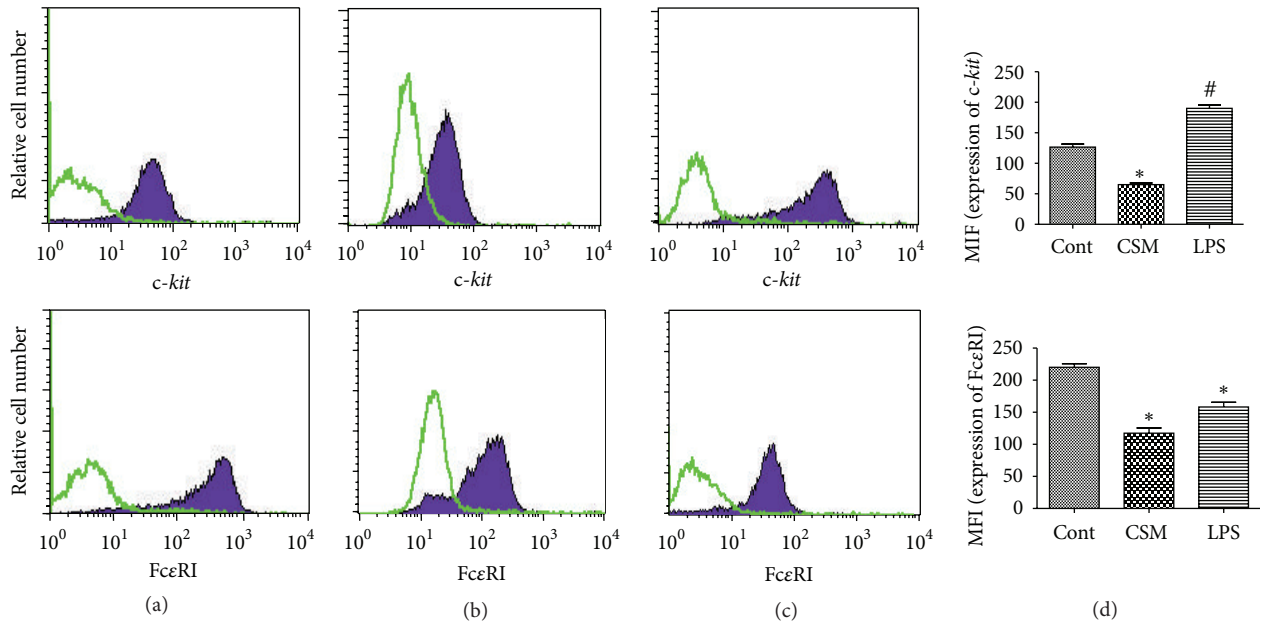


Figure 2: CSM modulates surface expression of FcεRI and *c-Kit*. BMMCs were cocultured in presence or absence of CSM (1.5%) or LPS during the third week of bone marrow culture. The surface expression of *c-kit* (upper panels) and FcεRI (lower panels) was detected by flow cytometry (blue histograms): control (a), CSM (b), and LPS (c). Green histograms represent isotype controls. (d) Quantification of 3 representative FACS analyses showing the mean fluorescence intensity (MFI) for each group. Values are expressed as mean ± S.E.M ($n = 3$). $^{*,\#}P < 0.05$ is significantly different (increased/decreased) compared to control.

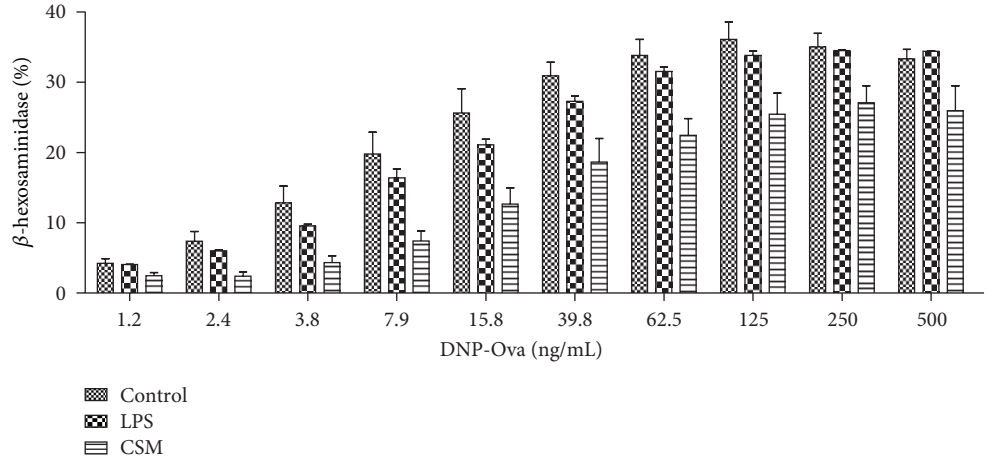


Figure 3: Long-term exposure of mast cells to CSM inhibits allergic degranulation. BMMCs were cultured in regular culture medium, in the presence of CSM (1.5%) or LPS (100 ng/mL) in the third week of bone marrow culture. Then, cells were sensitized with DNP-specific IgE, followed by activation with dinitrophenyl-conjugated human ovalbumin (DNP-Ova). Degranulation was assessed by the release of β-hexosaminidase in the supernatants from cells. Data are mean ± SEM of quadruplicate samples ($n = 4$).

CSM significantly suppressed the IgE-receptor mediated production of IL-4, IL-5, IL-6, IL-13, and TNF-α by mast cells (Figures 4(a)–4(e)). Culturing with LPS did not significantly change IL-4, IL-5, IL-6, IL-13, and TNF-α production (Figures 4(a)–4(e)).

4. Discussion

In the current studies, we further investigated the effects of CSM on the development and function of primary cultured bone-marrow-derived mast cells. We show that BMMC exposed to CSM during development from progenitor cells inhibited mast cell development as determined by toluidine staining and expression on *c-kit* and FcεRI. Furthermore, the release of both Th1 and Th2 cytokines in response to FcεRI activation was reduced. Interestingly, the TLR4 agonist LPS did not affect these parameters and even slightly increased IL-4 production.

Mast cells are important in allergic airway diseases, but they have remained poorly studied in nonallergic inflammatory airway diseases like COPD. Mast cells are of particular interest due to their ability to promote airway remodeling and

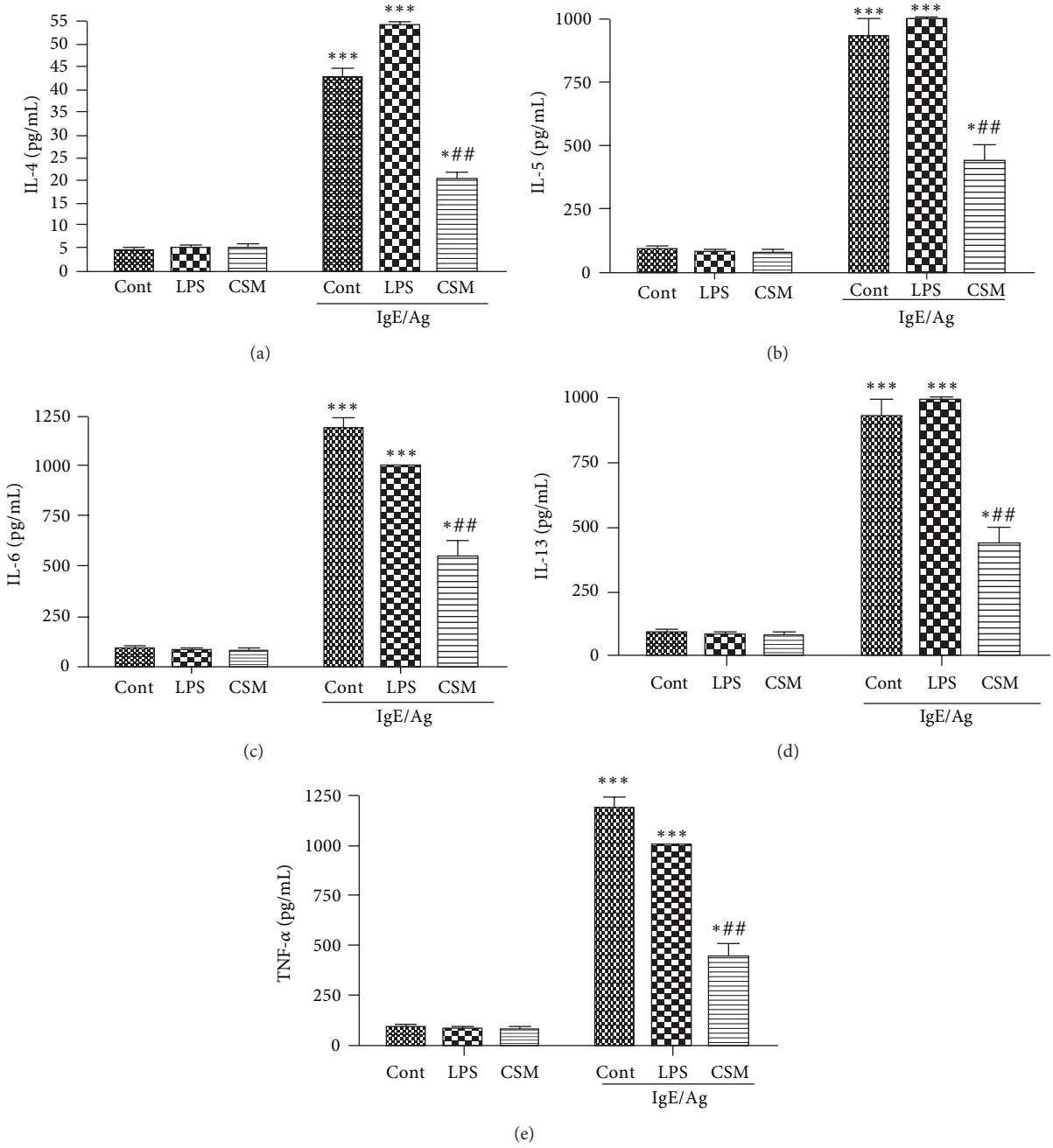


Figure 4: Long-term exposure of mast cells to CSM inhibits cytokine production by mast cells. BMMCs (control, CSM, and LPS cultured for 4–6 days) were sensitized with DNP-specific IgE, followed by activation with dinitrophenyl-conjugated ovalbumin (DNP-Ova) for 9 h. The levels of cytokines IL-4 (a), IL-5 (b), IL-6 (c), IL-13 (d), and TNF-α (e) in the supernatants were estimated by ELISA. Data are mean ± SEM of quadruplicate samples. The asterisks represent significant differences between nonactivated and activated cells ($^{***}P < 0.001$). Hatches represent significant differences between control mast cells and mast cells cocultured with CSM or LPS ($^{\#}P < 0.05$ and $^{\#\#}P < 0.001$).

mucus hypersecretion. Clinical data show increased levels of mast cell tryptase and degranulated mast cells in the lavage and bronchial tissue of smokers [30–33]. Moreover, CS exposure facilitates allergic sensitization in mice [34].

We have previously reported that short-term exposure of mature mast cells to CSM attenuated their response to allergic stimuli [28]. Kim et al. also showed an inhibitory effect of CSM on mast cell activation which suggests that CS may contribute to the reduced allergic symptoms observed in smokers [26].

Mast cells are functionally dynamic effector cells in innate and adaptive immunity [24]. Two mast cell surface receptors *c-Kit* and FcεRI mediate activation via innate and adaptive immune mechanisms, respectively [17, 23, 24]. *c-Kit* is expressed on both mature mast cells and on the earliest mast cell progenitors [35, 36]. *c-Kit* is expressed

both as a soluble form and on the cell membranes [36]. Although *c-Kit* represents a major growth and differentiation factor for both murine and human mast cells [35, 37] it also promotes *c-Kit*-dependent mast cell mediator release [38] as well as the release of mast cells mediators via IgE-dependent mechanisms [39]. IgE-dependent allergic diseases are initiated by multivalent binding of allergens to IgE that is bound to FcεRI on mast cells [40]. FcεRI plays a critical role in allergic reactions. It is the major surface receptor through which mast cells direct immunologically specific secretory effects such as the release of preformed cytoplasmic granule-associated mediators and the generation and release of lipid mediators and cytokines [41]. Thus, the suppression of *c-Kit* and FcεRI on the mast cells by CSM could account for decreased responsiveness of mast cells to IgE/Ag activation. Our study suggests that LPS may not be involved in the mechanism by which CSM affects mast cell maturation and activation.

Mast cells express functional TLRs [42] which may account for the protection conferred by mast cells against bacterial and parasitic infections [43]. Activated mast cells release an array of potent inflammatory mediators by rapid discharge of preformed mediators in granules, the generation of inflammatory lipids from arachidonic acid, and the production of numerous Th2-type cytokines and chemokines [44]. All these responses are evoked by allergens via FcεRI, while stimulation of mast cells via TLR2 and TLR4 receptors results primarily in generation of cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13, and TNF-α [43]. Saluja et al. recently showed that prolonged exposure of mast cells to LPS amplifies FcεRI-mediated degranulation, lipid mediator generation, and cytokine production [45]. This is in contrast to the effects found in this study, where LPS treatment did not increase IgE-mediated mast cell activation. The discrepancy may be due to the different mouse strains used in both studies and the time of treatment during mast cell development.

In conclusion, our study suggests that CSM, independent of TLR4 signaling, suppresses the maturation and function of mast cells. This suppressive effect of cigarette smoke on mast cells may account for the reduced allergic response seen in animal models of cigarette-smoke-induced emphysema [46].

Abbreviations

BMMC: Bone-marrow-derived mast cell
CSM: Cigarette smoke medium
COPD: Chronic obstructive pulmonary disease
FACS: Fluorescence-activated cells sorting
SCF: Stem cell factor
TLR: Toll-like receptor.

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Review Article

Lipotoxicity: Effects of Dietary Saturated and Transfatty Acids

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The ingestion of excessive amounts of saturated fatty acids (SFAs) and transfatty acids (TFAs) is considered to be a risk factor for cardiovascular diseases, insulin resistance, dyslipidemia, and obesity. The focus of this paper was to elucidate the influence of dietary SFA and TFA intake on the promotion of lipotoxicity to the liver and cardiovascular, endothelial, and gut microbiota systems, as well as on insulin resistance and endoplasmic reticulum stress. The saturated and transfatty acids favor a proinflammatory state leading to insulin resistance. These fatty acids can be involved in several inflammatory pathways, contributing to disease progression in chronic inflammation, autoimmunity, allergy, cancer, atherosclerosis, hypertension, and heart hypertrophy as well as other metabolic and degenerative diseases. As a consequence, lipotoxicity may occur in several target organs by direct effects, represented by inflammation pathways, and through indirect effects, including an important alteration in the gut microbiota associated with endotoxemia. Interactions between these pathways may perpetuate a feedback process that exacerbates an inflammatory state. The importance of lifestyle modification, including an improved diet, is recommended as a strategy for treatment of these diseases.

1. Introduction

Fat is an important component of the normal human diet. It is a source of energy and provides essential fatty acids and fat-soluble vitamins. However, several fatty acids in fats, especially saturated fatty acids (SFAs) and trans fatty acids (TFAs) may have adverse effects on human health [1–3].

In the human diet, SFAs are derived from animal sources, while TFAs originate in meat and milk from ruminant animals and result from bacterial biohydrogenation of unsaturated fatty acids in the rumen [4]. In addition, partial hydrogenation of unsaturated fatty acids in vegetable oils during the industrial production of certain foods produces TFA [5]. Small amounts of TFA are produced during the processes used to deodorize or refine vegetable oils to make the products more stable and robust and thus easier to handle or store [6, 7].

Most TFAs have physical properties similar to SFAs [8]. More specifically, monounsaturated TFA isomers with 18-carbon chain lengths (trans-18 : 1) are among the most predominant TFAs present in the human diet [9, 10]. It is well established that intake of SFA and TFA is a significant risk factor for cardiovascular diseases (CVD) as well as inflammation, insulin resistance, and obesity. These fatty acids also induce endothelial dysfunction and an unfavorable blood lipid profile, including increased LDL-c and decreased HDL-c levels [2, 11, 12].

High SFA and TFA intake, the typical dietary pattern of western populations, favors a proinflammatory status that contributes to development of insulin resistance. Roles for SFA and TFA intake have been demonstrated in several inflammatory pathways and result from imbalances in the highly interconnected lipid signaling pathways that contribute to disease progression in chronic inflammation, autoimmunity, allergy, cancer, atherosclerosis, hypertension, and heart hypertrophy as well as metabolic and degenerative diseases [13, 14].

The focus of this paper was to elucidate the influence and role of dietary SFA and TFA intake in lipotoxicity in the liver and the cardiovascular, endothelial, and gut microbiota

systems as well as in insulin resistance and endoplasmatic reticulum stress.

2. Insulin Sensitivity and Resistance

Insulin is an anabolic hormone that exerts several important metabolic effects. Insulin regulates glucose homeostasis at several levels, including decreasing hepatic glucose synthesis and increasing peripheral glucose uptake, primarily in muscle and adipose tissue. Moreover, this hormone stimulates lipogenesis and the synthesis of protein in hepatic and adipose tissues, while reducing lipolysis and proteolysis [15].

Events that occur after insulin binds to its receptor are highly regulated and specific and can be influenced by numerous factors such as the dietary composition, including the quantity and type of fatty acids [16, 17].

Although several mechanisms have been implicated in the development of insulin resistance [16], more studies are necessary to elucidate the link between the mechanisms of insulin resistance and fatty acid intake.

Increased lipid availability reduces insulin-stimulated glucose consumption in skeletal muscle. This effect is generally explained as a fatty acid-mediated inhibition of insulin signaling [15]. Moreover, in a recent investigation it was shown that a palatable hyperlipidic diet, rich with SFA, causes obesity and affects brain glucose metabolism in rats [18].

In a clinical study, short-term elevation of free fatty acids (FFAs) induced insulin resistance, which occurs primarily at the cellular level in skeletal muscle [17].

A chronic increase in plasma FFA levels is harmful as shown by the important effects of these dietary components in pancreatic beta cell lipotoxicity. Fatty acid derivatives can interfere with the function of these cells and ultimately lead to their death through lipoapoptosis [19].

Fatty acids in excess not only induce hepatic insulin resistance but also impair insulin clearance *in vitro* and *in vivo* in animals [20, 21] and humans [22]. This leads to the typical hyperinsulinemia observed in insulin-resistant states and in nonalcoholic fatty liver disease (NAFLD) [23, 24].

Several studies performed by our group have demonstrated that long-term interdisciplinary therapy reduces fat intake, in particular SFA, in obese adolescents. The intervention resulted in decreased visceral fat and tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels and increased levels of interleukin-10 (IL-10) and adiponectin, accompanied by a reduction in homeostatic model assessment-insulin resistance (HOMA-IR) and the occurrence of associated diseases [25–28]. In the context of these results, we proposed that the altered insulinemic status could be considered to play a key role in the development of several cardiometabolic risk comorbidities [12, 29–31].

In one review article, fatty acids were said to affect insulin secretion as a function of the chain length. Thus, insulin secretion increases as a function of longer carbon chains and decreases as a function of the degree of unsaturation. These findings suggest that SFA and TFA influence insulin resistance [32].

In an animal model, although TFA ingestion had no effect on fasting plasma glucose, insulin levels, or oral glucose tolerance, it significantly decreased insulin-stimulated glucose uptake in muscles compared to polyunsaturated fatty acids (PUFAs) [33].

In a cross-sectional study of individuals with high cardiometabolic risk, the association of TFA intake with insulin resistance was demonstrated. The authors speculated that TFA interferes with insulin signaling predominantly via intracellular kinases, which alter insulin receptor substrates [34].

Activation of intracellular kinases, such as inhibitor of nuclear factor-κB kinase (IKK) and c-Jun N-terminal kinase (JNK), alters insulin receptor substrates and decreases insulin sensitivity. Additionally, it is important to note that activation of transcription factors can contribute to reduced glucose uptake by the expression of proinflammatory cytokines, such as TNF-alpha and IL-6, causing impairment in the insulin receptor phosphorylation (Figure 3) [35].

The deleterious role of SFA in glucose and lipid metabolism has been previously shown. A partial explanation is that SFA increases production of cytokines such as TNF-α and IL-6 through hypertrophic adipocytes and infiltrating macrophages, and these cytokines cause the deterioration of insulin sensitivity [35].

Treatment of primary mouse hepatocytes and pancreatic cells with palmitic acid, an SFA, caused sustained JNK activation and insulin resistance. Moreover, the palmitic acid treatment inhibited glucose-induced insulin gene transcription. This effect may be mediated by interference of autocrine insulin signaling through the phosphorylation of insulin receptor substrates 1 and 2 at sites that interfere with their binding to activated insulin receptors [36]. It has been proposed that long-chain saturated fatty acids, such as palmitic acid, can trigger insulin resistance in both primary hepatocytes and pancreatic β-cells in a JNK-dependent manner. The JNK phosphorylation site on IRS-2 may be functionally equivalent to Ser-307 of IRS-1 (Figure 3). Moreover, JNK might also be involved in negative regulation of insulin synthesis or signaling within the pancreatic β-cells, the central site of blood glucose regulation. Thus, palmitic acid can be considered to be a potent activator of JNK in cultured hepatocytes and β-cells, leading to IRS-1 and IRS-2 Ser/Thr phosphorylation [36].

3. Hepatic Injury

The emergence of obesity has led to substantial prevalence of NAFLD. Worldwide, the prevalence of NAFLD ranges between 10% and 39%. However, the prevalence of NAFLD is increased in certain populations: the condition affects approximately 50% of diabetics, 57% to 74% of the obese, and up to 90% of morbidly obese people. Therefore, in the last decade, this disease has been recognized as an emerging clinical problem in obese patients [37, 38]. The pathogenesis of NAFLD is unclear, but there is evidence that insulin resistance, inflammation, and genetic and dietary factors, as

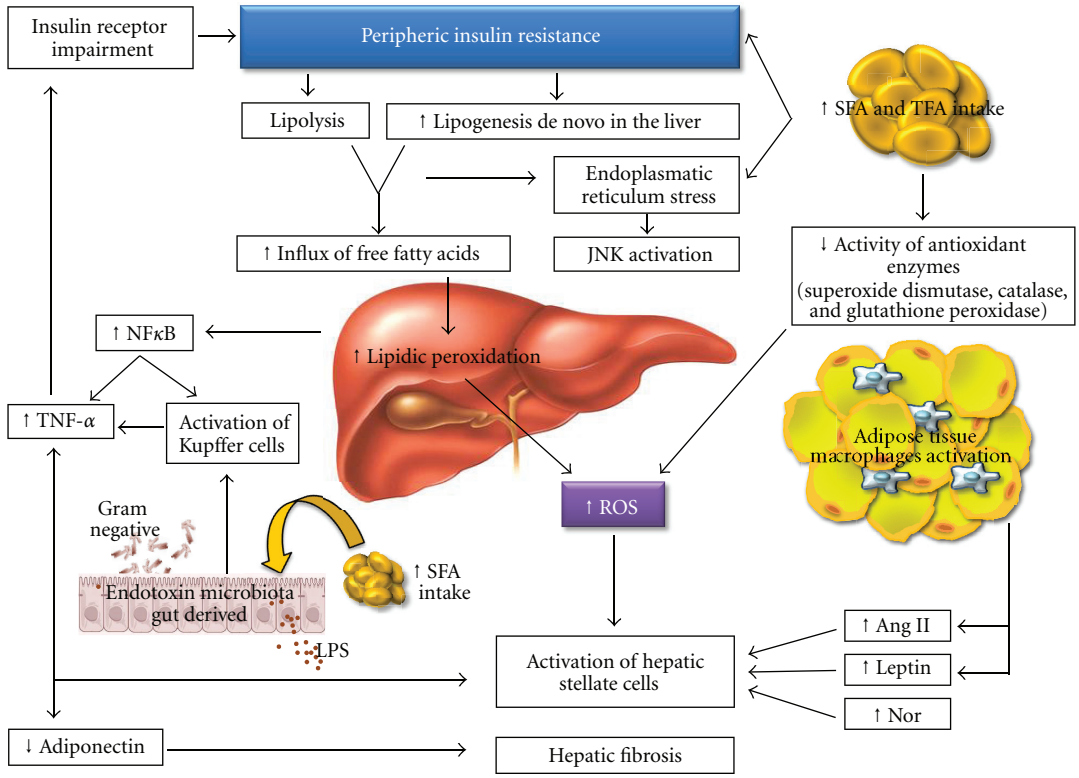

Figure 1: Schematic representation of SFA and TFA excess intake effects in hepatic injury and in endotoxemia. Ang II: angiotensin II, JNK: Jun N-terminal kinase, LPS: lipopolysaccharides, NF-κB: nuclear factor kappa-B, Nor: Noradrenaline, ROS: reactive oxygen species, SFA: saturated fatty acids, TFA: trans fatty acids, and TNF-α: tumor necrosis factor-alpha.

well as lifestyle, exert key roles in the development of NAFLD [39].

Several studies have revealed an association between obesity and NAFLD progression. In NAFLD patients, the adipocytes of the visceral tissue demonstrate elevated lipolytic activity, promoting a high influx of FFA into the portal vein [37, 38, 40]. Moreover, the literature shows an association between visceral adipocytes and the hepatic cellular inflammatory process [41]. It is believed that the physiopathology of NAFLD may be driven by several forms of hepatic injury. The first hypothesis involved in the development of NAFLD is related to insulin resistance. The influence of genetic and environmental factors can promote peripheral insulin resistance, which leads to increased levels of nonesterified fatty acids (NEFAs). Subsequently, a high influx of these fatty acids into hepatocytes promotes increased hepatic de novo lipogenesis. When the rate of lipogenesis exceeds the rate of β-oxidation of fatty acids and the exportation of VLDL, hepatic fat accumulation is observed (Figure 1) [40].

The exact mechanisms that promote the progression from steatosis to steatohepatitis have not yet been completely elucidated. However, it is clear that apoptosis can be a pathophysiologic marker of nonalcoholic steatohepatitis in some steatotic patients.

It has been proposed that the accumulation of FFA, especially saturated fatty acids, in the hepatocytes can promote apoptosis by diverse pathways. These may include ROS-induced stress that affects the mitochondrial membranes, endoplasmic reticulum, and lysosomes. Lipid peroxidation increases the levels of reactive oxygen species, which may be partially responsible for hepatocyte dysfunction [42] (Figure 1).

Apoptosis of the hepatocytes can occur via extrinsic or intrinsic pathways. The extrinsic pathway is induced by death ligands such as Fas (a key death receptor belonging to the tumor-necrosis-factor- (TNF-) receptor family) and TRAIL (TNF-related apoptosis-inducing ligand). In contrast, the intrinsic pathway of cell death is activated by the intracellular stress of membrane-bound organelles, such as ER, lysosomes, and mitochondria [43].

Saturated fatty acids activate complex intracellular pathways, including the activation of Toll-like receptor 4 (TLR-4), which subsequently stimulates TNF-α production. TNF-α consequently activates the JNK pathway [40], leading to the upregulation of the proapoptotic BH3-only protein PUMA (p53-upregulated modulator of apoptosis). PUMA then associates with BIM (Bcl-2-interacting mediator of cell death) to activate BAX, a proapoptotic protein of the BCL-2 (B-cell lymphoma 2) family. The end result of this pathway is mitochondrial dysfunction, activation of the caspase cascade, and cell death [43].

The TFA obtained in the diet accumulates in cellular triacylglycerols and phospholipids. Chronically excessive triacylglycerol accumulation in tissues such as the liver, muscle, and pancreatic beta cells leads to a protective response involving adaptation of the adipocytes, and this response

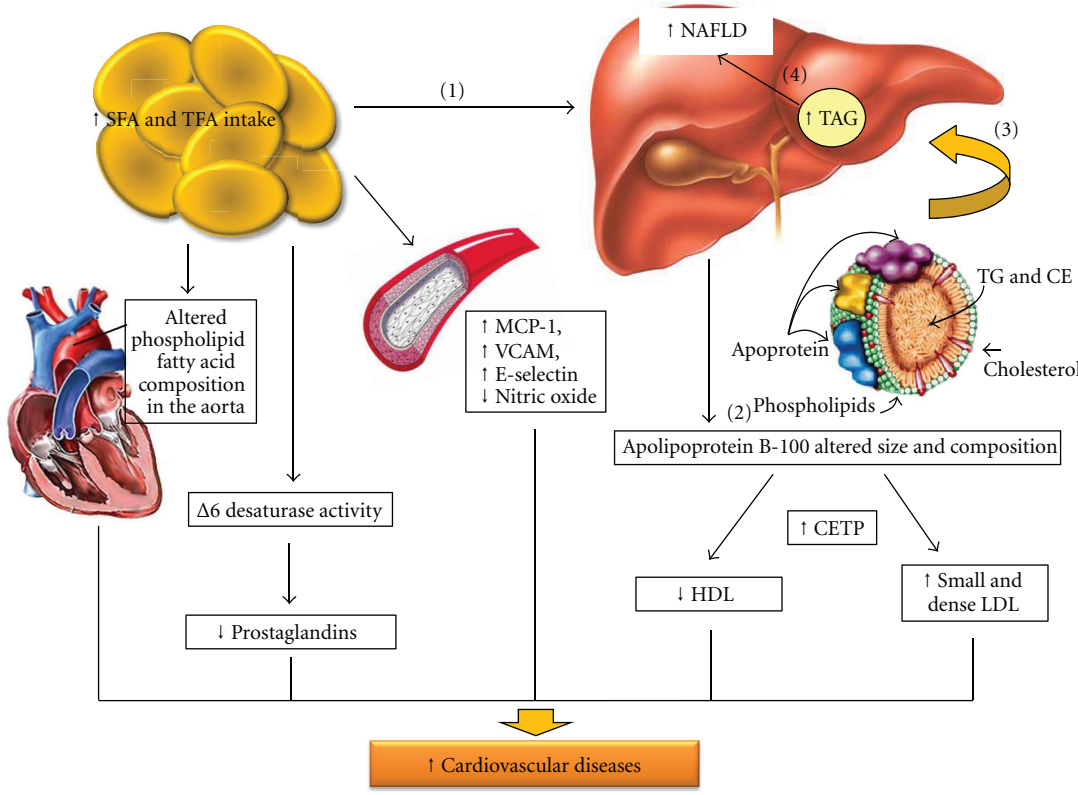

Figure 2: Schematic representation of SFA and TFA excess intake effects in hepatic injury, lipid metabolism, and cardiovascular risk. CE: cholesterol esterified, CETP: cholesteryl ester transfer protein, HDL: high density cholesterol, LDL: low density cholesterol, MCP-1: monocyte chemotactic protein-1, NAFLD: nonalcoholic fatty liver disease, SFA: saturated fatty acids, TFA: trans fatty acids, TG: triacylglycerol, and VCAM: vascular cell adhesion molecule.

includes activation of several inflammatory pathways that promote adipose tissue insulin resistance [34].

Toll-like receptors (TLRs) are classical pattern recognition receptors of the innate immune response. Two of these receptors, TLR4 and TLR2, are activated by SFA but inhibited by docosahexaenoic acid [44].

TLR4 activates both MyD88-dependent and -independent pathways. The MyD88-dependent pathway is initiated by the recruitment of TIRAP, an adaptor between the TIR domain of TLR-4 and MyD88, followed by activation of the IRAK family of protein kinases and subsequent phosphorylation of TRAF6. In the MyD88-independent pathway, the activation of TLR-4 requires the participation of TRAM to recruit TRIF, which then activates TRAF6 and TRAF3. TRAF6, directly or via TAK1, stimulates the IKK complex which promotes phosphorylation of IkB leading to activation of nuclear factor κB (NF-κB) (Figure 3) [45].

FFAs activate TLR-4 receptors in macrophages and adipocytes, which results in increased proinflammatory cytokine gene and protein expression [46]. Recently, it has been demonstrated that saturated fatty acids can induce TNF-α expression by macrophages by activating the MyD88-independent pathway [47].

TLR4 activation plays a central role in the inflammatory process, activating inflammatory cytokine gene transcription and also inducing endoplasmic reticulum stress. The activation of TLR4 by LPS induces a potent endoplasmic reticulum stress and unfolded protein response through the PERK/eIF2α and IRE1α/XBP1 pathways (Figure 4) [48]. NFkB, a transcription factor known as a mediator of immune and antiapoptotic responses, is activated by the accumulation of membrane proteins in the ER. Specifically, the accumulated proteins lead to the production of reactive oxygen intermediates, which activate NFkB by degradation of IkB. Recently, it has been proposed that the phosphorylation of eIF2a is required for the triggering of NFkB. In summary, the apoptotic process involves the activation of the gene for C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153) (Figure 4); the activation of the cJUN NH2-terminal kinase (JNK) pathway, which is mediated by the formation of the inositol-requiring 1 (Ire1)-TNF receptor-associated factor 2 (TRAF2)-apoptosis signal-regulating kinase1 (ASK1) complex; and the activation of the ER-associated caspase-12. In humans, these apoptotic pathways eventually lead to the activation of caspase-3 [49].

The second hypothesis of NAFLD physiopathology refers to the release of fatty acids from dysfunctional and insulin-resistant adipocytes. This release causes lipotoxicity, mainly to visceral adipose tissue [40]. The visceral adipose tissue is infiltrated by inflammatory cells, including macrophages and other immune cells, which increase secretion of

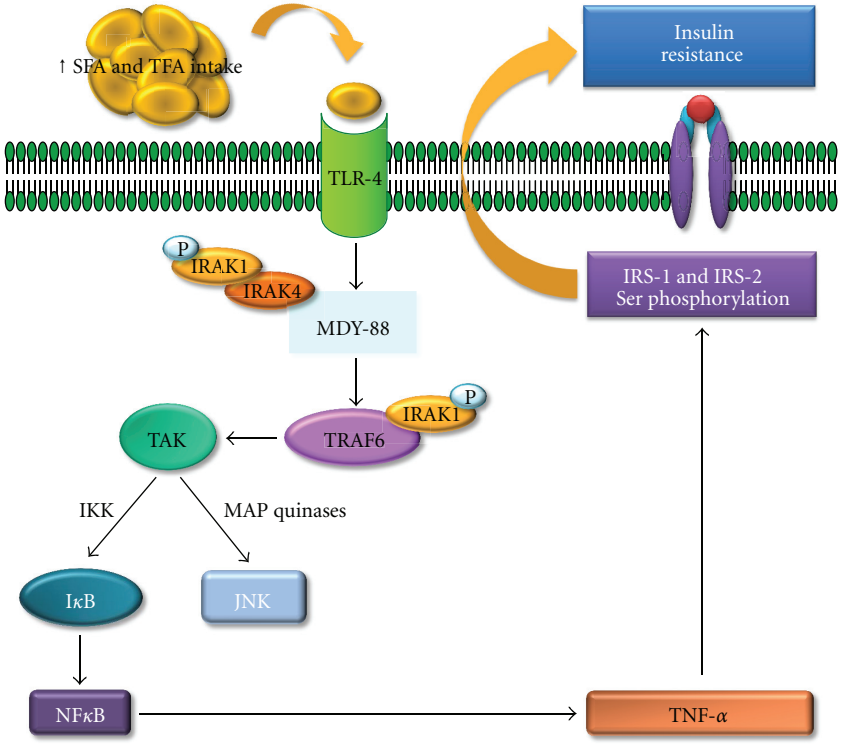

Figure 3: Schematic representation of SFA and TFA excess intake effects in the mechanisms of insulin resistance development. IKB: inhibitor of nuclear factor-κB, IKK: inhibitor of nuclear factor-κB kinase, IRAK-1: interleukin-1 receptor-associated kinase 1, IRAK-4: interleukin-1 receptor-associated kinase 4, IRS-1: insulin receptor substrate-1, IRS-2: insulin receptor substrate-2, JNK: Jun N-terminal kinase, MAP kinases: mitogen activated protein kinases, MDY-88: myeloid differentiation primary response gene (88), NF-κB: nuclear factor kappa B, P: phosphorus, TAK: thylakoid arabidopsis kinase, TLR-4: Toll-like receptor-4, TNF-α: tumor necrosis factor-alpha, TRAF-6: receptor-associated factor 6.

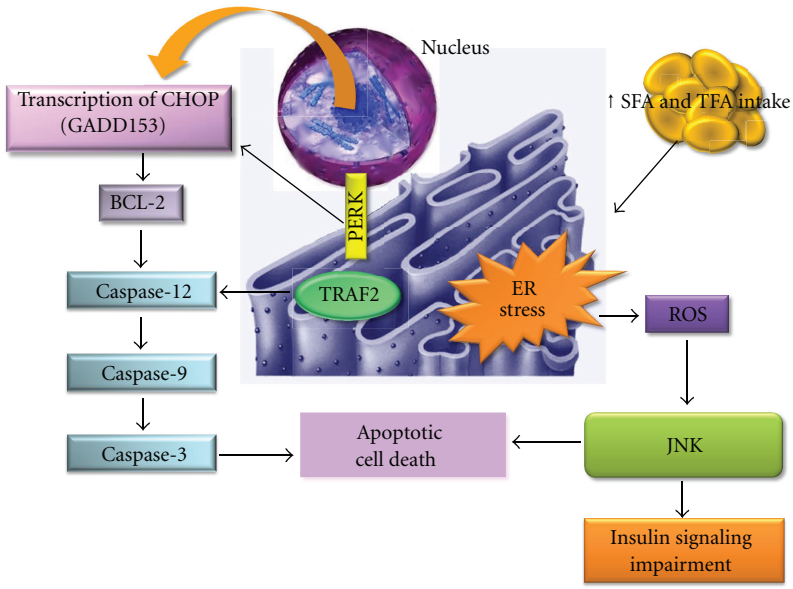

Figure 4: Schematic representation of SFA and TFA excess intake effects in endoplasmatic reticulum stress. BCL-2: B-cell lymphoma 2, CHOP: CCAAT/-enhancer-binding protein homologous protein, ER: endoplasmatic reticulum, GAAD 153: DNA damage-inducible gene 153, JNK: Jun N-terminal kinase, PERK: protein kinase RNA-like endoplasmic reticulum kinase, ROS: reactive oxygen species, SFA: saturated fatty acids, TFA: trans fatty acids, and TRAF2: TNF receptor-associated factor 2.

inflammatory adipokines such as leptin, IL-6, TNF-α, and angiotensinogen, as well as reducing secretion of adiponectin, an anti-inflammatory adipokine [50]. Insulin resistance of the visceral adipose tissue is associated with increased lipolytic activity, which increases the levels of FFA in the portal circulation, potentially resulting in hepatotoxicity (Figure 1) [40, 41].

Experimentally, it has been demonstrated that the decreased secretion of adiponectin in obesity alters lipid metabolism and insulin sensitivity in the liver. However, administration of recombinant adiponectin to adiponectin-deficient obese mice fed a high-fat diet dramatically alleviated hepatomegaly, steatosis, and inflammation [51].

At the clinical level, adipose tissue insulin resistance contributes to type 2 diabetes mellitus and CVD. On the other hand, decreasing plasma FFA concentration by administration of acipimox, a nicotinic acid analogue that inhibits adipose tissue lipolysis, rapidly improves muscle insulin sensitivity [52].

The potential ability of FFA to alter skeletal muscle glucose metabolism was first proposed more than 50 years ago [53] and has been widely investigated. The FFA-induced effects on this tissue do not appear to be the result of the accumulation of intramyocellular lipids per se. Rather, skeletal muscle insulin resistance is closely correlated with the presence of a variety of toxic metabolites derived from incomplete oxidation of fatty acids, such as acylcarnitines and long-chain fatty acyl CoAs, ceramides, and/or diacylglycerols [54, 55].

Oxidative stress is believed to be an important factor in the development of NAFLD [56]. The importance of fatty acids in this process is clear from the observation that biological membranes adjust their composition according to the fatty acid content of dietary fat [57]. Dietary fatty acids can influence the susceptibility of cells to oxidative stress, perhaps due to changes in the fatty acid composition of the cellular membranes [58].

Intake of high levels of SFA is associated with increased lipid content in the liver [18, 59–61] and with liver dysfunction. This dysfunction is thought to be caused by an increase in the production in reactive oxygen species, which leads to damage of the hepatic mitochondria. In addition, SFA intake exceeding 10% of total energy promotes insulin resistance, which plays a key role in the development of NAFLD [62].

The association between high intake of SFA and cholesterol with NAFLD has been previously demonstrated [63, 64]. SFAs promote endoplasmic reticulum stress as well as hepatocyte injury. Accumulation of SFA in the liver leads to an increase in markers associated with endoplasmic reticulum stress, such as reactive oxygen species and caspase activation. These biomarkers are associated with liver dysfunction. Moreover, the positive correlation between SFA intake and insulin resistance, which plays a key role in the development of NAFLD, has been demonstrated. These correlations suggest that limiting SFA intake is a valuable nutritional strategy for the prevention and treatment of NAFLD [62].

Papandreou et al. [64] demonstrated that the SFA intake was directly proportional to the degree of hepatic steatosis. In their study, multiple regression analysis of factors associated with fatty liver showed that HOMA-IR and SFA were the most significant factors for this condition after adjustment for age, gender, and diet. We have also observed an association among SFA intake, NAFLD, and orexigenic neuropeptides [26].

Diets rich in fatty acids, mainly SFA and TFA, as well as carbohydrate-rich diets, favor an acute increase in insulin resistance independent of adiposity. High SFA intake may also promote steatohepatitis directly by modulating hepatic triacylglycerol accumulation and oxidative activity and indirectly by affecting insulin sensitivity and postprandial triacylglycerol metabolism [64].

In clinical studies performed by our research group, we showed a positive correlation between calories derived from SFA intake and visceral fat in NAFLD patients [23, 26]. These data suggested that composition of the diet exerts an important role in the development of NAFLD and its treatment and that it is essential to consider excessive SFA intake as a critical risk factor for development of NAFLD [26].

Another potentially important mechanism for lipotoxicity of TFA in the liver is their effects on hepatic antioxidant enzymes. Reactive oxygen species (ROS) form as natural byproducts from the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress, ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. Endogenous antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), are essential to defend against ROS. The removal of reactive oxygen substances is accomplished by enzymatic and nonenzymatic reactions in biological systems. In enzymatic reactions, SOD converts superoxide anions to hydrogen peroxide (H_2O_2), and H_2O_2 can be rapidly degraded by CAT and GPx to H_2O [65–67].

High-fat diets can cause the formation of toxic intermediates that can inhibit the activity of antioxidant enzymes, resulting in the accumulation of O_2 radicals and H_2O_2, which subsequently form hydroxyl radicals [68, 69]. TFAs are associated with a decrease in the efficiency of the antioxidant-enzymatic system and therefore with the increase of oxidative stress in rat livers. TFA may impart their effect by enhancing intrinsic signaling mechanisms leading to a chronic, pro-inflammatory state. Consumption of diets high in TFA may induce long-term progressive changes in the antioxidant enzyme's activities [70].

Finally, another pathway by which excessive TFA intake could cause hepatic injury is through effects on lipid metabolism. *In vitro*, TFAs alter the secretion, lipid composition, and size of apolipoprotein B-100 (apoB-100) particles produced by hepatic cells [71] (Figure 2). Specifically, these cells fail to synthesize the apolipoprotein, which is required to package and export fat from the liver. Therefore, the liver accumulates triacylglycerol. In a study in which male Wistar rats were fed a high-fat diet including 20% fresh soybean oil diet, 20% oxidized soybean oil diet, or 20% margarine diet for 4 weeks, the highest inflammatory response in the liver was induced by the margarine diet. The authors demonstrated that oxidized edible oils fed to rats for four

weeks increased lipid peroxidation in the liver compared with rats fed nonoxidized oils. These results suggest that a strong relationship exists between the consumption of TFA in the oxidized oils and lipid peroxidation. This study provides evidence for a direct effect of TFA on liver dysfunction caused by disturbances in hepatic lipid metabolism. The resulting NAFLD is a key component of cardiometabolic risk. This evidence suggests that TFA may influence the risk factors for CVD [70].

Thus, the link between dysfunctional adipocytes and the liver involves several pathways that combine to promote the development of lipotoxic liver disease, a term that more accurately describes the pathophysiology of nonalcoholic steatohepatitis.

In fact, hepatic steatosis is considered to be a hepatic manifestation of cardiometabolic risk. This condition is associated with obesity, insulin resistance, hypertension, and dyslipidemia. Clinical studies corroborate the relationship between NAFLD and CVD. Specifically, obese NAFLD patients were shown to have a greater intima-media thickness (IMT), a subclinical marker of the atherosclerotic process, compared to non-NAFLD patients [72]. In this context, our research group performed a study that involved one year of interdisciplinary intervention in obese adolescents. The results indicated that the improvement of HOMA-IR was an independent predictor of carotid IMT changes in this population [30]. As previously discussed, hyperinsulinemia from increased insulin secretion and decreased insulin clearance correlates with the severity of hepatic steatosis, and chronically elevated plasma insulin levels may promote atherogenesis [73, 74]. Hyperglycemia, *per se*, and the typical atherogenic dyslipidemia in NAFLD driven by oversecretion of VLDL are established factors for CVD. However, whether NAFLD and CVD are mechanistically related or merely both associated with lipotoxicity remains to be established.

4. Cardiovascular Risk

Studies suggest multiple possible mechanisms that might mediate the association of TFA with CVD. Three main pathways for these physiological effects have been proposed: serum lipid concentrations, systemic inflammation, and endothelial cell function [3, 75].

Consumption of industrial TFA increases the blood concentrations of low-density lipoprotein (LDL), triacylglycerols, and Lp(a) lipoprotein while decreasing the levels of high-density lipoprotein (HDL) and reducing the particle size of LDL cholesterol. Furthermore, consumption of TFA can increase the ratio of total cholesterol to HDL cholesterol, a powerful predictor of CVD risk [3, 76]. Thus, TFAs have markedly adverse effects on serum lipid profiles.

As described previously, there is an important relationship between intake of TFA and incidence of CVD. However, the effects exceed those predicted by the changes in serum lipids alone, suggesting that TFAs influence other risk factors for CVD [77]. Specifically, in addition to their effects on lipid/lipoprotein profiles, TFA consumption is known to influence multiple risk factors including increased systemic inflammation [78], increased thrombogenesis, and reduced endothelial function [2], all of which, in combination or individually, contribute to increased cardiovascular risk. Experimental studies suggest that TFAs exert their multiple effects by influencing metabolic and signaling pathways in hepatocytes, monocytes, adipocytes, and endothelial cells. The precise molecular pathways through which TFAs influence these cell types are not well established [77].

The effect of TFA on systemic inflammation can be partially explained by the influence of these fatty acids on the prostaglandin balance. The effects on these processes can influence thrombogenesis and impair the activity of Δ desaturase, the enzyme responsible for the conversion of linoleic acid to arachidonic acid and other n-6 PUFA. Thus, this inhibition alters essential fatty acid metabolism [79]. Moreover, in an animal model of excess TFA consumption, changes in the phospholipid fatty acid composition in the aorta were observed [80] (Figure 2). TFAs have been associated with the activation of systemic inflammatory responses, including substantially increased levels of IL-6, plasminogen activator inhibitor-1 (PAI-1), TNF-α, TNF receptors, and monocyte chemoattractant protein-1, and with increased levels of several markers of endothelial activation, such as soluble intercellular adhesion molecule 1, soluble vascular-cell adhesion molecule 1, and E-selectin [2, 3, 81] (Figure 2).

In controlled trials, however, TFA did not increase all inflammatory markers [78, 82]. Oxidative stress may also explain the high risk of CVD associated with industrial TFA intake [83].

Oxidative stress induced by free radicals has been associated with the development of several diseases including CVDs, most likely through a vascular proinflammatory response [84]. However, further research is necessary to fully elucidate the implications of the effects of TFA on some markers of oxidative stress. Although the possible mechanisms that link TFA and oxidative stress are unknown, efforts to eliminate partially hydrogenated oils from the diet remain necessary and important to reduce the burden of CVD [70].

The third pathway linking TFA and CVD refers to the possible influence of TFA on endothelial cell function. Endothelial nitric oxide synthase (eNOS) synthesizes nitric oxide (NO) in response to many stimuli, such as fluid shear stress and insulin. These stimuli increase NO production in endothelial cells through an insulin receptor substrate-1 (IRS-1-) and phosphatidylinositol 3-kinase (PI3-kinase-) dependent pathway that causes phosphorylation of endothelial nitric oxide synthase (eNOS) by Akt [85].

A review of the influences of fatty acids on endothelial cell function suggested that increased ingestion of fatty acids impairs endothelial cell insulin signaling and NO production through the activation of the IKK/NF-κB pathway. Furthermore, an experimentally induced elevation of the concentration of plasma FFA in humans alters endothelial function [46].

It has also been shown that the dietary SFA palmitate attenuates endothelial insulin signaling and NO production by first activating NF-κB signaling, which results in a reduction in IRS-1/pAkt/peNOS signaling [77].

The reduction of SFA intake is considered a primary goal for decreasing the risk of CVD. A low SFA diet was demonstrated to be associated with the reduced progression of coronary atherosclerosis [86, 87].

The effect of SFA intake on the plasma lipid risk factors and effects on CVD are similar to those described for TFA intake. However, SFA ingestion is particularly associated with activation of the TLR pathways.

In a clinical investigation, TLR-4 and TLR-2 expression and activity were increased in the monocytes of patients with cardiometabolic risk. The pathways regulated by these receptors could contribute to the patients' high risk for CVD [88].

5. Endoplasmic Reticulum Stress

Lipid peroxidation is defined by a biochemical cascade that results in oxidative degradation of PUFA. When the lipid peroxidation occurs in biological membranes, it causes impaired membrane function and structural integrity, decreases in membrane fluidity, and inactivation of several membrane enzymes [89]. Niu et al. [90] reported that phospholipids derived from TFA had a higher membrane cholesterol affinity than their *cis*-analogues. Thus, TFA ingestion could alter cell membrane structure, organization, and composition in an ROS-mediated manner.

A recent animal experiment indicated that TFA reduced the membrane fluidity of fat cells and impaired cell function. The suggested mechanism involved production of additional reactive oxygen species associated with the increase in lipid peroxidation in the groups fed the TFA diet [91]. A high-fat diet induces endoplasmic reticulum stress (ER), which activates IKK and JNK, thereby impairing insulin signaling [92].

Recent evidence suggests that lipotoxicity in hepatocytes involves ER stress and JNK-mediated apoptosis [93, 94].

Disturbances in the normal functions of the ER lead to an evolutionarily conserved cell stress response, the unfolded protein response, which is aimed initially at compensating for damage but can eventually trigger cell death if ER dysfunction is severe or prolonged. Although the mechanisms by which ER stress leads to cell death are not completely understood, some of them have been described in the literature. A study of mice deficient in caspase-12 showed that while the cells of these mice were resistant to ER stress-induced apoptosis, apoptosis of the cells occurred normally in response to other death stimuli [95]. Based on these data, it was proposed that other pathways leading to cell death by ER stress should be explored. Increases in apoptotic proteins, such as BIM, BAK, and PUMA, were observed during ER stress, suggesting a connection between stress signals and the proapoptotic switch that occurs when cellular homeostasis is irreversibly altered, finally leading to cell death [96, 97]. The ER responds to the burden of unfolded proteins in its lumen by activating intracellular signal transduction pathways, generically termed the unfolded protein response (UPR). Another suggested mechanism is that the three UPR branches provide opposing signals and that the relative timing of their induction shifts the balance between cytoprotection and apoptosis as unmitigated ER stress persists. Specifically, IRE1 signaling attenuates upon prolonged ER stress, and PERK (protein kinase RNA-like endoplasmic reticulum kinase) signaling induces its own deactivation via GADD34 expression (Figure 4). Both pathways thus contain intrinsic timers that are likely to contribute to the life-or-death decision [98].

Important roles for ER-initiated cell death pathways have been recognized for several diseases, including hypoxia, ischemia/reperfusion injury, neurodegeneration, heart disease, and diabetes [99].

Studies suggest that cytokines, as well as elevated lipids, especially long-chain SFA, may induce ER stress in pancreatic β-cells and liver cells. SFA-induced β-cell death has been shown to be related to the activation of caspases [94, 100]. Elevated lipids also induce apoptosis in a number of cell types, suggesting that ER stress may be an early component of lipotoxicity [94].

A study of cultured H4IIE liver cells investigated the influence of SFA and TFA in the apoptosis process and the role of the ER stress-induced activation of caspases. The authors observed that SFA induced ER stress and increased both caspase-9 and caspase-3 activity (Figure 4). The authors hypothesized that saturation, *per se*, plays a role in lipotoxicity in liver cells [94].

6. Gut Microbiota

The human gut contains a massive number of microorganisms or microbiota. Several mechanisms have been proposed to link gut flora to obesity, including the role of the gut microbiota in increasing energy extraction from indigestible dietary polysaccharides [101] and elevating plasma lipopolysaccharide levels, resulting in chronic low-grade inflammation [102].

The intestinal flora exerts an important role in normal gut function and maintenance of health, and the dietary composition can influence the sequence and the nature of colonization. Cani et al. [103] found that a high-fat diet resulted in a significant change in the composition of the dominant bacterial populations within the gut microflora, including a decrease in the number of *Bifidobacteria*, Eubacterium, rectal *Clostridium coccoides* group, and *Bacteroides*, thus favoring an increase in the gram-negative to gram-positive ratio. This change in gut microflora composition was associated with a significant increase in plasma lipopolysaccharide (LPS) levels, fat mass, body weight gain, liver hepatic triglyceride accumulation, insulin resistance, and diabetes [103, 104]. In addition, de Wit et al. [105] observed that a high saturated fatty acid diet enhanced an overflow of dietary fat to the distal intestine, which affected the gut microbiota composition. This alteration was associated with obesity development and hepatic steatosis.

The gut microbiota of obese individuals or those consuming a high content of saturated fatty acids contains predominantly gram-negative bacteria rich in LPS. Toll-like receptors in the cell membranes recognize LPS in the circulation (endotoxemia) and activate specific kinases, which lead to insulin resistance. These pathways also activate NF-κB, which results in the expression of inflammatory genes. Similar to

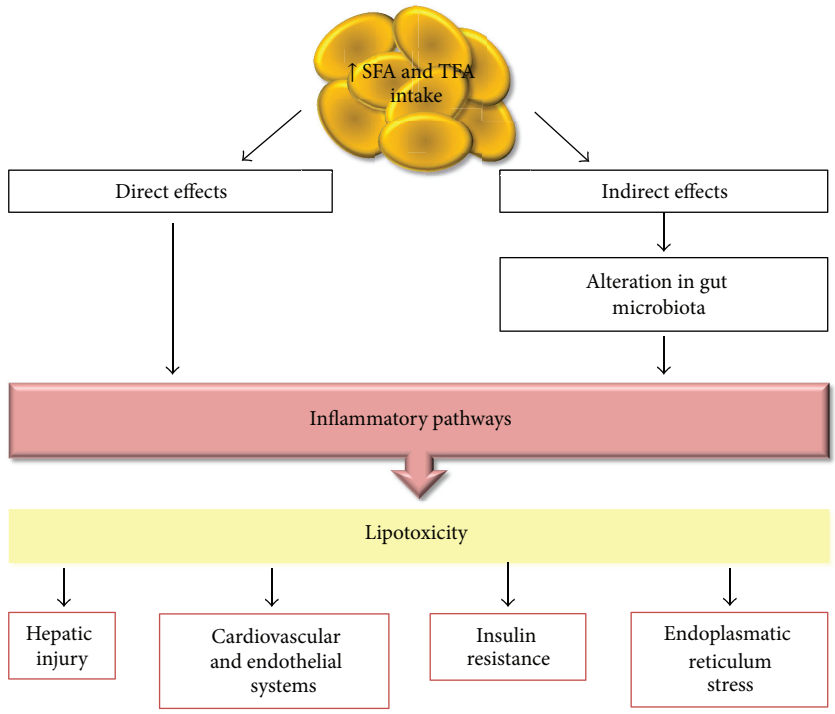

Figure 5: Schematic representation of SFA and TFA excess intake effects in the development of lipotoxicity in several target organs.

LPS, saturated fatty acids are also recognized by membrane receptors that trigger proinflammatory signaling pathways [102, 106].

Recently, our group demonstrated a positive correlation between plasma endotoxin concentration and both proinflammatory cytokines (especially IL-6) and insulin resistance in obese adolescents. Importantly, after long-term (one year) interdisciplinary therapy, endotoxemia, proinflammatory status, and insulin resistance were decreased [25]. These results showed the effectiveness of making lifestyle changes (i.e., nutritional modification) in reducing the proinflammatory state in obese individuals [107, 108].

7. Conclusion

These experimental and clinical findings indicate that excess intake of both SFA and TFA can promote lipotoxicity in several target organs by direct effects, represented by inflammatory pathways, and indirect effects, including important alterations in the gut microbiota with implications for the endotoxemia process (Figure 5). Interplay between these pathways perpetuates a feedback process in which an inflammatory state elevates the risk factors for diverse diseases.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Green Tea Extract Supplementation Induces the Lipolytic Pathway, Attenuates Obesity, and Reduces Low-Grade Inflammation in Mice Fed a High-Fat Diet

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The aim of this study was to evaluate the effects of green tea *Camellia sinensis* extract on proinflammatory molecules and lipolytic protein levels in adipose tissue of diet-induced obese mice. Animals were randomized into four groups: CW (chow diet and water); CG (chow diet and water + green tea extract); HW (high-fat diet and water); HG (high-fat diet and water + green tea extract). The mice were fed *ad libitum* with chow or high-fat diet and concomitantly supplemented (oral gavage) with 400 mg/kg body weight/day of green tea extract (CG and HG, resp.). The treatments were performed for eight weeks. UPLC showed that in 10 mg/mL green tea extract, there were 15 μg/mg epigallocatechin, 95 μg/mg epigallocatechin gallate, 20.8 μg/mg epicatechin gallate, and 4.9 μg/mg gallocatechin gallate. Green tea administered concomitantly with a high-fat diet increased HSL, ABHD5, and perilipin in mesenteric adipose tissue, and this was associated with reduced body weight and adipose tissue gain. Further, we observed that green tea supplementation reduced inflammatory cytokine TNFα levels, as well as TLR4, MYD88, and TRAF6 proinflammatory signalling. Our results show that green tea increases the lipolytic pathway and reduces adipose tissue, and this may explain the attenuation of low-grade inflammation in obese mice.

1. Introduction

Obesity is a serious health problem in developed countries, and the prevalence of obesity has increased dramatically for several decades. Both genetic and environmental factors are implicated in the development of obesity, in particular food overconsumption. Being severely overweight or obese is associated with major health risks such as cardiovascular disease, diabetes, nonalcoholic fatty liver disease, and cancer [1]. An important feature of obesity is its association with chronic low-grade inflammation. Adipose tissue is the largest endocrine organ in the body and is characterised by cytokine and chemokine production and acute-phase inflammatory signalling [2–4]. As an established in 3T3-L1 adipocyte cells

comprise main features relating to innate immunity [5]; this stage also is known to involve the toll-like receptors (TLRs) [6–9]. Stimulation of TLRs causes an immediate defensive response, including the production of an array of antimicrobial peptides and cytokines [10]; this response includes adaptor molecules, such as myeloid differentiation primary response gene 88 (MyD88) and the tumour necrosis factor receptor-associated factor 6 (TRAF6) [11].

The adipose tissue is involved in metabolic, physiological, and immunological regulation including the cytokines. Adipose tissue fat stores are mainly dependent upon fatty acid (FA) supply, FA esterification to triglycerides (TG), and TG breakdown, or lipolysis. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) both have the capacity to degrade TG by cleaving the ester bond, thus governing the lipolysis pathway in adipose tissue [12]. Adipose tissue lipolysis has received much attention over the past 10 years because of its altered regulation in obesity. Therefore, prevention and treatment of obesity should focus on anti-inflammatory effects, and various treatments have emerged, including phytoterapic therapy.

Green tea contains high levels of polyphenols, which may have a number of positive health effects in the prevention of lifestyle-related diseases [13]. Tea is one of the most popular beverages worldwide. Habitual consumption of green tea (*Camellia sinensis*), a popular beverage used in traditional Chinese medicine, has been associated with decreased risks for obesity [14], diabetes [15], hypertension [16], dyslipidemia [17], and CVD mortality [18] in several epidemiological studies. In selected clinical trials, green tea supplementation has been shown to significantly improve features of metabolic syndrome, such as decreased abdominal adiposity indicated by waist circumference in obese subjects [19].

Tea and tea components have been reported to possess various biological and pharmacological effects, such as antibacterial actions [20] and lowering plasma lipids and glucose levels [21, 22]. Green tea catechins are efficacious in cell and animal models of obesity, and the proposed modes of action include: decreased adipocyte differentiation and lipogenesis; increased beta-oxidation; and decreased lipid absorption [23]. However, relatively little is known about the underlying mechanism of action, in the regulation of body weight, lipolytic action and its relationship with inflammatory status. The aim of this study was to examine the effects of green tea extract on the body fat mass and lipolytic enzymes in adipose tissue of mice fed a high-fat diet and to observe whether reduction of fat mass is associated with diminished low-grade inflammation.

2. Experimental Methods

2.1. Animal, Diet, and Green Tea Supplementation. The Experimental Research Committee of the São Paulo Federal University approved (no. 1673/07) all procedures and the care of the animals used in this study. A total of 24 male Swiss mice ranging in age from 8 weeks were used. They were housed four per cage, receiving a chow diet and water *ad libitum*, in an animal room under a 12 h light-dark cycle, at 22 ± 1°C and 60 ± 5% humidity. After the acclimatisation period (1 week), the animals were randomly divided into four groups: (1) control mice (CW) fed on chow diet and placebo supplementation (0.1 mL water/day); (2) (CG) chow diet and green tea supplementation (0.1 mL water + 400 mg green tea extract per kg body weight/day); (3) (HW) a high-fat diet and placebo supplementation (0.1 mL water/day) for 2 months; (4) (HG) high-fat diet and green tea supplementation (0.1 mL water + 400 mg green tea commercial extract per kg body weight/day). The fatty acid composition of chow or high-fat diet diets is detailed in previous study from our group (Table 1) [24].

Table 1: Composition of standard chow and high-fat diet.

Ingredients	Standard chow		High-fat diet	
	g/kg^{-1}	Kcal/kg^{-1}	g/kg^{-1}	Kcal/kg^{-1}
Cornstarch (Q.S.P.)	398	1590	116	462
Casein	200	800	200	800
Sucrose	100	400	100	400
Dextrinated starch	132	528	132	528
Lard	—	—	312	2808
Soybean oil	70	630	40	360
Cellulose	50	—	50	—
Mineral mix	35	—	35	—
Vitamin mix	10	—	10	—
L-Cysteine	3	—	3	—
Choline	2.5	—	2.5	—
Total	1000	3948	1000	5358

2.2. Composition of Green Tea by Ultra-Performance Liquid Chromatography (UPLC). We evaluated composition of green tea commercial extract by Ultra-performance Liquid Chromatography- Mass Spectrometry. An Acquity UPLC system (Waters, Milford, MA, USA) consisting of a binary solvent manager and a sample manager was coupled to an Acquity TQD Mass Spectrometer (Micromass Waters, Milford, MA, USA). Analyses were performed on a bridged ethylene hybrid (BEH) C18 analytical column (50 mm × 2.1 mm, 1.7 μm, at a temperature of 25°C, injecting 5 μL of extract and standards. A gradient was applied at a flow rate of 0.2 mL min^{-1} using two mobile phases—(A) purified water with 0.1% formic acid; and (B) methanol—starting with 5% B, ramping to 100% B in 8 min, maintained until 8.50 min, returning to the initial conditions. Detection was carried out in the negative ion mode with an ESI source under the following conditions: capillary −3000 V, cone −30 volts, temperature 150°C; ranging between m/z 100–1000. Data acquisition was carried out by MassLynx software. Our data showed that, in Green tea extract, there were 15 μg/mg epigallocatechin, 95 μg/mg epigallocatechin gallate, 20.8 μg/mg epicatechin gallate, and 4.9 μg/mg gallocatechin gallate.

2.3. Biochemical Measurements. Eighteen hours after the last oral gavage of green tea extract and after a 12-hour fast, the animals were decapitated, blood was collected, and serum

samples were collected after allowing the blood to clot on ice. Serum was stored frozen at −80°C for analysis. Lab Test Kits were used to assess fasting total cholesterol, high-density lipoprotein (HDL-c), and triacylglycerol (TG) levels. The samples were analysed using an enzymatic method. LDL-c and VLDL-c were calculated according to the Friedewald equation ((LDL-c = total cholesterol-(HDL-c)-(TG/5)) and (VLDL = TG/5)) [25]. The Zen-Bio Kit was used to assess free fatty acid.

2.4. TNF-α, Adiponectin, and IL-10 Protein Level Determination by ELISA. Following decapitation, mesenteric adipose tissue was removed, dissected, homogenised, and centrifuged at 12,000 g for 40 min at 4°C; the supernatant was saved, and the protein concentration was determined using the BCA assay (Bio-Rad, Hercules, California) with bovine serum albumin (BSA) as a reference. Quantitative assessment of adiponectin, TNF-α, and IL-10 proteins was carried out by ELISA (DuoSet ELISA, R and D Systems, Minneapolis, MN) following the recommendations of the manufacturer. All samples were run as duplicates, and the mean value is reported.

2.5. Protein Analysis by Western Blotting. After euthanasia, the epididymal, retroperitoneal, and mesenteric adipose tissue was dissected and weighed. Mesenteric adipose tissue was homogenised in 1.0 mL solubilisation buffer at 4°C (1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulphonyl fluoride (PMSF), and 0.1 mg aprotinin/mL) with a Polytron (model 713T; Fisatom Equipamentos Científicos, São Paulo, SP, Brazil). Insoluble material was removed by centrifugation for 30 min at 9,000 g in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4°C. The protein concentration of the supernatants was measured by the BCA assay. Proteins were denatured by boiling (5 min) in Laemmli sample buffer [26] containing 100 mM DTT, run on 8, 10, or 12% SDS-PAGE gels in a Bio-Rad miniature slab gel apparatus. The electrotransfer of proteins from gels to nitrocellulose membranes was performed for ~1.30 h/4 gels at 15 V (constant) in a Bio-Rad semidry transfer apparatus. Nonspecific protein binding to the nitrocellulose was reduced by preincubation for 2 h at 22°C in blocking buffer (1% bovine serum albumine, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4°C with antibodies against TLR4, myeloid differentiation primary response gene (88) (MyD88), TNF receptor associated factor (TRAF6), hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), comparative gene identification-58 (CGI-58 or ABHD5), perilipin A, and alpha-tubulin obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted 1 : 1000 with blocking buffer supplemented with 1% BSA and then washed for 30 min in blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h at 22°C. For evaluation of protein loading, membranes were stripped and reblotted with an anti-alpha-tubulin antibody as appropriate. Specific bands were detected by chemiluminescence, and visualisation/capture was performed by exposure of the membranes to RX films. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software-Scion Corporation, Frederick, MD, USA).

2.6. Statistical Analysis. The statistical analysis was performed using the GraphPad Prism statistics software package version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The data are expressed as the means ± SEM. Implementation of the Kolmogorov-Smirnov test revealed that the results of experiments were distributed normally. The data were analysed using ANOVA two ways for comparison between four groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body Mass and Tissue Weight. The relative weight (tissue weight/total body weight) of epididymal adipose tissue was increased in group HW compared to the GW group and decreased in the HG group compared to the HW group. The mesenteric adipose tissue showed an increase in group HW compared to the GW group and decrease in the HG group compared to the HW group. In retroperitoneal adipose tissue, only the CG group decreased compared with the GW group. Liver and gastrocnemius tissues showed no significant difference between groups (Table 2).

3.2. Lipid Profile and Serum Adiponectin. Serum triglycerides and total cholesterol did not differ between any of the groups. The serum concentration of LDL in the HW group was increased compared to the GW group. The concentration of serum HDL in the HG group showed an increase compared to the HW group. The serum concentration of FFA did not differ between any of the four groups. The serum adiponectin in CG group increased compared to the GW group, and the HG group increased compared to the HW group (Table 3).

3.3. Cytokines in the Adipose Tissue. The cytokine concentration of adiponectin in the mesenteric adipose tissue in the CG group increased compared to the GW group, and the HG group increased compared to the HW group. The content of IL-10 showed a significant increase in group CG compared to the GW group. The TNF-α levels in the mesenteric adipose pad of the HW group showed a significant increase compared to the CW group. However, supplementation of green tea decreased this effect (HG versus HW groups; Table 4).

3.4. Quantification of Lipolytic Proteins. The LSH protein levels showed an increase in mice fed with chow diet supplemented with green tea (CG group) compared to no supplementation (CW group). No difference was observed between the chow and high-fat diet without supplementation. However, there were increased HSL protein levels in the HG group (green tea supplemented) compared to the HW group ($P < 0.05$; Figure 1(a)). The ATGL protein levels only showed an increase in the mice fed with chow diet and

Table 2: Body weight and absolute and relative tissue weight.

Parameters	CW	CG	HW	HG
Initial weight (g)	27.9 ± 0.9	27.7 ± 1.2	28.3 ± 1.5	25.9 ± 0.6
Final weight (g)	45.4 ± 3.2	33.5 ± 1.5*	40.8 ± 1.3	33.9 ± 1.4#
Delta weight (g)	17.5 ± 1.3	5.8 ± 1.1*	12.5 ± 0.9	8.0 ± 0.7#
Epididymal (g)	0.81 ± 0.07	0.62 ± 0.09	1.45 ± 0.43$	0.52 ± 0.08#
Epididymal (%)	1.78 ± 0.16	1.85 ± 0.17	2.89 ± 1.12	1.41 ± 0.19#
Retroperitoneal (g)	0.44 ± 0.07	0.19 ± 0.03*	0.54 ± 0.08	0.39 ± 0.08
Retroperitoneal (%)	1.05 ± 0.21	0.57 ± 0.11*	1.35 ± 0.20	1.14 ± 0.22
Mesenteric (g)	0.45 ± 0.07	0.33 ± 0.06	0.86 ± 0.28$	0.23 ± 0.04#
Mesenteric (%)	0.96 ± 0.12	1.05 ± 0.23	2.16 ± 0.72$	0.65 ± 0.11#
Liver (g)	1.77 ± 0.09	1.55 ± 0.05	1.79 ± 0.18	1.37 ± 0.06
Liver (%)	3.99 ± 0.33	4.73 ± 0.30	4.42 ± 0.49	4.05 ± 0.17
Gastrocnemius (g)	0.19 ± 0.01	0.17 ± 0.02	0.21 ± 0.01	0.18 ± 0.01
Gastrocnemius (%)	0.43 ± 0.04	0.53 ± 0.09	0.51 ± 0.03	0.54 ± 0.05

*$P < 0.05$ chow diet and green tea (CG) group versus chow diet and water (CW) group ($n = 12$). $$P < 0.05$ high-fat diet and water (HW) group versus CW group ($n = 12$). #$P < 0.05$ high-fat diet and green tea (HW) group versus HW group ($n = 12$). g: grams; %: percentage.

Table 3: Serum concentrations of triacylglycerol (TAG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), free fatty acids (FFA), and adiponectin in different experimental groups.

Parameters	CW	CG	HW	HG
TAG (mmol/L)	1.58 ± 0.04	1.57 ± 0.03	1.60 ± 0.04	1.46 ± 0.05
CT (mmol/L)	3.12 ± 0.08	3.29 ± 0.13	3.58 ± 0.17	3.54 ± 0.06
HDL (mmol/L)	1.54 ± 0.10	1.85 ± 0.16	1.81 ± 0.10	2.31 ± 0.13#
LDL (mmol/L)	0.83 ± 0.04	0.71 ± 0.06	1.11 ± 0.09$	0.87 ± 0.08
FFA (μM)	1.21 ± 0.08	1.26 ± 0.08	1.02 ± 0.11	1.16 ± 0.13
Adiponectin (ng/mL)	82.25 ± 1.76	106.49 ± 2.91*	85.82 ± 2.53	112.02 ± 7.64#

*$P < 0.05$ chow diet and green tea (CG) group versus chow diet and water (CW) group ($n = 12$). $$P < 0.05$ high-fat diet and water (HW) group versus CW group ($n = 12$). #$P < 0.05$ high-fat diet and green tea (HW) group versus HW group ($n = 12$).

supplemented with green tea (Figure 1(b)). In the HW group, the ABHD5 (or CGI-58) protein levels were reduced when compared to the GW group. However, supplementation with green tea strikingly increased the ABHD5 protein levels in obese mice (HG group) when compared to the HW group. No significant difference was observed in the chow-diet groups (Figure 1(c)). The perilipin protein levels increased in mice fed with chow diet supplemented with green tea (CG group) compared to no supplementation (CW group). No difference was observed between the chow and high-fat diets without supplementation. However, green tea increased perilipin protein levels in the HG group compared to the HW group (Figure 1(d)).

3.5. Quantification of Inflammatory Proteins. The TLR4 protein levels in diet-induced obese mice (HW group) were significantly greater than chow-diet mice (CW group). Green tea treatment decreased this effect significantly (HG versus HW groups, $P < 0.05$; Figure 2(a)). The MyD88 protein levels in the HW group increased compared to the CW group. However, when obese mice were supplemented with green tea (HG group), this effect was attenuated (Figure 2(b)). The TRAF6 protein levels in diet-induced obese mice (HW group) were significantly greater than chow-diet mice (CW group). Green tea treatment significantly decreased this effect (HG versus HW groups, $P < 0.05$; Figure 2(c)).

4. Discussion

Numerous studies have been conducted to increase our understanding of the cause and treatment of obesity. In this sense, an alternative strategy is necessary such as phytotherapy treatment. Chronic systemic inflammation directly contributes to the development of obesity [27]. For instance, overweight and obese women generally have elevated serum levels of inflammatory cytokines, such as TNF-α [28, 29]. Therefore, suppressing chronic inflammation may be a good strategy to prevent and/or treat obesity. Interestingly, previous studies suggest the positive impacts of green tea polyphenols could be via its ability to suppress chronic inflammation [30, 31]. In addition, the impacts of green tea consumption on weight loss have been reported in clinical [32–36] and laboratory studies [37]. Antiobesity effects of green tea are probably due to its capacity to elevate thermogenesis and fat oxidation [38, 39]. Thus, we hypothesise that green tea supplementation reduces body-fat mass by regulating lipolytic pathway-related genes; such changes will result in downregulation of cytokine production and proinflammatory molecule protein levels.

Table 4: Content cytokines in mesenteric adipose tissue.

Adipokines	CW	CG	HW	HG
Adiponectin (pg/μg of protein)	0.24 ± 0.03	0.37 ± 0.02*	0.18 ± 0.01	0.40 ± 0.04#
IL-10 (pg/μg of protein)	1.91 ± 0.26	11.27 ± 1.33*	3.47 ± 0.50	5.34 ± 0.47
TNF-α (pg/μg of protein)	2.09 ± 0.79	1.92 ± 0.61	5.80 ± 0.47$	2.62 ± 0.61#

*$P < 0.05$ chow diet and green tea (CG) group versus chow diet and water (CW) group ($n = 12$). $$P < 0.05$ high-fat diet and water (HW) group versus CW group ($n = 12$). #$P < 0.05$ high-fat diet and green tea (HW) group versus HW group ($n = 12$).

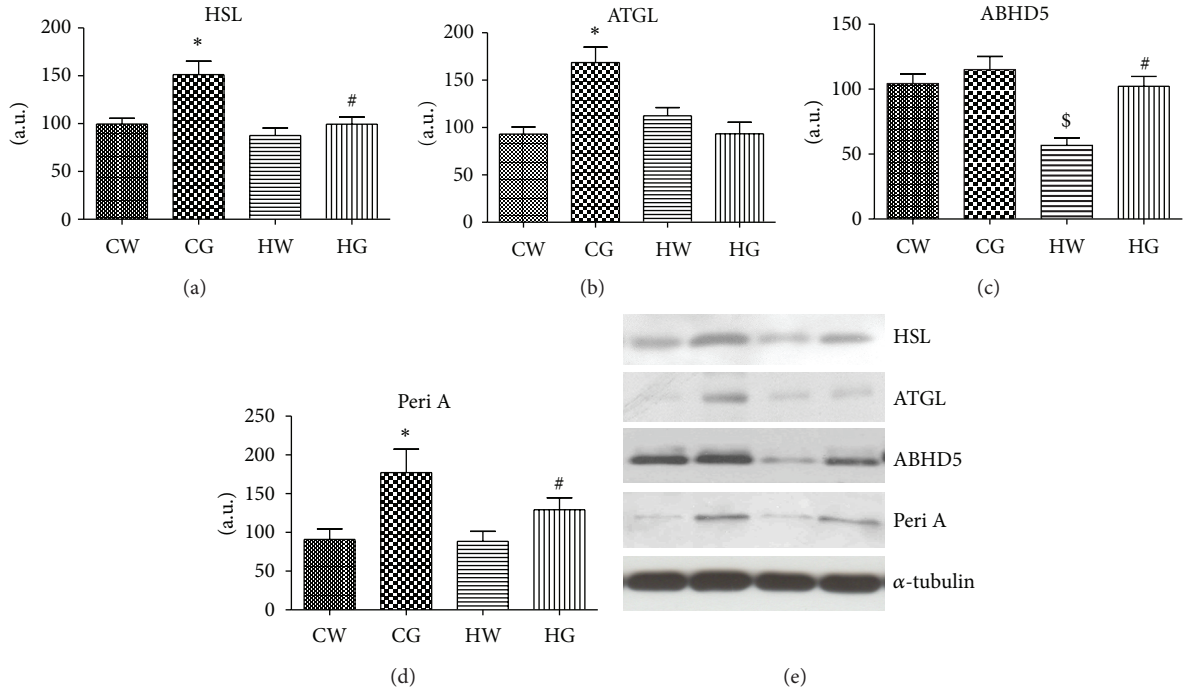

Figure 1: Protein levels of HSL, ATGL, ABHD5, and perilipin A. Mesenteric adipose tissue extracts were immunoblotted with anti-HSL (a), anti-ATGL (b), anti-ABHD5 (c), and Peri A (d). The results of scanning densitometry are expressed as arbitrary units. Bars represent means ± SEM of $n = 6$ mice, *$P < 0.05$ chow and green tea (CG) group versus chow and water (CW) group, $$P < 0.05$ high-fat and water (HW) group versus CW group, and #$P < 0.05$ high-fat and green tea (HG) group versus HW group. In (e), the representative bands of the molecules are shown. The membrane was stripped and immunoblotted with anti-α-tubulin antibody and used as loaded protein (lower panel in (e)).

In the study, we measured body weight of the animals at the beginning and end of the study. Our results demonstrate that a high-fat diet induced body-weight gain (as observed by delta weight) and epididymal and mesenteric adipose tissue pads. However, green tea promoted a reduced delta weight and adipose tissue pads. Further, green tea extract led to increased lipolytic pathway protein levels, adiponectin, and anti-inflammatory cytokine IL-10 and reduced proinflammatory cytokine TNF-α.

The therapeutic uses of tea are confined to alternative medicine. Although the anticarcinogenic, anti-inflammatory, and antimicrobial properties of tea have been known for many years, clinical medicine has not included its use in treatments, almost certainly due to the lack of knowledge about its exact mechanisms of action [21, 22]. In human experiments, acute ingestion of green tea extract, which is mainly composed of catechins, has been reported to increase the proportion of whole-body fat utilisation by augmenting oxidation and lipolysis [23, 38, 39]. Lee et al. [40] demonstrated in an in vitro study that EGCG modulates the increase in lipolysis by directly increasing the gene expression of HSL, demonstrating its important role in lipid metabolism. Habitual consumption green tea extract has been reported to reduce body weight and body fat [32–36]; this may occur via increased lipolysis in adipose tissue, and our data support this.

The anti-inflammatory effect of green tea has been attributed to the polyphenol content [30, 31]. In Asian countries, green tea, which contains a class of polyphenols known as tea catechins, has been habitually consumed as one of the most popular beverages. Tea polyphenols have been shown to inhibit proteasome function, thereby terminating inflammation. Although tea polyphenols have been claimed to be the most potent constituents of tea, there is increasing evidence that these compounds are not the only constituents responsible for the beneficial effects on health from tea [41].

Our results demonstrate that green tea is able to decrease the protein content of TNF-α in adipose tissues and stimulate

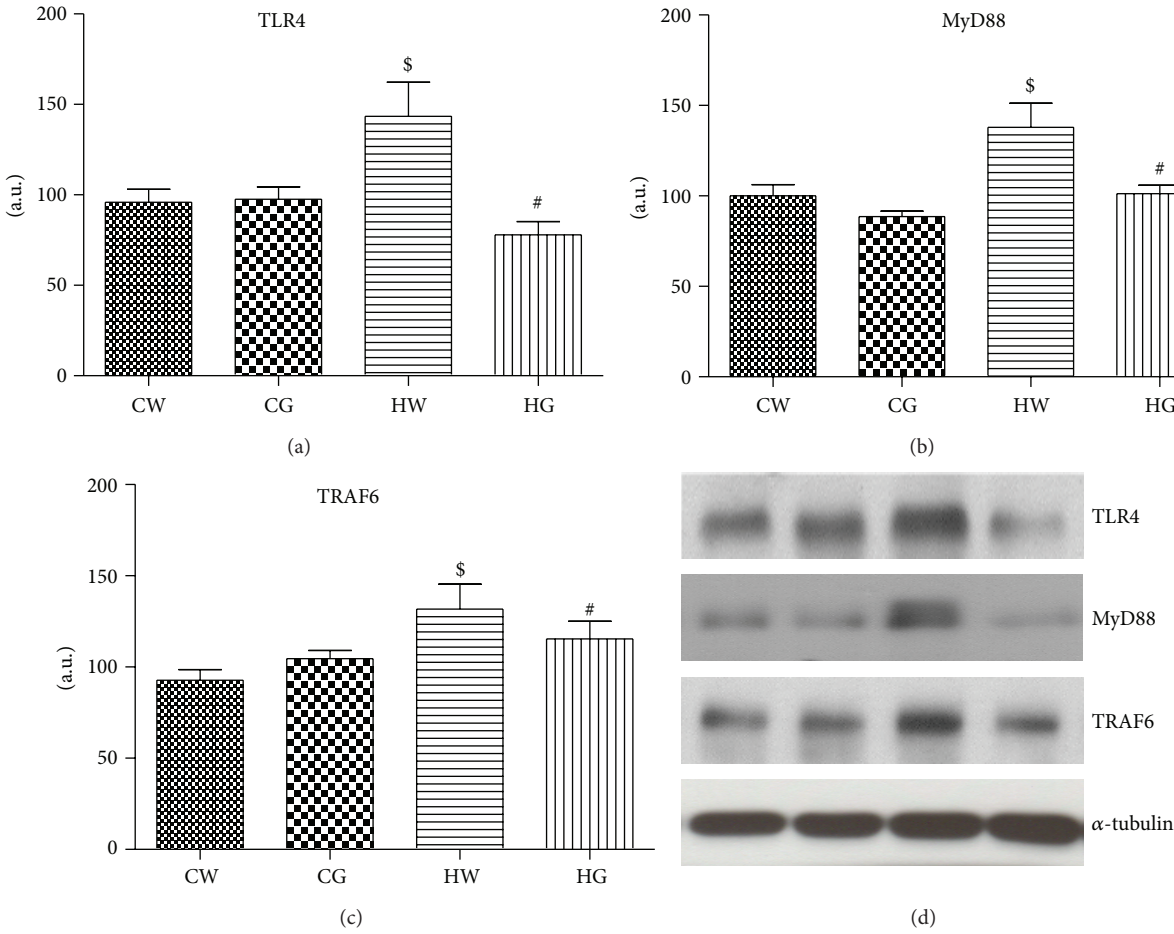

Figure 2: Protein levels of TLR4, MyD88, and TRAF6. Mesenteric adipose tissue extracts were immunoblotted with anti-TLR4 (a), anti-MyD88 (b), and anti-TRAF6 (c). The results of scanning densitometry are expressed as arbitrary units. Bars represent means ± SEM of $n = 6$ mice, $^{\#}P < 0.05$ when compared to the high-fat diet and green tea (HG) group versus high-fat diet and water (HW) group. In (d), the representative bands of the molecules are shown. The membrane was stripped and immunoblotted with anti-α-tubulin antibody and used as loaded protein (lower panel in (d)).

lipolytic enzymes. These conditions may favour reduced body weight and adipose tissues. In addition, we found that green tea reduced TLR4 expression, blocking proinflammatory effects. Youn et al. [42] showed that EGCG in cultured cells of the immune system had an anti-inflammatory effect, which was partly explained by the inhibition of the TLR. In summary, our results show that green tea extract intake increases expression of lipases, reduces adipose fat mass, and in parallel reduces inflammatory molecules and cytokines. Futures studies are needed to better understand the mechanism involved in the beneficial effects promoted by green tea extract intake, especially in mice fed a high-fat diet.

Conflict of Interests

All authors declare no conflict of interests.

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Clinical Study

Different Activation of TRAF4 and TRAF6 in Inflammatory Bowel Disease

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In recent years, interests combining the exploration of tumor necrosis factor receptor-associated factor 4 (TRAF4) and TRAF6 in immune cells and transgenic mice are emerging. Although it has been found that TRAF4 and TRAF6 share the same TRAF binding sites, comprehensive study of TRAF4 and TRAF6 in inflammatory bowel disease (IBD) is still lacking. This paper shows similar and different expression patterns of TRAF4 and TRAF6 in patients with IBD. The results indicate that TRAF4 and TRAF6 are overexpressed in IBD. TRAF4 and TRAF6 play different roles in the pathogenesis of IBD. Moreover, TRAF4 may be an indicator of endoscopic disease activity of UC and TRAF6 preactivation can be detected in noninflamed colonic segments.

1. Introduction

Tumor necrosis factor receptor-associated factors (TRAFs) act as adapter molecules controlling signaling pathways, such as nuclear factor kappa B (NF-κB), interleukin-1 receptor (IL-1R), toll-like receptor (TLR), and transforming growth factor-β (TGF-β) [1, 2]. For a long time, TRAF6 has shown conserved function in activation of autoimmunity and inflammation. It is important that TRAF6 contributes to the CD40-mediated activation of NF-κB and c-Jun kinase (JNK). Association of TRAF6 with CD40 is essential for CD40-mediated IL-6 expression [3], which could explain the requirement for membrane-bound CD40 ligand to induce IL-6 production by immunocytes [4]. The survival, regulation, and activation of immunocyte and epithelial cell, signaling through cell surface receptors to activate NF-κB and mitogen-activated protein kinases (MAPKs) through TRAF6, are critical regulations of immune response [5]. Unlike TRAF6, the molecular mechanism of TRAF4 in multiple signaling pathways triggered by TNFR-related proteins remains enigmatic. Moreover, the subcellular localization and functions of TRAF4 have been controversial for years. It has been indicated that TRAF4 augments NF-κB activation through glucocorticoid-induced TNFR (GITR) expression on T cells, B cells, and macrophages [6]. As a unique TRAF family member mediating signal transduction by TNF, IL-1R, or TLR, it is found that TRAF4 acts as a positive effector of bone morphogenetic protein (BMP) and the TGF-β signaling pathway [7].

The intestinal epithelium and immune cells in the gut establish active sites of immune reactivity. Breakdown of homeostasis between intestinal microbiota and the mucosal immune system, together with both environmental and genetic factors, leads to inflammatory bowel disease (IBD). NF-κB and TLR are considered as nodal points in the suppression and/or recruitment of immune responses in IBD [8]. Interestingly, although mechanisms of TRAFs in IBD are not yet fully studied, TRAF-related inflammatory mediators as TGF-β or CD40 play critical roles in a wide array of cellular functions in IBD [9, 10].

Recent studies in immune cells and transgenic mice regarding the role of TRAF4 and TRAF6 have revealed that they share the same binding sites, yet comprehensive study of TRAF4 and TRAF6 in IBD is still lacking [11]. Based on the hypothesis that TRAF4 and TRAF6 may be activated prior to the clinical or endoscopic activation in patients with IBD, we sought to measure TRAF4 and TRAF6 expressions to explore their potential roles in IBD patients.

2. Materials and Methods

2.1. Patients and Samples. Patients were enrolled according to clinical and endoscopic diagnosis. Patients with pregnancy, colorectal resection for UC, disease involving only small bowel, poor bowel preparation as visible area of intestinal mucosa <90%, or use of steroids, immunosuppressants, or infliximab before colonoscopic sampling were excluded. No diagnosis altered after at least 3 months of followup. Endoscopic score was evaluated using simplified endoscopic score in Crohn's disease (SES-CD) or Baron score for patients with CD or UC, respectively. Healthy controls were included without sign or symptoms of bowel disease.

Human intestinal biopsies and blood samples were collected at Division of Gastroenterology and Hepatology, Shanghai Jiao-Tong University School of Renji Hospital Medicine, in accordance, with guidelines of the Research Ethics Committee of Renji Hospital, Shanghai Jiao Tong University, School of Medicine. All patients and healthy controls agreed to provide written consents.

Human tissue specimens were taken from both macroscopically inflamed and non-inflamed regions of the colon. Biopsies from the colon of healthy donors were also analyzed. Tissue specimens were put into liquid nitrogen within 10 minutes after biopsy for protein extraction or kept in RNAlater (Qiagen) for RNA isolation. Human peripheral blood was separated into plasma and peripheral blood mononuclear cells (PBMCs). Plasma was obtained using commercially EDTA-treated tubes (Gongdong Medical Technology Co., Ltd.) and PBMCs were isolated according to Lymphoprep (Axis-Shield PoC AS, Norway) protocol. Briefly, diluted blood was overlayed over 3 mL Lymphoprep and centrifuged at 800 ×g for 20 mins. PBMCs were removed from a distinctive band at the sample interface after centrifugation. Then, PBMCs were kept in RNAlater (Qiagen) for RNA isolation according to manufacturer's protocol.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). Plasma was obtained following centrifugation of whole blood for 15 minutes at 2,000 ×g. Samples were stored at −80°C prior to analysis via Elisa. Samples were analyzed using kits against TRAF4 and TRAF6, according to the manufacturer's specifications (Lanji Biochemical and Diagnostics, Shanghai, China) and a microtiter plate reader was used to read absorbance at 450 nm. Experiments were performed in triplicate.

2.3. RNA Isolation, cDNA Synthesis, and Real-Time PCR. Total RNA in PBMCs and tissue samples were isolated with TRIzol Reagent (Ambion) according to the manufacturer's protocol for cells and tissue. The quantity and quality of RNA were detected using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). Primers were designed with Primer 5.0 (ABI) software and consequently synthesized by Sango Biotech (Shanghai) Co., Ltd. The primer set for TRAF4 was 5′-AGGAGTTCGTCTTTGACACC-ATC-3′ (forward) and 5′-CTTTGAATGGGCAGAGC-ACC-3′ (reverse), with a product of 162 bps. The primer set for TRAF6 was 5′-CCTTTGGCAAATGTCATCTGTG-3′ (forward) and 5′-CTCTGCATCTTTTCATGGCAAC-3′ (reverse), with a product of 140 bps. The primer set of GAPDH was 5′-GTGAAGGTCGGAGTCAACGG-3′ (forward) and 5′-CCTGGAAGATGGTGATGGGAT-3′ (reverse), which provided a product of 226 bps.

cDNAs were produced with PrimeScriptTM RT reagent Kit (Takara Biotechnology Dalian Co., Ltd.). Briefly, reverse transcripts were incubated at 37°C for 15 minutes and 85°C for 5 seconds. SYBR Premix Ex Taq kit was purchased from TakaRa and real-time PCR reactions were done using a StepOne Plus device (Applied Biosystems) at 95°C for 10 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds according to instruction of the SYBR Premix Ex Taq kit. The expression levels of the target genes were normalized to GAPDH with 2-ΔΔCt method [12].

2.4. Western Blot Analysis. For Western blot analysis, PMBCs and tissue samples were lysed in RIPA buffer (Sigma) containing protease inhibitors (Roche) and agitated on ice for 30 minutes. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Pierce, Wohlen, Switzerland). Protein electrophoresis was performed according to the protocol of Mini-PROTEA III (Bio-Rad). Briefly, proteins were separated in 10% polyacrylamide gels (Tris/glycine) and transferred onto polyvinylidene fluoride membrane (Millipore). Membranes were sequentially labeled by primary and secondary antibodies. Western blots were probed with antibodies against TRAF4 (rabbit polyclonal anti-TRAF4; 1 : 1000, Santa Cruz), TRAF6 (mouse monoclonal anti-TRAF6, 1 : 1000, Santa Cruz), and β-actin (mouse monoclonal anti-β-actin; 1 : 2500, Santa Cruz). Secondary antibody was purchased from GE Healthcare life Science. Detection was enhanced by SuperSignal West Pico Chemiluminescent Substrate (Pierce). Experiments were performed in triplicate.

2.5. Statistical Analysis. Statistical significance was determined using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). A $P < 0.05$ was considered significant with either ANOVA analysis or Tukey's multicomparison. A two-sided Fisher's exact test or χ^2 test was performed to analyze discrete variables.

3. Results

3.1. Characteristics of Included Subjects. From February 2007 to February 2010, 40 CD patients, 42 UC patients, and 40 healthy controls were included in our present study. Patients with IBD indicated significantly lower body mass index (BMI) than healthy controls ($P < 0.0001$). The UC group contained significantly more smokers than CD patients (22 : 8, $P = 0.0023$). Three patients with ileitis, 15 patients with ileocolitis, and 22 patients with colitis were enrolled in CD group. Thirteen patients with proctosigmoiditis, 20 patients with left sided colitis, and 9 patients with pancolitis were enrolled in UC group. Characteristics of included subjects were described in Table 1.

Table 1: Characteristics of included subjects.

	CD ($n = 40$)	UC ($n = 42$)	HC (40)
Gender (female/male)	21/19	20/22	20/20
Age (years)	33.58 (28.87–38.28)	41.64 (37.18–46.10)	35.48 (30.06–40.89)
BMI (kg/m^2)	19.29 (18.81–19.76)***	20.00 (19.41–20.59)***	22.84 (22.10–23.58)
Smoking (yes/no)	8/32	22/20**	3/37
Extent			
Ileitis	3		
Ileocolitis	15		
Colitis	22		
Proctosigmoiditis		13	
Left-sided colitis		20	
Pancolitis		9	
Therapy			
5-ASA/SASP	36	42	
Glucosteroids	22	16	
AZA	8	6	
Infliximab	6	1	
Surgery	1	1	
Endoscopic score†	3.200 (1.650–6.000)	2.000 (1.000–3.000)	

CD: Crohn's disease; UC: ulcerative colitis; HC: healthy controls; BMI: body mass index; 5-ASA: 5-aminosalicylic acid; SASP: sulfasalazine; AZA: azathioprine; $^{***}P < 0.0001$, significance is the difference from healthy controls; $^{**}P < 0.01$, significance is the difference from patients with Crohn's disease; †values are medians and 25%–75% percentile, simplified endoscopic score in Crohn's disease (SES-CD) is used to validate endoscopic severity in Crohn's disease, and Baron score is used to validate endoscopic severity in ulcerative colitis, respectively.

3.2. TRAF4 and TRAF6 Expressions in Plasma. To investigate the diagnostic value of TRAF4 and TRAF6 in IBD, we detected levels of soluble TRAF4 and TRAF6 in plasma of IBD patients. We found that TRAF4 and TRAF6 were significantly higher both in patients with CD and UC than in healthy controls (Figures 1(a) and 1(b)). However, only overexpression of soluble TRAF4 showed a significantly positive correlation with endoscopic disease activity index (Baron score) in UC patients (spearman's $r = 0.458$, $P = 0.002$).

Furthermore, we observed that TRAF4 showed a significantly diagnostic value in differentiating active IBD patients from healthy controls ($P < 0.0001$, Figures 2(a) and 2(b)). Although TRAF6 also showed a significantly diagnostic value in differentiating active CD, UC from healthy controls, the lower area under the curve (AUC) predicted a less diagnostic value than TRAF4 (Figures 2(c) and 2(d)).

3.3. TRAF4 and TRAF6 Gene Expressions in Peripheral Blood Mononuclear Cells. To identify gene expressions of TRAF4 and TRAF6 in PBMCs in patients with CD and UC, we isolated RNA from PBMCs. Similar to their expression in plasma, TRAF4 and TRAF6 showed significantly higher levels both in patients with CD and UC than in healthy controls (Figures 1(c) and 1(d)) (all $P < 0.0001$).

3.4. Different Upregulation and Preactivation of TRAF4 and TRAF6 Expressions in Colonic Tissues. Given that segmental changes can exhibit inflammation in the colon in IBD patients and that intestinal segments in endoscopic remission can appear as histologic colitis, we determined the expressions of TRAF4 and TRAF6 both in inflamed and non-inflamed intestinal mucosae. Unfortunately, 3 patients with only ileitis and nine patients with pancolitis were excluded based on exclusion criteria. 5 CD patients and three UC patients were also excluded for refusal to biopsy.

Quantitative real-time PCR (qRT-PCR) was used to determine the gene expressions of TRAF4 and TRAF6 in inflamed and non-inflamed intestinal mucosae of IBD. It was found that TRAF4 and TRAF6 expressions were significantly higher in inflamed intestinal mucosa of patients compared to normal mucosa of healthy controls (all $P < 0.0001$) (Figures 3(a) and 3(b)). Interestingly, TRAF6 expressions were also significantly higher in non-inflamed tissue of IBD patients than in healthy controls, which may indicate potential preactivation of TRAF6 in IBD (Figure 3(b)).

Western blotting was used to measure protein expressions of TRAF4 and TRAF6 in inflamed and non-inflamed intestinal mucosae of IBD (Figure 4). Our data indicate that only TRAF6 expressions were significantly higher in non-inflamed tissue of IBD patients than in healthy controls, although TRAF4 and TRAF6 protein expressions were significantly higher in inflamed intestinal mucosa of patients than in normal mucosa of healthy controls (all $P < 0.0001$) (Figures 3(c) and 3(d)). Similar to their gene expressions, TRAF4 and TRAF6 protein expressions were significantly increased in inflamed intestinal mucosa compare to the non-inflamed mucosa or healthy controls. Also, TRAF6 protein expression was significantly higher in inflamed intestinal mucosa of IBD patients compared to healthy controls.

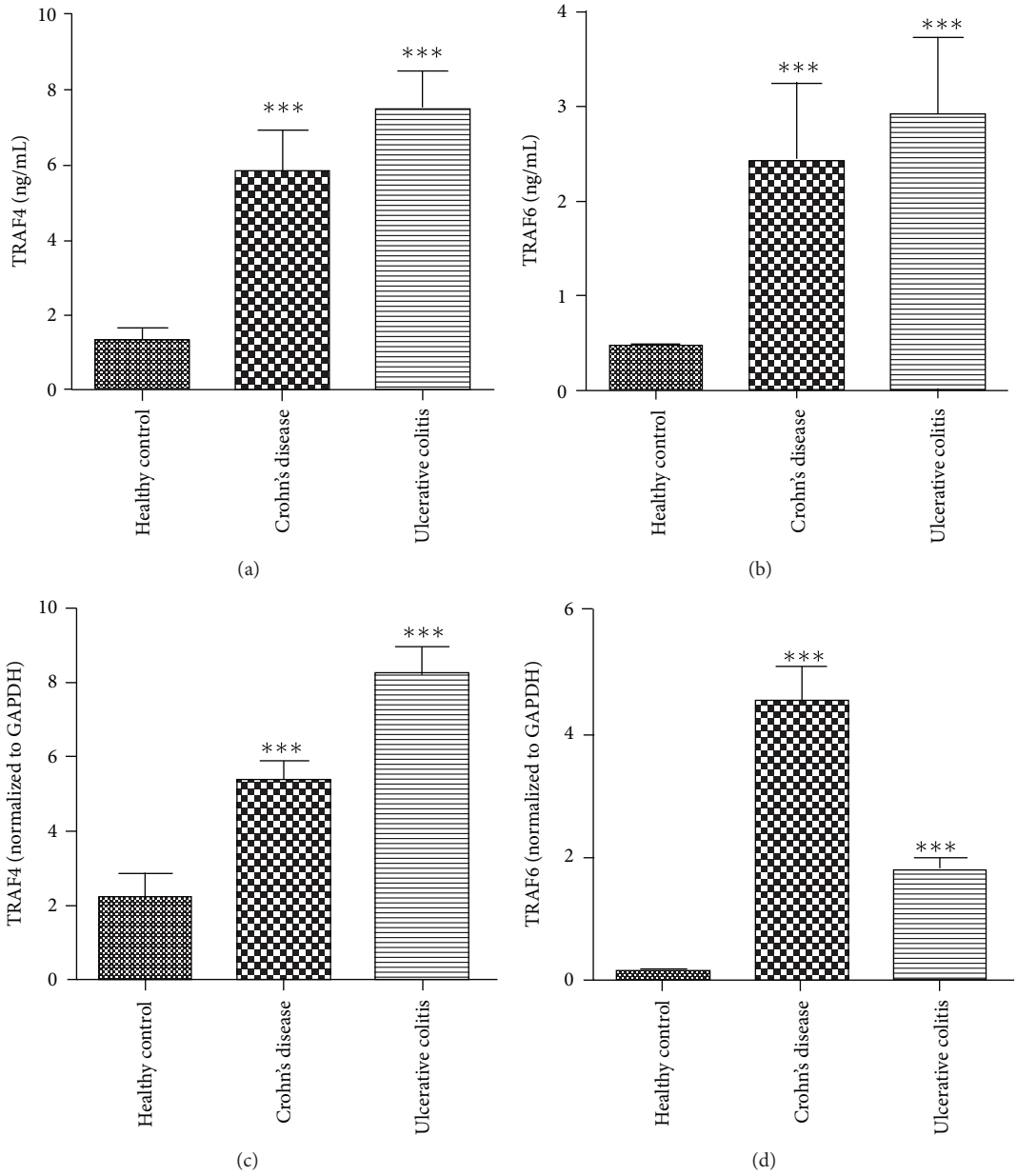

Figure 1: Soluble TRAF4 (a) and TRAF6 (b) protein levels in plasma; TRAF4 (c) and TRAF6 (d) gene expression in peripheral blood mononuclear cells of patients with Crohn's disease and ulcerative colitis. $^{***}P < 0.0001$.

4. Discussion

In the current study, we demonstrated that two members of the TRAF family, TRAF4 and TRAF6, were activated in patients with IBD. Although both TRAF4 and TRAF6 showed potentially diagnostic value in differentiating active CD and UC from healthy controls, only TRAF6 could be preactivated in non-inflamed tissue of IBD patients. Although TRAFs have similar overall structural features including a C-terminal receptor-binding domain and a leucine-zipper domain, their own structural difference leads to distinctive interaction with receptors [13, 14]. Growing evidence indicates that TRAFs are regulated not only by their own structural features but also by the nature of their interactions, recruitment, or localization.

The understanding of TRAF functions increased much more rapidly for TRAF6 than for TRAF4. Early overexpression studies clearly indicated that TRAF6 contributes to the CD40-mediated activation of NF-κB and other signaling molecules [15]. NF-κB activity has been upregulated in lamina propria immune cells and in epithelia of the inflamed gut in IBD [16]. The NF-κB pathway regulates inflammation, regulatory T-cell production, and DC function. However, activation of important transcriptional regulators including NF-κB and the stress-activated protein kinases (SAPKs) mediated by TRAF6 requires binding to CD40. CD40

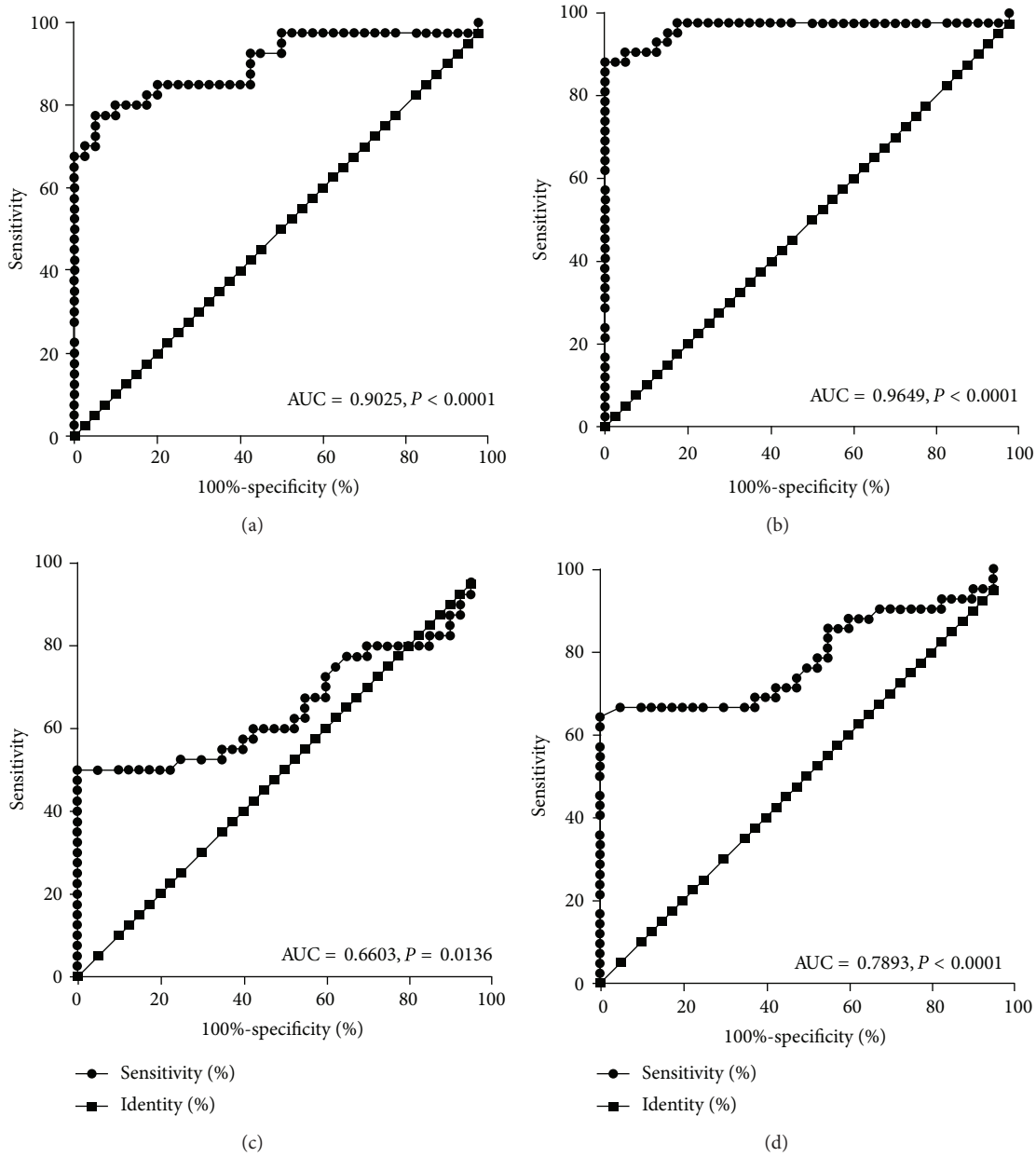

Figure 2: Receiver operating characteristic (ROC) curves indicate diagnostic value in differentiating active CD, UC from healthy controls. TRAF4 showed a significantly diagnostic value in differentiating active CD patients from healthy controls (a) and active UC from healthy controls (b). TRAF6 also showed a significantly diagnostic value in differentiating active CD (c) and UC (d) from healthy controls. However, the lower area under the curve (AUC) predicted a less diagnostic value than TRAF4 (c) and (d). Abbreviations: AUC: area under the curve.

mutants and transgenic mice have been established to address the roles of TRAF6. The cytoplasmic CD40 binding domain for TRAF6 is necessary for the CD40-mediated activation of IL-6 production in monocytes and macrophages [17], and the proinflammatory IL6 biologic network is upregulated in active IBD [18]. Moreover, experiments with immune cells or fibroblasts isolated from TRAF6-deficient mice indicate that TRAF6 is required for the CD40-mediated activation of not only NF-κB but also JNK and p38 MAPK signals [19, 20]. Although CD40-mediated JNK activation in B cells seems to require cytoplasmic TRAF6, TRAF6 mutants defective in CD40 binding were able to activate the JNK pathway and upregulate CD80, indicating that TRAF6 may be able to contribute to certain JNK signals without the binding of CD40 [21]. JNK and MAPK signaling pathways are involved in governing lymphocyte influx into the gut in IBD patients by regulating lymphocyte adhesion and transmigration [22]. Furthermore, only one study screened potential alterations of the TRAF6 gene in a large number of CD and UC patients but failed to identify apparent disease-causing mutations [23].

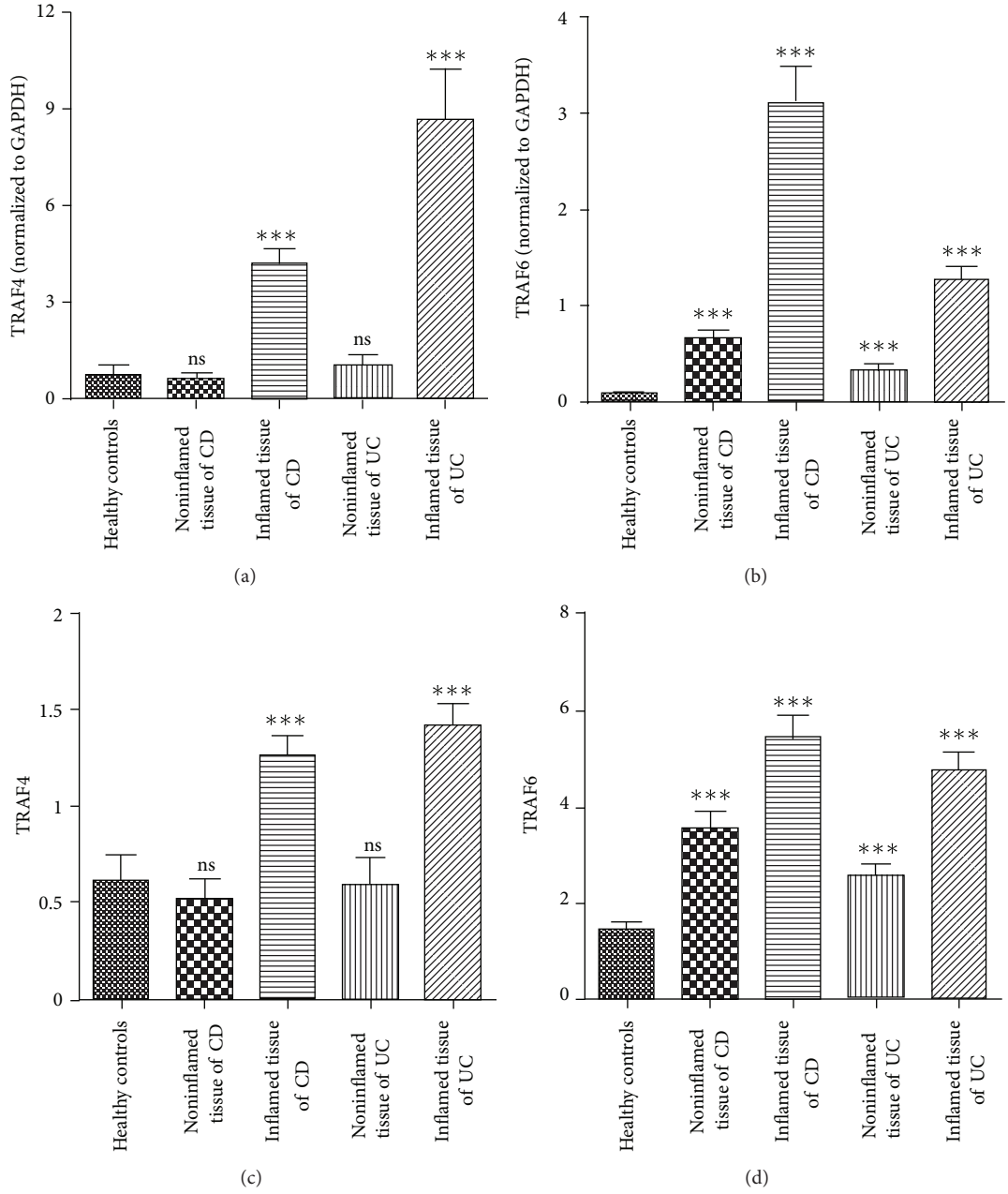

Figure 3: Differences in gene and protein expressions of TRAF4 and TRAF6 in the inflamed and non-inflamed colonic mucosa in patients with Crohn's disease or ulcerative colitis and in normal control tissues. (a) TRAF4 gene expression; (b) TRAF6 gene expression; (c) TRAF4 protein expression, (d) TRAF6 protein expression. ns: not significant $^{***}P < 0.0001$.

Their data suggested that TRAF6 may have essential roles in human biology that it might not tolerate any significant structural alterations.

TRAF4 possesses several unique characteristics that are different from other members of TRAFs. The subcellular localization and molecular mechanisms of action of TRAF4 are controversial. Few studies have implicated TRAF4 in IBD or colitis. Dendritic cells from TRAF4-deficient mice exhibited reduced migration both *in vitro* and *in vivo* experiments [24]. This result indicates that TRAF4 could participate in immune functions by facilitating immune cell migration. Interestingly, TRAF4 increases NF-κB activation through the GITR via a TRAF-binding site located in the cytoplasmic domain of GITR. This domain is responsible for the inhibition of Treg cells and the promotion of T-cell activation. Although no NF-κB activity has ever been detected via TRAF4 overexpression, TRAF4 has been implicated as an upstream molecule that regulates the JNK pathway via interaction and activation of Misshapen (Msn), a member of the SPS1 protein kinase family [25]. Moreover, TRAF4 positively regulates transforming growth factor (TGF)-β via potentiating bone morphogenetic protein (BMP) and Nodal

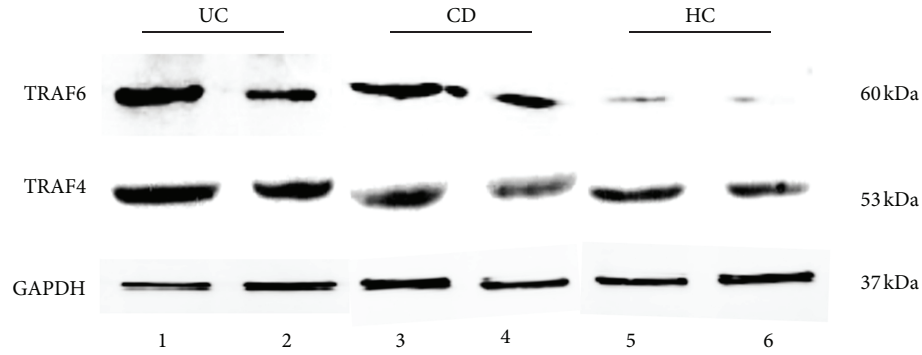

Figure 4: Western blot analyses of TRAF4 and TRAF6 protein expressions in colon. Abbreviations: CD: Crohn's disease; UC: ulcerative colitis; HC: healthy controls; 1, 3: inflamed colonic mucosa; 2, 4: non-inflamed intestinal mucosa under endoscopy; 5, 6: normal colonic mucosa in healthy controls under endoscopy.

signaling [26]. TGF-β plays a role in the pathogenesis of IBD by activating its specific receptors [27]. All TRAFs except TRAF4 have been identified to directly or indirectly act with CD40. Accordingly, TRAF4 is upregulated in B cells following CD40 signaling, which suggests that TRAF4 affected downstream of CD40 pathway [28]. This may explain our result that TRAF4 expressions were not significantly different in non-inflamed tissue of IBD patients compared to healthy controls.

Our understanding of TRAF4 and TRAF6 functions in the present study indicates that overlapping and unique functions will ultimately be attributed to each of the TRAFs. To our knowledge, this paper may be the first one to combine analyzing TRAF4 and TRAF6 in IBD patients. However, there are still some limits to the present study. First, the present study did not include histological criteria to distinguish non-inflamed tissue from inflamed tissue, which may confuse the microscopically inflamed tissues with non-inflamed tissues. Second, the present study could not include laboratory data as ESR or CRP to assess activity of disease, which may lead to the potential bias of overreliance on clinical manifestations and endoscopic assessment. Collectively, our data showed similar and different expression patterns of TRAF4 and TRAF6 in patients with IBD. To date, correlations between TRAF4 and TRAF6 have not been explored to elucidate signaling cascades. Future studies to determine multifaceted roles may offer targets in the treatment of IBD.

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Research Article

Time Course of Muscle Damage and Inflammatory Responses to Resistance Training with Eccentric Overload in Trained Individuals

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The purpose of this study was to observe the time course of muscle damage and inflammatory responses following an eccentric overload resistance-training (EO) program. 3 females (23.8 ± 2.6 years; 70.9 ± 12.7 kg; 1.6 ± 0.08 m) and 5 males (23.8 ± 2.6 years; 75.1 ± 11.2 kg; 1.8 ± 0.1 m) underwent thirteen training sessions (4 × 8–10 eccentric-only repetitions—80% of eccentric 1RM, one-minute rest, 2x week^{-1}, during 7 weeks, for three exercises). Blood samples were collected prior to (Pre) and after two (P2), seven (P7), nine (P9), eleven (P11), and thirteen (P13) sessions, always 96 hours after last session. The reference change values (RCV) analysis was employed for comparing the responses, and the percentual differences between the serial results were calculated for each subject and compared with $RCV_{95\%}$. Four subjects presented significant changes for creatine kinase at P2, and another two at P13; six for C-reactive protein at P2, and three at P11; two for neutrophils at P2, P4, and P13, respectively; and only one for white blood cells at P2, P4, P7, and P9, for lymphocyte at P7, P9, and P13, and for platelet at P4. We conclude that EO induced high magnitude of muscle damage and inflammatory responses in the initial phase of the program with subsequent attenuation.

1. Introduction

Resistance-training protocols with eccentric overload (EO) have been investigated about their efficiency on strength and muscle cross-sectional area increases [1–4]. Experimental observations reported suggest that this training variation might be superior for both strength and hypertrophy development when compared to conventional concentric/eccentric training protocols [1–3]. EO is imposed once the maximal voluntary force is greater in this kind of muscle action [5, 6], thus promoting a relatively lower workload for the eccentric phase during the conventional programs.

Meanwhile, the high-intensity unaccustomed eccentric component is characterized by inducing greater muscle damage incidence with significant acute decreases in muscle function [7], acute-phase inflammatory responses [8–12], and increases in plasmatic activity of myofibrillar proteins such as creatine kinase, lactate dehydrogenase, and myoglobin [12–15]. Instead, there have also been reports that a bout of eccentric exercise at several weeks interval results in a marked reduction in the symptoms associated with muscle damage [9]. This adaptation of eccentric exercise-inducing protection against subsequent tissue damages has been referred as repeated bout effect [16].

Observing the data on the initial drop in performance induced by the eccentric activity [7, 15, 17], recent researches have aimed at a very important aspect of the physical training process: the monitoring of biochemical and immunological markers and also the performance [18–20]. High training loads with insufficient recovery periods have been suggested

to induce overreaching and overtraining in team sport players [18, 21], and the monitoring of these responses along with the training period may be critical for the identification of such events.

Hematological and biochemical analyses are often used to identify fatigue and recovery in athletes during the training seasons [18], although relatively few studies have systematically examined the use of EO resistance-training protocols for monitoring such factors [2–4, 12]. To compare individual blood parameter values with reference intervals obtained from a physically active population may also be a useful tool to monitor the adaptive effects of exercise. However, this form of comparative analysis has certain limitations, as laboratory results may be influenced by biological variation [22], hindering the clinical results interpretation, particularly those from consecutive analysis performed for the same individual.

Considering this aspect to compare the serial results, in the present study, we adopted the reference change values (RCV) or critical difference analysis. The RCV is a tool employed to verify if the difference between two consecutive samples is significant and biologically relevant, considering the various components of intrinsic variation affecting laboratory assays. These components include factors related to laboratory activity (preanalytical and analytical variation) and those related to normal intraindividual biological variation [23]. As such, the RCV defines the percentage of change that should be exceeded when considering the analytical and biological variation inherent to a test, in order to evaluate significant changes between two consecutive measurements.

Bearing this in mind, the aim of this study was to observe the time course of muscle damage and inflammatory responses to resistance-training with EO adopting the RCV to compare the serial results. Our initial hypothesis was to observe high magnitude of muscle damages on the initial phase of the program, together with greater inflammatory responses. However, a subsequent attenuation of these events by the repeated bout-effect occurrence was expected [9, 16].

2. Methods

2.1. Experimental Design. Thirteen training sessions were performed twice a week, performed at the same hour of the day, and under the supervision of the researchers involved. One familiarization session with the resistance exercise equipments happened three weeks before the program started. Blood samples were collected in eight distinct time points throughout the program and always before the individuals started the sessions. These time points were Pre (prior to the first session), P2 (after two sessions), P7 (after seven sessions), P9 (after nine sessions), P11 (after eleven sessions), and 96 hours after the last training session (P13).

2.2. Participants. Eight healthy subjects (3 female: age: 23.8 ± 2.6 years; body mass: 70.9 ± 12.7 kg; height: 1.6 ± 0.08 m; % body fat: 29.6 ± 4.3; and 5 male: age: 23.8 ± 2.6 years; body mass: 75.1 ± 11.2 kg; height: 1.8 ± 0.1 m; % body fat: 20.0 ± 4.9) participated in the study. The inclusion criteria included being engaged in resistance-training programs for at least one year, and no intake of exogenous anabolic-androgenic steroids, drugs, medication, or dietary supplements with potential effects on physical performance is recorded. Training programs usually performed by the subjects consisted of 3–5 sets of 6–12 repetitions with 1-2-minute rest interval between sets, performed 4-5 times per week. Subjects gave written informed consent after being advised about the purposes and the risks associated with the study. Subjects fasted for one hour prior to the blood collection, being also required to refrain from strenuous exercise and the consumption of alcohol, tobacco or caffeine 48 hours before the testing sessions. During the period of the study, none of the participants reported any kind of infection state that could affect his/her immune responses despite the inflammation induced by the exercise. Research Ethics Committee (019/2004) approved the experimental protocol.

2.3. Strength Tests. The one repetition maximal test (1RM) was employed a fortnight prior to the beginning of the study, aiming the prescription of the resistance-training intensity (% 1RM). Since the present study makes a distinction between the concentric and eccentric phase of the movement, it was necessary to employ a specific test to access the maximum weight to be bore by the eccentric-only muscle action. The typical 1RM test reflects only the maximum weight that can be lifted using a concentric action—designated as 1RMcon. This specific evaluation is referred as 1RM eccentric (1RMecc) [24], and it is adopted due to the observations that skeletal muscles are capable of developing much higher forces when they contract eccentrically compared to concentrically [24].

The 1RMcon testing was conducted using the methods described by Brow and Weir [25], and for the 1RMecc the methods described by Hollander and coworkers [24]. There were three-to-five single trials for the validation of the test, which was the subject using proper form and completing the entire lift in a controlled manner without assistance [26].

2.4. Training Program. The program consisted of thirteen sessions over seven weeks, performed on Tuesdays and Thursdays, always at the same time of the day and under the supervision of the researchers involved. Prior to each session, subjects completed a standardized warm up program of static stretching exercises, and a specific warm up of 8 repetitions with approximately 50% of the estimated 1RMcon for each exercise. Training protocol consisted of 4 sets of 8–10 eccentric-only repetitions with 80% of 1RMecc, and one minute of rest between sets. The concentric phase of the movement was performed with the assistance of the researchers, and the exercises employed were Bench Press, 45-degree Leg Press, and Bent-Over Rows.

2.5. Blood Samples. Blood samples were collected under standardized conditions: 2.0 mL of total venous blood was collected in vacuum tubes containing EDTA/K3 to determine hematological parameters, and 8.0 mL of venous blood was collected in tubes with a Vacuette (Greiner Bio-one) gel separator in order to obtain serum for biochemical measurements. Blood samples were collected 96 hours after the last

training bout, in the morning after 12 hours of fasting, in a seated position, transported at 4°C to the laboratory within 30 minutes, centrifuged under refrigeration at 1.800 ×g for 10 minutes, immediately separated, and protected from light.

2.6. Blood Analysis. Hematological analyses were conducted using a KX-21N (Sysmex, Brazil) automated hematology analyzer. The analysis included red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), erythrocyte distribution width (RDW), white blood cell (WBC) count, lymphocyte (LYNF) count, neutrophil (NEUTR) count, and platelet count (PLT).

Biochemical measurements were conducted with commercial kits (Wiener Lab; Rosario, Argentina) and with an Autolab (Boehringer, Mannheim, Germany) analyzer. The assay included creatine kinase (CK) activity, and C-reactive protein (CRP). To minimize analytical variations, the same technician tested all samples without changing reagent lots, standards, or control materials.

2.7. Statistical Analysis. The percentual differences between the serial results were calculated for each subject and compared with $RCV_{95\%}$ to detect significant changes [27]. The $RCV_{95\%}$ to detect significant changes was based on the following formula: $RCV_{95\%} = (2)^{1/2} * Zp(CV_A^2 + CV_I^2)^{1/2}$ where $2^{1/2}$ is to verify difference between 2 time points, Zp : Z score (probability 95% = 1.96), CV_A: analytical coefficient of variation, and CV_1: intraindividual coefficient of variation [28]. The $RCV_{95\%}$ for physically active subjects employed in this study was previously determined [23]. The $RCV_{95\%}$ considered were RBC = 8.3%; Hb = 8.0%; Ht = 8%; MCV = 2.3%; MCH = 2.8%; MCHC = 3.3%; RDW = 6.1%; WBC = 43.9%; LYNF = 40.5%; NEUTR = 65.3%; PLT = 21.5%; CK = 119.3%; CRP = 206%. Significant change for each analyte was considered when its percentage change in two subsequent measurements exceeded the determined $RCV_{95\%}$. The reference intervals were based on results published by our laboratory based on a physically active population: RBC (4.4–5.6 10^{12}/L), Hb (13.0–16.1 g/dL), Ht (39.5–48.0%), MCV (80.9–94.9 fL), MCH (26.1–31.6 pg), RDW (12.1–14.3%), WBC (4.5–10.1 10^9/L), LYNF (1.2–3.3 10^9/L), NEUTR (1.8–6.7 10^9/L), PLT (140–337 10^9/L), CK (<1309 U/L), and CRP (<19.8 mg/L) [29]. Pearson's coefficient of correlations was used in order to establish relationships between the analysis. Statistical comparisons were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

Table 1 shows significant changes in CK and CRP accessed throughout the training period. CK and CRP presented significant changes at specific time points, but not for all subjects. Four subjects presented significant changes in CK activity at P2 (+1719%, +1250%, +1281%, and +312% resp.), and other two at P13 (+391% and +139%, resp.). For CRP six subjects presented significant changes at P2 (+1100%, +243%, +3800%, +2500%, +1400%, and +2400%, resp.), one at P4 (+567%), other at P9 (+3200%), three at P11 (+300%, +3400%, and +3900%, resp.), and other at P13 (+1500). Pearson's coefficient of correlation at P2 for CK and CPR ($R = 0.31$), CK and NEUTR ($R = -0.24$), and CK and WBC ($R = -0.09$) was not significant ($P > 0.05$). Table 1 presents the significant changes in CK and CRP throughout the training period and the specific subjects who presented these alterations.

Only two subjects presented significant changes for NEUTR (Table 2). Subject 7 presented changes at P2 (+90%) and P4 (−71%), and subject 6 at P13 only (+60%). In addition, subject 7 also presented significant changes for WBC at P2 (+53%), P4 (−57%), P7 (+71%), and P9 (+49%), for LYNF P7 (+344%), P9 (+57%), and P13 (−44%), and for PLT at P4 (−25%). No significant changes were observed for RBC, PLT, Ht, MCH, MCHC, RDW, MCV, and Hb throughout the training period. Table 2 presents the significant changes in the aforementioned markers and the specific subjects presenting alterations.

4. Discussion

Our main findings were that four subjects presented significant changes for CK at P2, and other two at P13. For CRP six subjects presented significant changes at P2, one at P4, other at P9, three at P11, and other at P13. Only two subjects presented significant changes for NEUTR. Subject 7 presented changes at P2 and P4, and subject 6 at P13 only. In addition, subject 7 also presented significant changes for WBC at P2, P4, P7 and P9, for LYNF at P7, P9, and P13, and for PLT at P4. No significant changes were observed for RBC, PLT, Ht, MCH, MCHC, RDW, MCV, and Hb. No significant correlations were observed between CK and CRP as well as WBC and NEUTR at P2.

To the best of our knowledge, this is the first study to employ the RCV specifically for physically active subjects when comparing the temporal behavior of inflammatory and muscle damage responses to EO. Individual blood analyses provided by the RCV allowed the identification of the specific subjects seeming to be more susceptible for inflammatory processes and muscle damage. Conventional statistical analysis considers the probability of occurrence of one event for a group and not for specific subjects, what may mask or disregard the more responsive individuals. These results appear to be very important for future studies aiming at monitoring these events.

CK responses presented by four subjects at P2 supported our initial hypothesis to observe high magnitudes of muscle damage on the initial phase of the program. The subsequent attenuation of this event confirmed the occurrence of the repeated bout effect, with the exception of two subjects with significant increases of CK at P13 in relation to RCV, but within the RI for physically active subjects (Table 1). The repeated bout effect refers to the adaptation whereby a single bout of eccentric exercise protects against muscle damage

Table 1: Significant changes in CK and CRP and the specific subjects presenting alterations. RI: reference interval for physically active subjects [23]; Δ: difference between two consecutive analyses.

Analyte	Moment	Subject	Measured values: latter − former (Δ)	% change
CK $RCV_{95\%}$ = 119.3% RI < 1309 U/L	P2	3	2455 − 135 (2320)	+1719
	P2	4	1417 − 105 (1312)	+1250
	P2	5	2278 − 165 (2113)	+1281
	P2	7	465 − 113 (352)	+312
	P13	1	339 − 69 (270)	+391
	P13	8	404 − 169 (235)	+139
CRP $RCV_{95\%}$ = 206% RI < 19.9 mg/L	P2	1	1.2 − 0.1 (1.1)	+1100
	P2	3	2.4 − 0.7 (1.7)	+243
	P2	5	3.9 − 0.1 (3.8)	+3800
	P2	6	2.6 − 0.1 (2.5)	+2500
	P2	7	1.5 − 0.1 (1.4)	+1400
	P2	8	2.5 − 0.1 (2.4)	+2400
	P4	4	2 − 0.3 (1.7)	+567
	P9	2	3.3 − 0.1 (3.2)	+3200
	P11	1	0.4 − 0.1 (0.3)	+300
	P11	3	3.5 − 0.1 (3.4)	+3400
	P11	7	4 − 0.1 (3.9)	+3900
	P13	8	1.6 − 0.1 (1.5)	+1500

Table 2: Significant changes in NEUTR, WBC, LYNF, and PLT and the specific subjects presenting alterations. RI: reference interval for physically active subjects [23]; Δ: difference between two consecutive analysis.

Analyte	Moment	Subject	Measured values: latter − former (Δ)	% change
NEUTR $RCV_{95\%}$ = 65.3% RI = 1.8–6.7 (10^9 cel/L)	P2	7	9.4 − 4.9 (4.5)	+90
	P4	7	2.7 − 9.4 (−6.7)	−71
	P13	6	6.2 − 3.9 (2.3)	+60
WBC $RCV_{95\%}$ = 43.9% RI = 4.5–10.1 (10^9 cel/L)	P2	7	12.2 − 8.0 (4.2)	+53
	P4	7	5.2 − 2.2 (3.0)	−57
	P7	7	8.9 − 5.2 (3.7)	+71
	P9	7	13.3 − 8.9 (4.4)	+49
LYNF $RCV_{95\%}$ = 40.5% RI = 1.2–3.3 (10^9 cel/L)	P7	7	5.6 − 1.3 (4.3)	+344
	P9	7	8.8 − 5.6 (3.2)	+57
	P13	7	7.0 − 3.9 (3.1)	−44
PLT $RCV_{95\%}$ = 21.5% RI = 140–337 (10^9 cel/L)	P4	7	234 − 176 (58)	−25

from subsequent bouts [16]. The potential adaptations that explain the phenomena have been categorized as neural, mechanical, and cellular. Regarding the cellular adaptations there is evidence of longitudinal addition of sarcomeres and adaptations in the inflammatory response following an initial bout of eccentric exercise, limiting also the proliferation of damage.

Despite four individuals not presenting significant increases in CK at P2, we cannot completely state that they did not experience muscle injuries. Evidences in the literature report that CK plasma activity is not considered as a gold-standard evaluation of muscle damage because it does not present a significant linear correlation with muscle functions and ultrastructural changes in muscle following exercise [17, 30, 31]. Presently, muscle functions measurements are considered the most indicated methods for quantifying injuries because the event results in an immediate and prolonged reduction in these parameters, persisting over the entire span of the progression of the degenerative and regenerative processes [17, 32]. On the other hand, plasma activities of myofibril proteins are not evidenced over this entire time course of degenerative and regenerative processes, and even when evidenced, they may or may not be correlated with the magnitude of the functional decrements [17, 32]. We

recognize that one limitation of the present study was not having a quantification of muscle functions parallel to plasma CK activity in order to establish possible correlations between them. CRP responses do not correlate to CK, emphasizing that elevated CRP levels may be associated to damage in nonskeletal muscle tissue.

Similar to CK, CRP responses were characterized by two time points when subjects appeared to express higher responses. Despite the similarity of response at P2, no significant correlations with CK were found, confirming the observations made by previous studies [13]. Literature emphasizes that elevated CRP levels may simply mark the clearance of modified molecules or necrotic tissue in an effort to limit damage associated with the inflammatory process [33]. Providing a valid prediction of the progression of vascular lesions in the absence of acute infection, the increases in CRP levels may be associated to damage in nonskeletal muscle tissue [33], thus, explaining the poor correlation between CK and CRP responses found. It is important to observe that all subjects presented significant changes in relation to RCV but within the traditional RI for physically active subjects (Table 1).

Time course of muscle damage and inflammatory responses have also been investigated in two recent studies [34, 35]. The first study evaluated the process following an international rugby union game. An acute-phase inflammatory response reflected through immediate increases in serum cortisol and IL-6, followed by delayed increases in serum CK (14 hours) activity and CRP (38 hours) was observed. The findings suggested that a rugby match elicits disturbances in host immunity, which last up to 38 hours into the recovery period. The second research observed the process together with performance changes, following a soccer match (in the morning of the game day, immediately after, and 24, 48, 72, 96, 120, and 144 hours after match). Performance deteriorated 1-to-4 days after match, an acute-phase inflammatory response consisting of a postmatch peak of leukocyte count, 24-hour peak of CRP, and 48-hour peak of CK were observed.

According to the behaviors reported in the aforementioned studies, it becomes evident that the moment for collection of blood samples is a crucial aspect for their observation (i.e., the inflammatory process appears to have an acute-phase response with a subsequent attenuation). The moment of blood collection in the present study was 96 hours after the last training bout, which could not be the most adequate one for the observation of the time course for some markers. These observations may help us to explain the reasons why only two subjects presented significant changes for NEUTR at P13, and only one for WBC (at P2, P4, P7, and P9), LYNF (at P7, P9, and P13), and PLT (at P4). Other subjects may have expressed an early-stage (24–48 hours after last bout) acute-phase inflammatory responses, which was not detected 96 hours after the last bout.

Specific resistance-training inflammatory responses were reported by Simonson and Jackson [11]. Blood samples were drawn at before, after, 15 minutes after, and 30 minutes after exercise. All leukocyte subpopulations, except for basophils and eosinophils, increased at after exercise but the counts declined at after, 15, and 30 minutes after exercise. Only NEUTR did not return to preexercise levels by 30 minutes after exercise. The majority of resistance exercise-induced leukocytosis was due to an increase in circulating LYNF and monocytes. In the study, the authors suggested that by the lack of large alterations and rapid recovery from cell number, resistance training is not immunosuppressive. Meanwhile, we highlight that the data of the aforementioned studies were not analyzed according to the RCV for physically active subjects.

5. Conclusions

EO resistance training protocol induced high magnitudes of muscle damage and CRP responses on the initial phase of the program, with a subsequent attenuation of the event. Such behavior confirms the occurrence of the repeated bout effect for this particular training protocol, except for two subjects. Nevertheless, CRP responses do not correlate to CK, emphasizing that elevated CRP levels may be associated to damage in nonskeletal muscle tissue.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

The Treg/Th17 Imbalance in Patients with Obstructive Sleep Apnoea Syndrome

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Obstructive sleep apnoea syndrome (OSAS) is a chronic inflammatory disease regulated by T lymphocytes. Our purpose is to assess the pattern of Th17 cells and $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells in peripheral blood of patients with OSAS. Fourty-four OSAS men and 20 healthy volunteers were enrolled. Twenty-three patients were classified into mild to moderate group and 21 cases were classified into severe group according to the severity of OSAS. We detected the frequencies of Th17 and Treg and related serum cytokines secretion and expressions of key transcription factors. OSAS patients revealed significant increase in peripheral Th17 number, Th17-related cytokines (IL-17 and IL-6), and RORγt mRNA levels. They also presented a significant decrease in Treg number, Treg-related cytokines (TGF-β_1), and Foxp3 mRNA levels as compared with normal persons. As a result, the Th17/Treg ratios were markedly more upregulated in OSAS patients than those in control group. Furthermore, the Th17/Treg ratio was positively related to the severity of OSAS and serum levels of C-reactive protein. The development of OSAS may be associated with peripheral Th17/Treg imbalance and characterized by a proinflammatory cytokine microenvironment. These results opened an alternative explanation for the substantial activation of immune cells in OSAS and the development of related complications.

1. Introduction

Obstructive sleep apnoea syndrome (OSAS) is a highly prevalent disease and is recognized as a major public health burden. OSAS is characterized by repeated events of partial or complete upper airway obstruction during sleep that lead to disruption of normal ventilation, hypoxemia, and sleep fragmentation. Although the basic mechanisms mediating this association are likely multifactorial and remain to be fully elucidated, the cumulative burden of a chronic and low-grade systemic inflammation has emerged as the most likely contributor to the occurrence and magnitude of OSAS associated morbidity [1–4]. Accumulating evidence in the past decade has corroborated the close association between OSAS, inflammation and various cardiovascular morbidities [5, 6].

Sleep is characterized by a specific regulation of the endocrine and autonomic nervous system, and thereby sleep is supposed to exert a systemic control over immune function [7, 8]. OSAS is involving various immune cells, particularly T lymphocytes [9, 10]. Activation of T lymphocytes is among the crucial steps leading to the release of inflammatory mediators and adhesion molecules. T cells play a significant role in atherogenesis and plaque development via cytokine production and by directly contributing to vascular injury [11]. In brief, investigating how this breathing disorder modulates immune responses may facilitate understanding the pathogenesis of OSAS in relation with its complications.

Alberti et al. found a prevailing activation of the Th1-type cytokine pattern in OSAS patients by measuring plasma cytokine levels in OSAS patients [12]. Conversely, Dyugovskaya et al. reported a shift in Th1/Th2 cytokine expression towards Th2 dominance in patients with OSAS [10]. In contrast, Dimitrov et al. revealed that, compared with wakefulness, early nocturnal sleep induced a shift in the Th1/Th2 cytokine balance towards increased Th1 activity, as indicated by an increased ratio of IFN-γ/IL-4 producing T helper cells. However, the Th1 shift was only of moderate size and replaced by Th2 dominance during late sleep [13]. To date, only a few paper has explored the Th1/Th2 balance in OSAS patients with incompatible results.

There has recently been a minirevolution in the basic understanding of immunology following the discovery of two new subsets of T helper cells, T regulatory (Treg) and Th17, which have opposite effects on autoimmunity and inflammation. Th17 cells expressing retinoic acid related orphan receptor γt (RORγt) play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 and IL-6. Th17 cell is a key effector in the immune response and play critical roles in the development of autoimmunity by producing IL-17 and, to a lesser extent, TNF-α and IL-6. While Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) orchestrate the overall immune response and play a role in maintaining peripheral immune tolerance by contact-dependent suppression or releasing anti-inflammatory cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β_1 and regulating the activity of the effector T cells. Therefore, the Th17/Treg balance may control the development of autoimmunity and inflammation, and Th17/Treg imbalance is proved to be wide existed in human cancer, inflammatory and autoimmune diseases [14–17]. Therefore, we hypothesize that circulating Treg/Th17 imbalance may also present in OSAS patients, which is related with systemic inflammation and important in the pathogenesis of OSAS.

To the best of our knowledge, a possible role for the Th17/Treg axis in OSAS has never been elucidated in OSAS. Thus, the purpose is to test the hypothesis that the balance of Th17 and Treg frequencies in the peripheral circulation is disturbed in patients with varying degrees of OSAS. We evaluated plasma levels of Th17- and Treg-related cytokines and mRNA expression of relevant transcription factors (RORγt and Foxp3) in peripheral blood monouclear cells (PBMC). As a sensitive marker of inflammation, C-reactive protein (CRP) is believed to be both a by-product and a mediator of the low-grade inflammation that occurs in OSAS. Therefore, we explored the potential correlation of Th17/Treg imbalance with serum CRP level and the severity of OSAS.

2. Materials and Methods

2.1. Subjects and Protocol. Consecutive men (age $\geqslant$18 and <70 years) with newly confirmed OSAS by overnight polysomnography (PSG) were initially recruited. All of them had been referred to the Sleep Disorders Center, 3rd affiliated hospital of Sun Yat-sen University between Jan. 2008 to Dec. 2011, with symptoms suggesting sleep related breathing disorders and had never been previously diagnosed or treated for OSAS.

Patients with a history of chronic or recent ($\leqslant$1 month) clinically significant infectious or inflammatory condition including asthma, trauma, vaccination, any invasive medical/surgical ($\leqslant$3 months), or dental ($\leqslant$1 month) procedure were eliminated. Patients with morbid obesity (body mass index (BMI) > 35 kg/m^2), hypertension, coronary artery disease, heart failure, a history of stroke, diabetes mellitus, chronic obstructive or restrictive pulmonary disease, chronic renal disease, dyslipidemias, or pharmacologically treated depression were ineligible for the study. Current smokers and exsmokers who smoked within 12 months before the start of current study were excluded. Patients receiving medications or nutritional supplements and night shift workers also were ineligible. Patients with sleep disorders such as upper airway resistance syndrome, central sleep apnea syndrome, periodic limbs movement, or narcolepsy were also removed from final analysis.

We recruited control subjects from the community at the same time. Control subjects were nonsmoking healthy subjects who had an AHI lower than 5 and no complaint of sleep apnea. They have no chronic diseases mentioned above. Control subjects were matched to OSAS patients for gender (only male), age (within 4 years), and BMI (within 15%). Except for possible obesity, all control subjects had a normal physical examination and laboratory tests. All control subjects underwent PSG to exclude the presence of sleep-disordered breathing.

All subjects completed a standardized questionnaire including the Epworth sleepiness scale (ESS) score [18], demographic data, and clinical history. The study was approved by the institutional review board of the Institutional Review Board of Sun Yat-sen University. All patients gave their written informed consent.

2.2. Data Collection. At baseline, medical history was recorded and a physical examination was performed. Anthropometric data (age, BMI, neck circumferences) along with daytime habits, such as smoking or exercise, were recorded. BMI, a statistical measurement that compares a person's weight and height, was calculated as weight in kilograms divided by the height in meters squared. Neck circumference was measured at the cricothyroid level. Sleepiness was evaluated by ESS. To exclude any respiratory or cardiovascular disease, spirometric evaluation, arterial blood gas analysis while the patient breathed room air, chest radiograph, and resting 12-lead electrocardiogram were conducted. All data were extracted from the medical records using a specially designed case report form (CRF). Then the data were double-entered manually into a Microsoft Excel master sheet to build our database. All forms were checked by another researcher for errors. The variables recorded on the CRF and pertinent definitions were described as follows.

2.2.1. Sleep Study. Subjects underwent an overnight polysomnography (PSG) with the Embla-Monet 32 Sleep

System (Embla, USA) which consisted of recording for EEG, electrooculography, electromyography, ECG, nasal pressure transducer, thermistor for nasal airflow, thoracic and abdominal impedance belts, pulse oximetry, tracheal microphone for snoring, and sensors for leg and sleep position. PSG recordings were manually scored by a single registered technologist as described previously [19–21].

Apnea was defined as the pause of airflow at the nose and mouth lasting for >10 s. Hypopnea was defined as a decrease of ≥30% in thoracoabdominal motion associated with a fall in baseline oxygen saturation of >4%. The apnea-hypopnea index (AHI) was expressed as the number of episodes of apnea and hypopnea per hour of total sleep time (TST). The severity of hypoxia was assessed by calculating the lowest oxygen saturation (nadir SaO_2) and the percentage of TST spent with oxygen saturation at SaO_2 < 90%. OSAS was defined by AHI ≥ 5/h. OSAS subjects were classified into two groups according to their apnea-hypopnea frequency as mild to moderate OSAS (AHI > 5 ⩽ 30), and severe OSAS (AHI > 30).

2.2.2. Blood Samples and PBMC Isolation. Blood samples were obtained the morning after polysomnography, between 08:00 and 09:00 following an overnight fast. The 15–20 mL of peripheral blood (PB) samples were collected into collection tubes containing 0.2 mL sodium heparin. PBMCs were prepared by Ficoll-Hypaque density centrifugation for analysis of flow cytometry and real-time quantitative polymerase chain reaction (RT-qPCR). Plasma was obtained after centrifugation and stored at −80°C for the measurement of the cytokines.

2.2.3. Flow Cytometric Analysis of Treg and Th17 Cells. PBMCs were suspended at a density of 2×10^6 cells/mL in complete culture medium (RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 10% heat inactivated fetal calf serum; Gibco BRL, Gaithersburg, MD). The cell suspension was transferred to each well of 12-well plates. Cultures were stimulated with phorbol myristate acetate (PMA, 50 ng/mL) plus ionomycin (1 μM) for 4 h in the presence of monensin (500 ng/mL; all from Alexis Biochemicals, San Diego, CA). The incubator was set at 37°C under a 5% CO_2 environment. After 4 h of culture, the contents of the well were transferred to 5-mL sterile tubes and centrifuged at 1500 rpm for 5 min.

For the Th17 analysis, the cells were incubated with phycoerythrin (PE) antihuman CD4 (eBioscience, San Diego, CA) at 4°C for 20 min. For the Treg analysis, the cells were incubated with fluorescein isothiocyanate (FITC) anti-human CD4 and PE anti-human CD25 at 4°C for 30 min. Following surface staining, the cells were fixed and permeabilized according to the manufacturer's instructions, and then stained with FITC anti-human IL-17A for Th17 detection or PerCP-Cy5.5 (PC61.5; eBioscience) anti-human Foxp3 for Treg detection. Isotype controls were treated to enable correct compensation and confirm antibody specificity. All of the antibodies and reagents were from BD Pharmingen. Stained cells were analysed by flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson) equipped with CELLQest Pro 5.2 software (BD Biosciences, USA).

2.2.4. RORγt and Foxp3 Expression Determined by Real-Time Quantitative PCR. Total RNA was extracted from the PBMCs with TRIzol extraction (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, cDNA was synthesized using random hexamer primers and RNase H-reverse transcriptase (Invitrogen). The following primer pairs were used: Foxp3: F: 5′-CACGCATGTTTGCCTTCTTCAGA-3′, R: 5′-GTAGGG TTGGAACACCTG CTGGG-3′, and RORγt: F: 5′-GCA ATGGAAGTGGTGCTGGTT-3′, R: 5′-AGGATGCTTTGG CGATGAGT C-3′. Primers were entered into NCBI BLAST database to ensure that it was specific for the target mRNA transcription and then synthesized by Sangon Biotech Co. (Japan). PCR were performed using Syber Green real-time PCR regent box (Toyobo) in a total volume of 20 μL. PCR conditions for Foxp3 was 95°C for 1 min, 40 cycles of 95°C for 15 s, 61°C for 15 s, 72°C for 45 s, but the annealing temperature of RORγt was 64°C. Samples were analyzed utilizing the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated by using the comparative CT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization, and a no template sample was used as a negative control. All reactions were carried out in triplicate per sample.

2.2.5. Detection of Plasma Cytokines and hsCRP. The plasma levels of IL-17, IL-6, TGF-β_1, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA), following the manufacture's instructions (ELISA kits, all from R&D system). The minimal detectable concentrations were 2 pg/mL for IL-17, 0.7 pg/mL for IL-6, 5 pg/mL for TGF-β_1 and 7.8 pg/mL for IL-10. All samples were measured in duplicate.

As previously described [21], serum levels of highly sensitive CRP (hsCRP) were measured with a particle-enhanced Immunoturbidimetry (Beijing O&D Biotech Company Ltd, Cox Bio China, Beijing, China). The lower limit of detection for hsCRP was 0.06 mg/L.

2.2.6. Statistical Analysis. Values are expressed as mean ± standard deviation (SD) in the tables and figures. Differences between the values were determined using Student's *t*-test. Grouped data were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. When the equal variance test failed, a Mann-Whitney rank sum test was used. Spearman's correlation was used as a test of correlation between two continuous variables. A P value of <0.05 indicates significance. Statistical analysis was performed using a commercial software package (SPSS, version 15; SPSS; Chicago, IL).

Table 1: Baseline characteristics of study population.

Variables	Control	OSAS		
		Total	Mild to Moderate	Severe
Subjects, n	20	44	23	21
Age (years)	45.80 ± 23.01	46.43 ± 18.22	44.90 ± 20.64	48.07 ± 11.28
BMI (kg/m^2)	26.90 ± 4.25	28.15 ± 5.34	28.55 ± 6.29	27.82 ± 5.01
Neck circumference (cm)	41.15 ± 5.63	43.93 ± 5.72	43.58 ± 6.22	44.13 ± 5.19
AHI (episodes/h)	2.26 ± 1.30	40.41 ± 20.68*	23.12 ± 6.93‡	59.33 ± 12.59†§
Nadir SaO_2 (%)	94.78 ± 3.49	76.24 ± 7.56*	80.65 ± 8.19‡	71.56 ± 7.87†§
SaO_2 < 90% (%TST)	1.06 ± 0.93	17.63 ± 8.32*	7.72 ± 7.43‡	28.52 ± 10.37†§
ESS scores	4.93 ± 2.05	14.74 ± 1.74*	13.80 ± 1.92‡	15.95 ± 1.63§

Values are expressed as mean ± SD.
*$P < 0.05$, Control versus total OSAS;
$^†P < 0.05$, Mild to Moderate OSAS versus Severe OSAS;
$^‡P < 0.05$, Control versus Mild to Moderate OSAS;
$^§P < 0.05$, Control versus Severe OSAS.
Definition of abbreviations: AHI: apnoea hypopnoea index; BMI: body mass index; ESS, Epworth sleepiness scale; nadir: lowest oxygen saturation recorded; OSAS: obstructive sleep apnoea syndrome; TST: total sleep time.

3. Results

3.1. Basic Characteristics of Studied Subjects. A total of forty-four men with newly diagnosed OSAS were recruited. Five patients were diagnosed as mild OSAS and 18 patients were moderate OSAS. Thus, a total of twenty-three patients were enrolled as mild to moderate OSAS group and 21 patients were screened as severe OSAS group. Twenty health men were enrolled as the control group at the same time. Overall clinical characteristics are summarized in Table 1.

The mean AHI in OSAS group was 40.41 ± 20.68 and mean age of OSAS patients was 46.43 ± 18.22 years. As shown, EES score and PSG parameters were significantly higher in the OSAS group comparing with the control group ($P < 0.05$). However, no statistically significant differences were found between three groups from the standpoint of age and BMI ($P > 0.05$).

3.2. Th17 Frequency Was Increased in the Peripheral Blood of OSAS Patients. As shown in Figure 1(a), the prevalence of Th17 cells in PBMCs was expressed as a ratio of $CD4^+IL17^+$T cells/$CD4^+$T cells. The frequence of Th17 was evidently increased in the peripheral blood of OSAS patients (3.08 ± 0.68%) than those in normal controls (1.65 ± 0.49%; $P < 0.001$). Furthermore, the percentage of Th17 was markedly higher in patients with severe OSAS (3.42 ± 0.49%) than those in subgroup with mild to moderate OSAS (2.77 ± 0.68%; $P = 0.002$).

3.3. Treg Frequency Was Decreased in the Peripheral Blood of OSAS Patients. The prevalence of Treg cells was expressed as a ratio of $CD4^+CD25^+Foxp3^+/CD4^+$T cells. As shown in Table 2 and Figure 1(b), the frequency of Treg was significantly decreased in the peripheral blood of OSAS patients (1.50 ± 0.38%) compared to control group (2.81 ± 0.46%; $P < 0.001$). Moreover, significant difference was found between severe OSAS group and patients with mild to moderate OSAS (1.30 ± 0.31% versus 1.69 ± 0.34%, $P = 0.001$). Nevertheless, the percentage of $CD4^+CD25^+$T lymphocytes was similar in the three groups (data not shown).

3.4. Balance of Th17 and Treg Was Disrupted in OSAS Patients. When the balance of Th17 and Treg was further investigated, their relationship was expressed as a ratio of Th17/Treg. We demonstrated that the ratio of Th17/Treg was highest in patients with severe OSAS (2.80 ± 0.84), lower in those patients with mild to moderate OSAS (1.71 ± 0.67) and lowest in control subjects (0.58 ± 0.13) (Figure 1(c)). As a result, the Th17/Treg ratio was significantly increased in OSA patients comparing with control group (2.23 ± 0.94 versus 0.58 ± 0.13, $P < 0.001$).

3.5. Expression of Foxp3 and RORγt mRNA in PBMCs. RORγt is an important transcription factor for the differentiation of Th17, while Foxp3 is the master transcription factor in Treg. We thus analyzed the levels of transcription factors for Th17 (RORγt) and Treg (Foxp3) by RT-qPCR in PBMCs from included subjects. As shown in Figure 2, the levels of RORγt expression were much upregulated in the OSAS group (3.18 ± 1.19) than those in the health control group (1.42 ± 0.32, $P < 0.01$). And there was significant difference between the severe (4.25 ± 0.79) and mild to moderate subgroups (2.21 ± 0.37) within OSAS patients ($P < 0.05$). In contrast, the expression of Foxp3 was markedly lower in the OSAS patients (2.50 ± 1.38) as compared with the control group (4.10 ± 0.97, $P < 0.01$), while Foxp3 levels in severe OSAS group (1.33 ± 0.35) were significantly downregulated than that of the subgroup with mild to moderate OSAS (3.56 ± 1.07, $P < 0.01$).

3.6. Plasma Concentrations of Related Cytokines and hsCRP. Plasma levels of IL-17, IL-6, TGF-β_1, IL-10, and hsCRP were detected in normal controls and OSAS patients by means of ELISA tests (Table 2, Figure 3). IL-17 in severe patients (75.24 ± 11.40 pg/mL) and mild to moderate apnea (65.78 ± 13.97 pg/mL) were both significantly higher

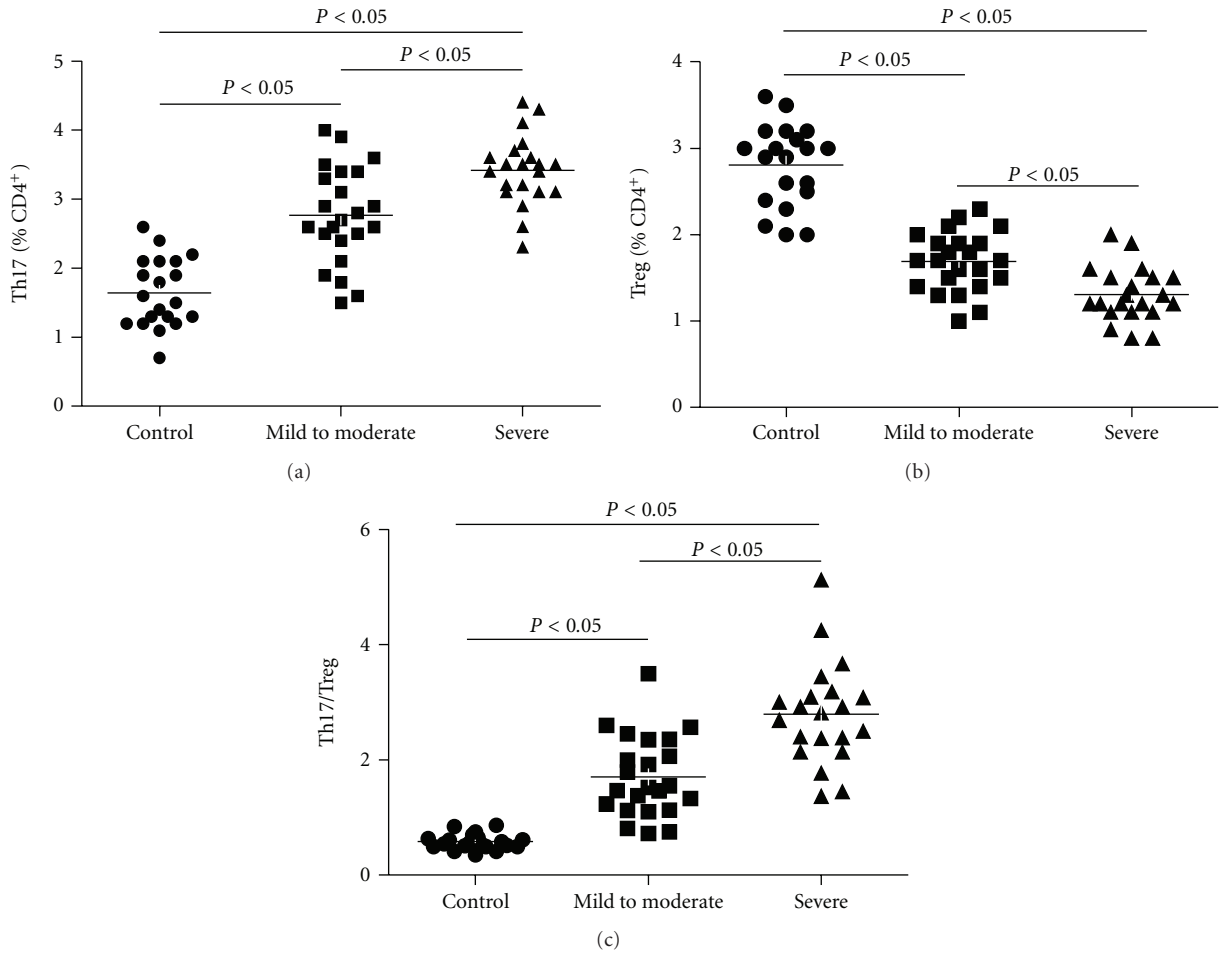

Figure 1: Frequencies of circulating Th17 and Treg cells, as well as the ratio of Th17/Treg in OSAS patients and control group. PBMCs from studied subjects were stained with labeled antibodies as described in Section 2. (a) Circulating Th17 frequencies increased in OSAS patients compared with controls; (b) circulating Treg cell subset decreased in OSAS patients compared with controls; (c) the ratio of Th17/Treg increased in OSAS patients compared with controls.

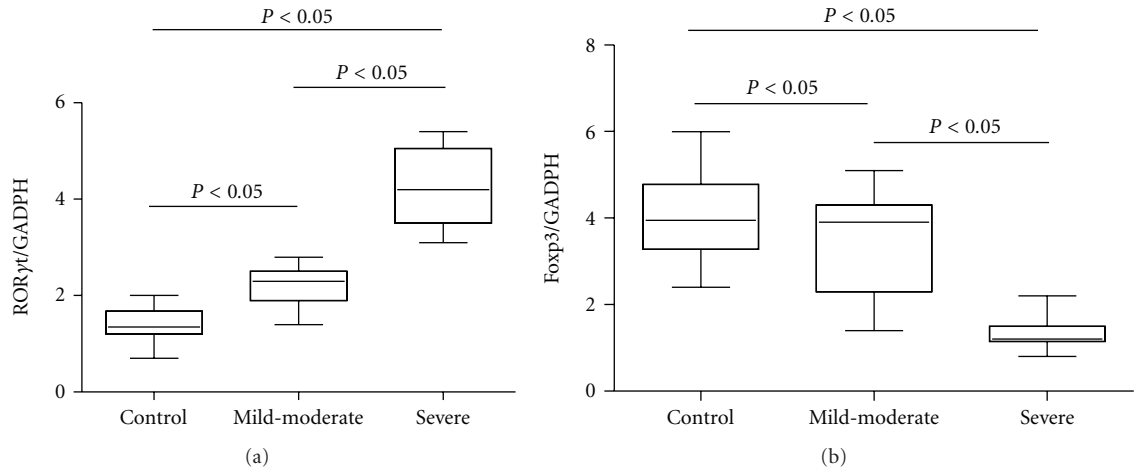

Figure 2: Expression of RORγt and Foxp3 in PBMCs from patients with OSAS and control groups. (a) Ratios of RORγt/GAPDH mRNA were compared in three groups. (b) Ratios of Foxp3/GAPDH mRNA were compared in three groups.

Table 2: Summary of RT-qPCR, flow cytometry, and ELISA results in study population.

Variables	Control	OSAS ($n = 44$)		
		Total	Mild to Moderate	Severe
Subjects, n	20	44	23	21
T-cell counts (Flow cytometry)				
Th17 (% of $CD4^+$)	1.65 ± 0.49	3.08 ± 0.68*	2.77 ± 0.68‡	3.42 ± 0.49†§
Treg (% of $CD4^+$)	2.81 ± 0.46	1.50 ± 0.38*	1.69 ± 0.34‡	1.30 ± 0.31§
Th17/Treg ratio	0.58 ± 0.13	2.23 ± 0.94*	1.71 ± 0.67‡	2.80 ± 0.84†§
mRNA expression of transcription factors (RT-qPCR)				
RORγt /GADPH mRNA	1.42 ± 0.32	3.18 ± 0.19*	2.21 ± 0.37‡	4.25 ± 0.79†§
Foxp3/GADPH mRNA	4.10 ± 0.97	2.50 ± 1.38*	3.56 ± 1.07‡	1.33 ± 0.35†§
Protein levels of cytokines (pg/mL) (ELISA)				
IL-10	52.17 ± 23.18	47.84 ± 10.72	46.79 ± 11.69	49.06 ± 8.51
TGF-β_1	146.81 ± 21.36	63.18 ± 16.10*	74.26 ± 11.40‡	52.19 ± 12.53†§
IL-17	55.12 ± 18.23	70.30 ± 13.65*	65.78 ± 13.97‡	75.24 ± 11.40†§
IL-6	42.56 ± 21.15	55.09 ± 17.02*	56.20 ± 22.41‡	53.75 ± 19.52§
hsCRP	60.33 ± 20.38	183.11 ± 73.02*	118.65 ± 16.72‡	253.71 ± 36.49†§

Values are expressed as mean ± SD.
The prevalence of Treg or Th17 cells was expressed by a percentage of $CD4^+CD25^+Foxp3^+/CD4^+$T or $CD4^+IL\text{-}17^+/CD4^+$ T lymphocytes.
*$P < 0.05$, Control versus total OSAS;
†$P < 0.05$, Mild to Moderate OSAS versus Severe OSAS;
‡$P < 0.05$, Control versus Mild to Moderate OSAS;
§$P < 0.05$, Control versus Severe OSAS.

compared with concentration in control group (55.12 ± 18.23 pg/mL, $P < 0.01$). The level of IL-6 in OSAS patients (55.09 ± 17.02 pg/mL) was significant higher that in control group (42.56 ± 21.15 pg/mL, $P < 0.01$), however it's comparable in severe subgroup and patients with mild to moderate OSAS ($P > 0.05$). As shown in Figure 3, while plasma TGF-β_1 in severe patients (52.19 ± 12.53 pg/mL) and mild to moderate patients (74.26 ± 11.40 pg/mL) with OSAS were markedly lower than those in the control group (146.81 ± 21.36 pg/mL; $P < 0.01$). In contrast, the same differences were not found for IL-10 ($P > 0.05$).

The levels of hsCRP were increased in OSAS group (183.11 ± 73.02 pg/mL) compared with those in control group (60.33 ± 20.38 pg/mL; $P < 0.01$). Moreover, hsCRP was markedly higher in subjects with severe OSAS (253.71 ± 36.49 pg/mL) when compared to subjects with mild to moderate OSAS (118.65 ± 16.72 pg/mL; $P < 0.01$).

3.7. Correlations between Peripheral Th17 Frequency and RORγt mRNA, Plasma IL-17 Level. In 44 patients with OSAS, the plasma concentration of IL-17 was positively correlated with peripheral blood frequencies of Th17 ($r = 0.525$, $P = 0.000$) (Figure 4(a)). There were significantly positive correlations between Th17 frequencies and the level of RORγt mRNA in PBMC from OSA patients ($r = 0.483$, $P = 0.001$) (Table 3, Figure 4(b)).

3.8. Correlations between Peripheral Treg Frequency and Foxp3 mRNA, Plasma TGF-β_1 Level. And TGF-β_1 concentration was positively correlated with peripheral blood frequencies of Treg ($r = 0.427$, $P = 0.004$) in OSAS patients (Figure 4(d)). Significantly positive correlations were found between peripheral Treg percentage and the expression of Foxp3 mRNA in PMBCs from OSAS patients ($r = 0.435$, $P = 0.003$) (Figure 4(c)). The correlations between other concentrations were all negative.

3.9. Correlation of Circulating Th17/Treg Ratios with hsCRP and Disease Severity in OSAS Patients. We next sought to analyze the correction of the ratio of Treg to Th17 cells to the disease severity in OSAS patients (Table 3). The plasma level of hsCRP has been proved to have a close relationship with severity and complication in OSAS patients. In current OSAS patients, serum level of hsCRP was positively correlated with AHI ($r = 0.458$, $P < 0.001$). In contrast, serum level of hsCRP was positively related with Th17 frequence ($r = 0.372$, $P = 0.013$) and negatively related with Treg frequence ($r = -0.433$, $P = 0.003$). And also, hsCRP level was positively correlated with the ratio of Th17/Treg ($r = 0.475$, $P = 0.001$) in OSAS patients.

As shown in Table 3, AHI index was positively correlated with circulating Th17/Treg ratio ($r = 0.321$, $P = 0.029$), but not with single percentage of Th17 or Treg in PBMC from OSAS patients ($P > 0.05$). This result suggests that decreased ratio of Treg to Th17 cells may be an indicator for the disease severity of OSAS indicated by AHI. There was no significant correlation between the other PSG parameters and the ratio of Th17 to Treg in the OSAS patients ($P > 0.05$). The relationship between BMI or ESS scores and Th17/Treg ratios in OSAS patients was also studied, but no correlations were identified ($P > 0.05$).

Table 3: Correlation coefficients between circulating Th17, Treg, or ratio and BMI, PSG parameters, and inflammatory cytokines in OSAS patients ($n = 44$).

Parameters	Th17		Treg		Th17/Treg	
	r	P	r	P	r	P
BMI	0.124	NS	0.140	NS	0.260	NS
AHI	0.284	NS	−0.290	NS	0.321	0.029
Nadir SaO_2 (%)	0.189	NS	−0.238	NS	0.194	NS
$SaO_2 < 90\%$ (%TST)	0.207	NS	−0.274	NS	0.262	NS
ESS scores	0.175	NS	−0.266	NS	0.178	NS
RORγt/GADPH mRNA	0.483	0.001	−0.114	NS	0.076	NS
Foxp3/GADPH mRNA	−0.234	NS	0.435	0.003	−0.053	NS
IL-10 (pg/mL)	0.089	NS	0.120	NS	0.095	NS
TGF-β_1 (pg/mL)	−0.291	NS	0.427	0.004	−0.211	NS
IL-17 (pg/mL)	0.525	<0.001	−0.224	NS	0.288	NS
IL-6 (pg/mL)	0.068	NS	0.106	NS	0.183	NS
hsCRP (pg/mL)	0.372	0.013	−0.433	0.003	0.475	0.001

Definition of abbreviations:
AHI: apnoea hypopnoea index; BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; ESS: Epworth sleepiness scale; nadir: lowest oxygen saturation recorded; OSAS: obstructive sleep apnoea syndrome; TST: total sleep time.

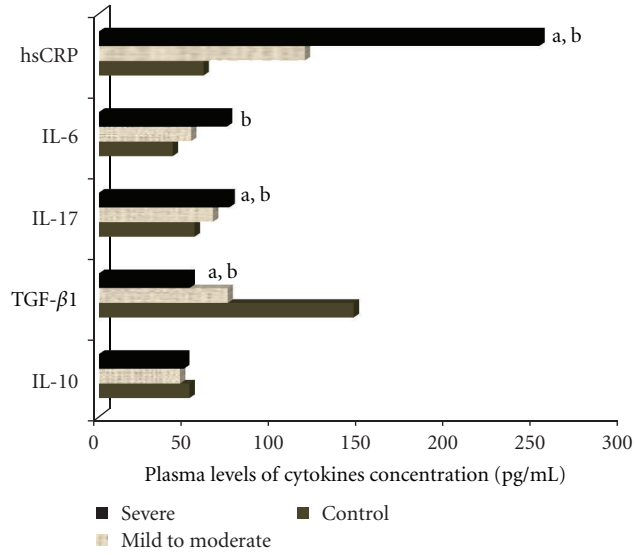

Figure 3: Represents the plasma levels of cytokines concentration in three groups (mild to moderate OSAS, severe OSAS and control group). The IL-17 and IL-6 concentrations in patients with OSAS were significantly higher when compared to concentrations in control group. While plasma TGF-β_1 concentrations in the OSAS group were significantly lower than those of control group. And plasma IL-10 concentrations were comparable in three groups. [a]$P < 0.05$, Mild to Moderate OSAS versus Severe OSAS; [b]$P < 0.05$, Control versus Severe OSAS.

4. Discussion

The present data provide direct evidence that OSAS patients exhibited a significant increase in peripheral Th17 frequency, Th17-related cytokines (IL-17) and transcription factor (RORγt) levels, and dramatic decrease in Treg frequency, Treg-related cytokines (TGF-β_1) and transcription factor (Foxp3) levels when compared to the control group. It's the first clue that circulating Treg/Th17 balance is impaired in these patients. More important, circulating Treg/Th17 ratio negatively correlated with the plasmatic hsCRP level and severity of OSAS, as determined by AHI. These results suggest a potential role for a higher ratio of circulating Th17/Treg, which indicates the systemic inflammation in the pathogenesis of OSAS and subsequent complications.

To the best of our knowledge, this is the first study to examine the circulating Th17 and Treg cells and their balance in OSAS patients. Bollinger et al. demonstrated that $CD4^+CD25^+$ natural regulatory T cells (nTreg) number and function follow a rhythm across the 24-h period and sleep deprivation severely disturbs the functional rhythm of nTreg cells [8]. In agreement, we found that the frequencies of Treg cells and the expression of Foxp3 were significantly lower in patients with OSAS when compared to normal persons. By

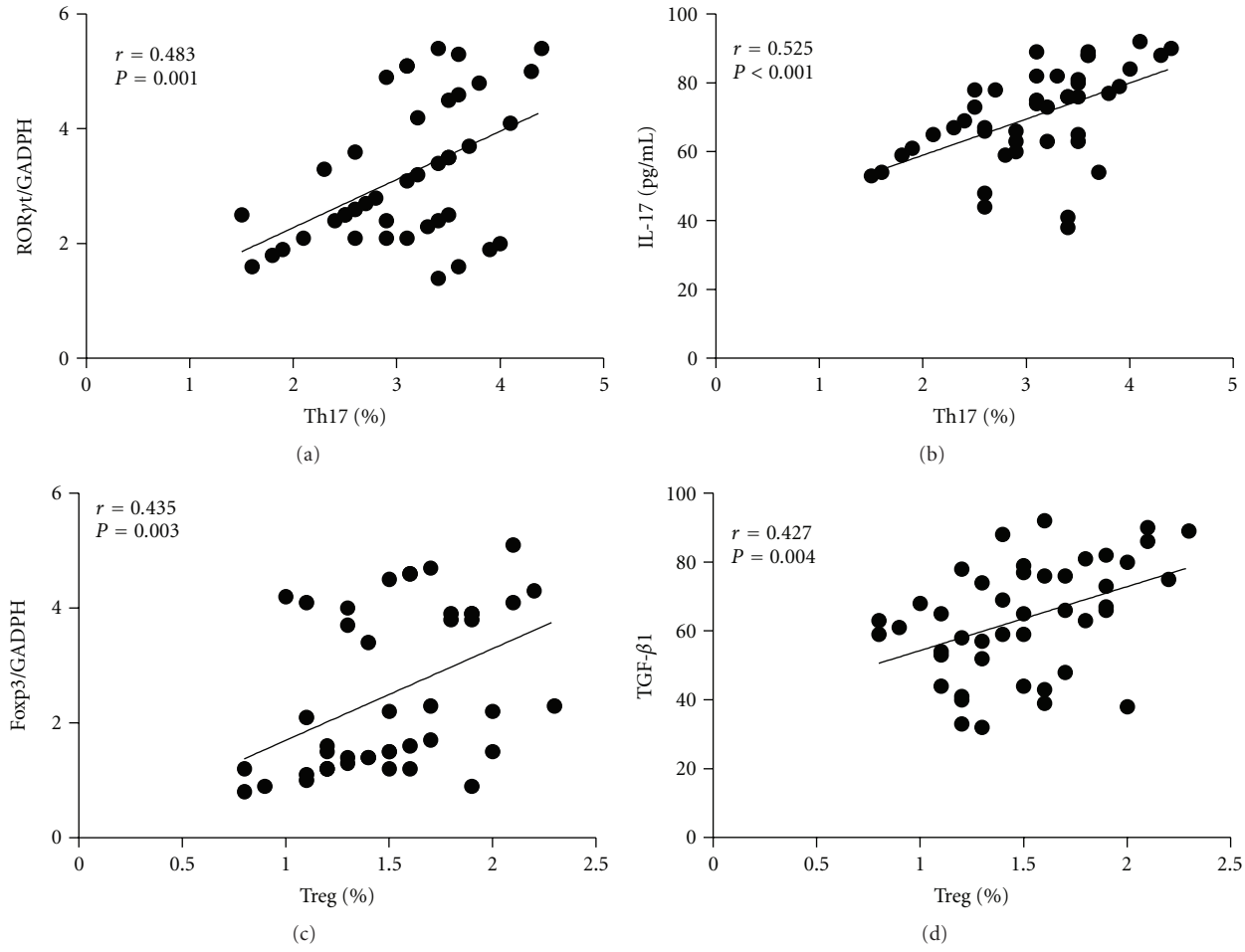

Figure 4: Spearman correlation of circulating cytokines and the frequences of Treg or Th17 cells in OSAS patients. (a) Ratios of RORγt/GAPDH mRNA positively correlate with Th17 percentage. (b) IL-17 concentrations positively correlate with Th17 percentage. (c) Ratios of Foxp3/GAPDH mRNA positively correlate with a proportion of Treg cells. (d) TGF-β_1 concentrations positively correlate with a proportion of Treg cell.

far, there is no research investigating the role of peripheral frequence of Th17 cells in any sleep disorders. In this study, we found that patients with OSAS exhibited a significant increase in peripheral Th17 number, Th17-related cytokines (IL-17) and RORγt levels when compared to normal persons. As a result, a Treg/Th17 functional imbalance exists in OSAS patients, suggesting a potential role for Treg/Th17 imbalance in the pathogenesis of OSAS.

Th17 cells and Treg cells not only share the same origin but also are mutually antagonistic in function. Th17 cells have been established as an important T-helper effectors lineage, which mediates protection against extracellular pathogens. On the negative side, Th17 cells also appear to be the driving force in the pathogenesis of autoimmune and inflammatory disorders. In contrast to Th17 cells, $CD4^+CD25^+$ and $FOXP3^+$ (Tregs) play a critical role in maintaining self-tolerance and in preventing organ-specific autoimmunity, allergy, and allograft rejection. T cell development exhibits a degree of plasticity that meets local requirements and thereby transgresses lineage barriers. Although these two T cell subsets have a reciprocal relationship and differentiation pathways, it was recently reported that Tregs are able to differentiate into IL-17-producing cells, and that human peripheral blood and lymphoid tissue contain a significant number of $CD4^+Foxp3^+$ T cells that have the capacity to produce IL-17 upon activation [22].

Especially, for the first time, we report that the ratio of Th17/Treg cells, but not peripheral Th17 or Treg frequence alone, had a linear negative correlation with OSAS severity indicated by AHI. In accordance to our results, Freire et al. found no association of AHI with total lymphocyte, neutrophil, or peripheral blood cell count alterations [9]. Recently, many investigators raised the notion of a Th17/Treg balance and reported an imbalance in patients with various autoimmune and inflammatory diseases [14–17]. A higher Th17/Treg ratio may characterize a more severity in autoimmune, inflammatory and allergic diseases. It illustrates that the balance or interplay between various types of immune cells may be the better predictors for clinical outcome of patients [23]. Our data may be compatible

with this new theory. The ratio of Treg/Th17 may reflect a specific skewed balance of anti- and proinflammatory T cell subsets in OSAS patients, while absolute numbers reflect the general activation. Because of the reciprocal developmental pathway for the generation and the opposite effects of Th17 and Treg cells, Treg/Th17 subsets may therefore have been evolved to regulate systematic inflammation, analogous to the dichotomy of Th1/Th2 T-cell subsets. As a result, the Th17/Treg ratio became an important tool in describing and understanding various immunological conditions, such as tumor, inflammatory, autoimmune, and allergic disease or models.

Adenoid hypertrophy is the most common cause of upper airway obstruction and sleep-disordered breathing in children. Sade et al. evaluated the adenoidal lymphocyte subsets to describe the percentage of various lymphocyte subsets in hypertrophied adenoids of children and correlated them with symptom severity. They found a significant negative linear correlation between the Th17/Treg ratio and the patients' clinical scores independent of age and gender [24]. Apparently, our observation was opposite to the results of Sade. However, the pathogenesis and pathophysiology of OSAS in adult and children were not completely identical. Moreover, it may reflect the inconsistence between local and systemic inflammation in OSAS. In the context of acute and chronic infectious existing in local adenoids of children, a lower Th17/Treg ratio might decrease the total clearance of microorganisms and increase chronic immune activation and proliferation of lymphocytes, thereby causing hypertrophy of lymph nodes, including adenoids and tonsils. It has recently been hypothesized that adult OSAS might be a triggering factor for the development of autoimmune phenomena [25]. Under the circumstances, a higher Th17/Treg ratio may bring on the loss of tolerance and regulation, and finally a persistent, low-degree systemic inflammatory reaction characterized by autoimmune or allergic diseases.

Activation of T lymphocytes is among the crucial steps leading to the release of inflammatory mediators and adhesion molecules. Activation of inflammatory cells and their interaction with endothelial cells have been demonstrated in OSAS [26]. In a series of experiments, Dyugovskaya et al. showed that various subpopulations of cytotoxic T cells of patients with OSAS acquire an activated phenotype with the downstream consequence of increased cytotoxicity against endothelial cells [10, 11]. The research in the past five years have demonstrated that subsets of T lymphocytes, both effector T cells such as Th1 and Th2 lymphocytes, as well as Th17 and Treg, play critical roles in the development of atherogenesis and vascular remodeling [27, 28].

Several large-scale prospective studies have shown that the hsCRP level is an independent predictor of risk of myocardial infarction, stroke, peripheral vascular disease, and vascular death, making it a useful marker for cardiovascular risk stratification [29]. Previous works have demonstrated an independent relationship between OSAS and an elevated hsCRP [30, 31]. A close link between circulating Treg/Th17 ratio and serum hsCRP level had been proved in our paper. Kim et al. recently reported that FOXP3 DNA methylation levels positively correlated with hsCRP levels and AHI in 47 OSAS children [32]. A high FOXP3 DNA methylation may favor the downregulation of Foxp3 protein expression and thus reduce the number of Tregs, thereby favoring increased systemic inflammatory responses in pediatric OSAS. Accordingly, their results corroborated our observation that the ratio of Treg/Th17 was negatively correlated with hsCRP and AHI in OSAS patients. It should be careful in understanding and comparing previous results because only sensitive measurement of hsCRP was valid to demonstrate an association with AHI.

The cytokine milieu of the local environment plays a pivotal role in the differentiation from naive $CD4^+$ T cells to Treg or Th17 cells. In the present study, results showed that the concentrations of IL-6 and IL-17, both of which promote the differentiation of Th17 cells, were all significantly higher in OSAS patients, and IL-17 concentrations were positively correlated with Th17 cells frequencies. These suggested that the proinflammatory cytokine microenvironment, characterized by elevated IL-6 and IL-17 levels and decreased TGF-β_1 concentrations, could potentially support the continued generation of Th17 cells but meanwhile suppress the development of Treg cells, which led to the Th17/Treg numerical imbalance in OSAS patients [33, 34]. This numerical imbalance might consequently result in the functional imbalance of Th17/Treg which contributed to enhancing the formation of the inflammatory cytokine microenvironment, and eventually formed a positive feedback mechanism to amplify proinflammatory immune responses.

A potential reason for the immune system activation in OSAS patients may be the obesity that represents a major risk factor and usually accompanies OSAS [35]. Although the BMI levels remained comparable, there were clearly some overweight men with a mean BMI around 28 kg/m^2 were included in our cohort. Further research concerning this issue may enroll only non-overweight OSAS groups for analysis. And other measurements, such as hip-waist ratio or densitometry of abdominal fat, can replace BMI to assess obesity in OSAS patients.

5. Conclusion

Our data offered direct evidence for the skewed balance of Th17/Treg, pro- and anti-inflammatory T cell subsets in OSAS patients. We also identified that the ratio of Th17/Treg does not only correlate with inflammatory marker but is also associated with disease severity of OSAS. Thus, our results suggest that increased ratios of Th17/Treg subpopulations may play a role in the pathogenesis of OSAS. These results opened an alternative explanation for the substantial activation of immune cells as well as the development of OSAS and complications, which may have significant impacts on the prevention and treatment of OSAS patients.

The biological significance and clinical implications of the imbalance of serum Th17/Treg merit future confirmatory and mechanistic studies in larger cohorts. The further studies may be designed to observe the change of Treg/Th17 imbalance in OSAS during constant positive airway pressure

(CPAP) treatment and to investigate the impact of comorbidies on the Treg/Th17 balance in OSAS patients. Restoration of the immune imbalance may be a future therapeutic approach for inhibiting systemic inflammatory processes in order to ameliorate the cardiovascular risk factors associated with OSAS.

Abbreviations

AHI: Apnoea-hypopnoea index
BMI: Body mass index
CPAP: Continuous positive airway pressure
CRF: Case report form
ESS: Epworth sleepiness scale
Foxp3: Forkhead box P3
hsCRP: High-sensitivity C-reactive protein
OSAS: Obstructive sleep apnoea syndrome
PBMC: Peripheral blood monouclear cells
PSG: Polysomnography
RORγt: Retinoic acid related orphan receptor γt
TST: Total sleep time.

Conflict of Interests

None of the participating institutions and authors have conflict of interests regarding the study.

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Research Article

Th17 Cell Enhances CD8 T-Cell Cytotoxicity via IL-21 Production in Emphysema Mice

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Emphysema is a T-cell mediated autoimmune disease caused predominantly by cigarette smoking. Th17 cells and related cytokines may contribute to this disorder. However, the possible implication of Th17 cells in regulating inflammatory response in emphysema remains to be elucidated. In the current study, we tested the protein levels of IL-17 and IL-21 in peripheral blood and lung tissues from cigarette-smoke- (CS-) exposed mice and air-exposed mice, analyzed the frequencies of $CD4^+IL\text{-}17^+$(Th17) cells, $IL\text{-}21^+$Th17 cells, and $CD8^+IL\text{-}21R^+$ T cells in peripheral blood and lung tissues of mice, and their relationship with emphysematous lesions, and explored the impact of IL-21 on cytotoxic $CD8^+$ T cells function *in vitro*. It was found that the frequencies of Th17, $IL\text{-}21^+$Th17, and $CD8^+IL\text{-}21R^+$ T cells and the levels of IL-17 and IL-21 of CS-exposed mice were much higher than those of the air-exposed mice and correlated with emphysematous lesions. Additionally, the number of $IL\text{-}21^+$Th17 cells positively correlated with the number of $CD8^+IL\text{-}21R^+$ T cells. The *in vitro* experiments showed that IL-21 significantly augmented the secretion of perforin and granzyme B in $CD8^+$ T cells from CS-exposed mice. These data indirectly provide evidence that Th17 cells could be involved in the control of the local and system inflammatory response in emphysema by regulating $CD8^+$ cytotoxic T-cell function.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of disability and death worldwide [1–3]. It is predominantly caused by smoking and is characterized by poorly reversible airflow limitation. Pulmonary emphysema is a major component of COPD. Although COPD is more and more common, the molecular and cellular mechanisms that are responsible for the development of COPD are not well understood. Early studies have shown that COPD is marked by the accumulation of both $CD4^+$ and $CD8^+$ T cells in the airways and lung parenchyma, with $CD8^+$ T cells predominating [4]. Recent findings suggest that COPD is an autoimmune disease characterized by an association of antielastin antibody and Th1 response [5, 6]. Th1 cells contribute principally, but not exclusively, to the pathogenesis of COPD.

IL-17 (also known as IL-17A) was first cloned in 1993 and identified as cytotoxic T lymphocyte-associated antigen (CTLA)-8 [7]. IL-17F was later discovered and closely related with IL-17A. They are all expressed in activated $CD4^+$ memory T cells [8]. Increasing evidence has indicated that IL-17 is involved in inflammatory disorders of the lungs [9, 10]. IL-17 may play an important role in the pathogenesis of COPD because of its ability to induce neutrophilic airway inflammation by stimulation of neutrophil chemotaxis and mucin gene expression in bronchial epithelial cells [11, 12]. Overexpression of IL-17 in lungs of transgenic mice may induce lung inflammation with a COPD-like phenotype [13]. Th17 cells are the newly described subset of $CD4^+$ T cells and have significant role in the progression of several T cells driven by autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, which were previously thought to be exclusively mediated by Th1 cells [14, 15]. More recently, Th17 cells have been found in COPD and emphysema [16, 17]. However, the role of Th17 cells in regulating inflammatory response in emphysema remains to be demonstrated.

IL-21 is a pleiotropic cytokine of the γ-chain family, which engages the common cytokine receptor γ-chain

expressed on cells of both lymphoid and myeloid lineages. This cytokine was originally thought to be restricted to $CD4^+$ T cells (Th1 and Th2 cells) and NKT cells, but it is now clear that IL-21 is also produced by Th17 cells [18, 19]. IL-21 can serve to recruit Th17 cells into the inflamed tissue, and also deliver intracellular signal through IL-21R and influence T-cell activation and differentiation [20]. Recently, Zeng et al. [21] demonstrated that *in vitro* exposure to IL-21 can lead to the generation of $CD8^+$ T cell in increased numbers and with enhanced function. These data suggest that Th17 cells may play role in regulating $CD8^+$ cytotoxic T-cell function via IL-21/IL-21R.

In the present study, we hypothesized that Th17 cells and IL-21 are involved in the local and system inflammatory response using a murine model of emphysema induced by smoking. We tested IL-17 and IL-21 protein levels in both peripheral blood and lung tissues of cigarette-smoke-(CS-) exposed mice and air-exposed mice, and analyzed the expression of Th17, IL-21^+Th17, and $CD8^+$IL-$21R^+$ T cells in peripheral blood and lung tissues of mice, and their relationship with emphysematous lesions. Finally, we assessed the possible modulating effect of recombinant IL-21 (rIL-21) on $CD8^+$ T cells *in vitro*.

2. Materials and Methods

2.1. Animals and Cigarette Smoke Exposure Protocol. Specific pathogen-free inbred male BALB/c mice (8 weeks of age, 20–25 g body weight) were purchased from the Guangxi Medical University Laboratory Animal Center (Nanning, China). All animal procedures were reviewed and approved by the Laboratory Animal Ethics Committee of Guangxi Medical University. All mice were housed in sterilized cages and maintained on a 12 : 12 h light-dark cycle and received sterilized food and water ad libitum.

Mice ($n = 10$) were exposed whole-body to CS, as described previously [22]. Briefly, groups of ten mice (CS-exposed mice) were exposed to five cigarettes (Nanning Jiatianxia unfiltered cigarettes: 12 mg of tar and 0.9 mg of nicotine), 4 times a day with 30 min smoke-free intervals in a closed 0.75 m^3 room, 5 days a week for 24 weeks. Mice tolerated cigarette smoke exposure without evidence of toxicity (carboxyhemoglobin levels ~10% and no weight loss). An optimal smoke : air ratio of 1 : 6 was obtained. The control groups (air-exposed mice) were exposed to 24 weeks air. The serum carboxyhemoglobin of CS-exposed mice was $8.4 \pm 1.2\%$ versus $1.0 \pm 0.3\%$ in air-exposed mice ($n = 10$), which is similar to carboxyhemoglobin blood concentrations of human smokers [23].

2.2. Tissue Processing. 24 hours after the last air or smoke exposure, the mice were sacrificed with sodium pentobarbital anesthesia. Blood samples were collected via retroorbital bleeding and were divided into 2 parts. Approximately 200 μL of peripheral blood was obtained for flow cytometric analysis, and approximately 1500 μL of peripheral blood was separated serum for enzyme-linked immunosorbent assay analysis (ELISA). The left lungs were used for histology. One part of the right lungs (30 to 50 mg) was homogenized for ELISA, and remanent parts were used for the preparation of single-cell suspensions. Spleens were harvested aseptically and minced for culture.

2.3. Morphometry. The left lungs were inflated by instilling 10% formalin at a constant pressure of 25 cm H_2O (for 10 min) and then ligated and removed. Inflated lungs were fixed for 24 h before embedding in paraffin. After paraffin embedding, 5 μm sections were cut and stained with hematoxylin and eosin for histological analysis. For each animal, 10 fields at a magnification of 100x were captured randomly from the 4 different zones of the left lung. We determined enlargement of alveolar spaces by quantifying the mean linear intercept (Lm) and destruction of alveolar walls by measuring the destructive index (DI) in CS- and air-exposed mice, as described previously [24, 25]. Two investigators independently measured Lm and DI in a blinded manner.

2.4. Preparation of Lung Single-Cell Suspensions. Lung single-cell suspensions were prepared from part of the right lung, as detailed previously [26]. Briefly, the lung was thoroughly minced, digested, passed through a 70 μm cell strainer, washed and centrifuged twice with cold PBS at 1200 rpm for 10 min at 4°C, and resuspended in PBS. The mononuclear cells were isolated from the lung single-cell suspension by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden), washed and centrifuged twice with cold PBS at 1200 rpm for 10 min at 4°C, and kept on ice until labelling.

2.5. Lymphocyte Preparation. Erythrocytes were lysed with RBC lysis buffer (Sigma-Aldrich) for 10 minutes at room temperature and the remaining cells were washed twice with cold PBS and centrifuged at 1200 rpm for 10 minutes. Fresh peripheral-blood mononuclear cells (PBMCs) were used for intracellular cytokine staining within 1 h.

After mincing spleens, the cell suspensions were pipetted rapidly with a sterile Pasteur pipette into 3 mL of RPMI 1640 (Gibco, USA), filtered through nylon mesh to eliminate debris, and centrifuged at 1000 rpm for 5 min. The cell pellets of spleens were resuspended in PBS, and the lymphocyte fractions were isolated by Ficoll-Plaque (Solarbio Science & Technology, China) gradient centrifugation. Lymphocytes were maintained in a 24-well flat-bottom tissue culture plate with RPMI 1640 supplemented with 10% fetal calf serum (Gibco, USA) at 37°C in a humidified atmosphere with 5% CO_2.

2.6. Immunofluorescence Labeling and Flow Cytometry. The expression markers on T cells were determined by flow cytometry after surface staining or intracellular staining using phycoerythrin cyanine-5-conjugated anti-mouse CD4 (PE-Cy5-CD4), fluorescein isothiocyanate-conjugated anti-mouse CD8 (FITC-CD8), phycoerythrin-conjugated anti-mouse IL-17 (PE-IL-17), phycoerythrin-conjugated anti-mouse IL-21R (PE-IL-21R), and Alexa Fluor 647-conjugated anti-mouse IL-21 (Alexa Fluor 647-IL-21). These mice Abs were purchased from BD Biosciences or eBioscience

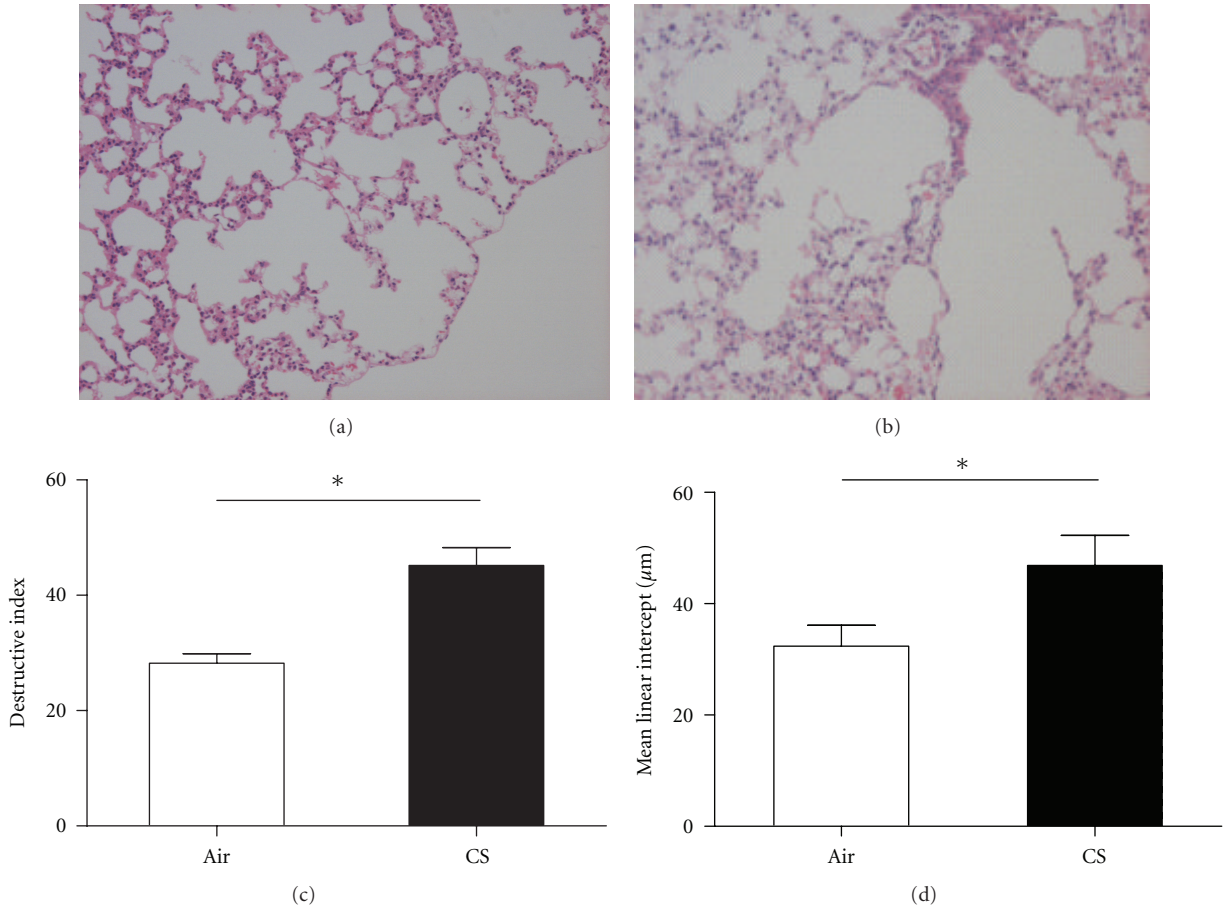

Figure 1: Photomicrographs of haematoxylin and eosin-stained lung tissue of air- and CS-exposed mice at 24 weeks (magnification, ×100). Smoke exposure clearly induced alveolar wall destruction and airspace enlargement in mice. (a) Air-exposed mice, (b) CS-exposed mice. Quantification of pulmonary emphysema. Morphometry of the lungs after chronic (24 weeks) air or CS exposure: (c) DI and (d) Lm values of mice. Results are expressed as means ± SD. $n = 10$ animals/group; $^*P < 0.001$.

(San Diego, CA). Briefly, PBMCs were stimulated with phorbol myristate acetate (PMA, 25 ng/mL, Sigma-Aldrich, USA) and ionomycin (10 μg/mL, Sigma-Aldrich, USA) in the presence of GolgiStop (BD Biosciences) for 5 h. The cells were washed and then fixed/permeabilized in the eBioscience fixation/permeabilization and permeabilization buffers according to the manufacturer's protocol [27], stained with fluorescent antibodies against CD4, CD8, IL-21R, IL-17, and IL-21. Flow cytometry was performed on a BD FACSCalibur flowcytometer and analyzed by using FCS ExpressV4 software.

2.7. Spleen CD8$^+$ T Cell Cultures and rIL-21 Stimulation. Bulk CD8$^+$ T cells from spleen were positively selected using paramagnetic microbeads conjugated to anti-mouse CD8 (Ly-2) monoclonal antibody according to the manufacturer's instructions (MACS, Miltenyi Biotec). The purity of CD8$^+$ T cells thus obtained was approximately 95%.

Purified CD8$^+$ T cells were cultured at 1×10^6 cells/mL in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, 100 g/mL streptomycin, 2 mM L-glutamine, and 50 M mercaptoethanol (RPMI 1640 complete medium 2 mL) in 96-well plates (200 μL) at 37°C, 5% CO_2, and 100% humidity with phytohemagglutinin (PHA, 10 ng/mL) and treated with or without 50 ng/mL of mrIL-21 for 3 days. A cytokine concentration of 50 ng/mL was chosen based on initial dose-response experiments with 10 to 100 ng/mL concentrations and published literature to achieve maximal effect on CD8 T-cell cytotoxic [21, 28]. The cells were washed once and restimulated with PMA/ionomycin in the presence of GolgiStop (BD Biosciences) for 5 h and then fixed/permeabilized with eBioscience fixation/permeabilization according to the manufacturer's protocol and stained with antibodies specific for intracellular perforin (PE-perforin) and granzyme B (APC-granzyme B).

2.8. Cytokine Measurement. The concentrations of IL-17 and IL-21 in the peripheral blood and the lungs, as well as perforin and granzyme B in culture supernatants, were measured by ELISA kits according to the manufacturer's protocols (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate.

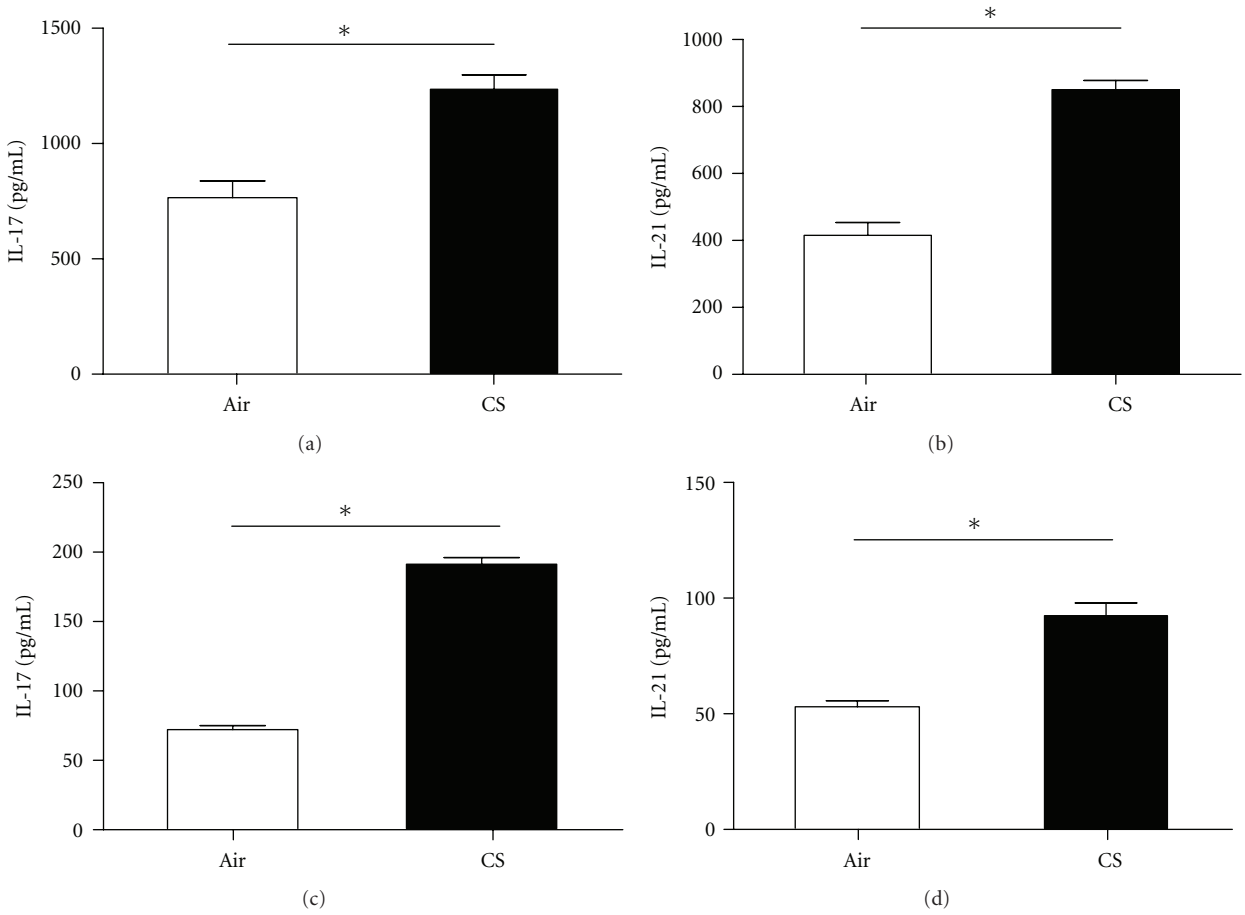

Figure 2: Protein levels of Interleukin- (IL-) 17 and Interleukin-21 (IL-21) in lungs and peripheral blood of air- and CS-exposed mice at 24 weeks by using ELISA. (a) Protein levels of IL-17 in lungs, (b) protein levels of IL-21 in lungs, (c) protein levels of IL-17 in peripheral blood, and (d) protein levels of IL-21 in peripheral blood. Results are expressed as pg/mL (mean ± SD). $n = 10$ animals/group; $^*P < 0.001$.

2.9. Statistical Analysis. All data were described as the mean ± SD. Independent-samples t-test and Pearson correlation were used for statistical analysis. Statistical analysis was performed by using SPSS statistical software version 16 (SPSS Inc., Chicago, IL), and P values <0.05 were considered as significant.

3. Results

3.1. Histological and Lung Morphometric Studies. Emphysema is a structural disorder characterized by destruction of the alveolar walls and enlargement of the alveolar spaces. Histologically, the lungs sections from the air-exposed mice showed normal alveolar structure and exhibited normal size airspaces with thin septa (Figure 1(a)). In contrast, the lungs sections from the CS-exposed mice showed an increased air space enlargement and destruction. Some airspaces seemed irregular in size, and septa were thin (Figure 1(b)). The DI was higher in CS-exposed mice (45.16 ± 3.13) compared with air-exposed animals (28.86 ± 2.07, $P < 0.001$; Figure 1(c)); exposure to cigarette smoke significantly induced airspace enlargement. The Lm was also higher in CS-exposed mice (46.87 ± 7.16 μm) compared with air-exposed animals (32.60 ± 3.21 μm, $P < 0.001$; Figure 1(d)).

3.2. IL-17 and IL-21 Protein Levels Were Significantly Elevated in CS-Exposed Mice. In the present study, using ELISA we noted that the lung levels of IL-17 and IL-21 were significantly increased in CS-exposed mice compared to air-exposed mice ($P < 0.001$, Figures 2(a) and 2(b)). Similarly, we also noted that the levels of IL-17 and IL-21 in the peripheral blood of CS-exposed mice were significantly higher than those of air-exposed mice ($P < 0.001$, Figures 2(c) and 2(d)).

To further confirm our results, we studied the relation between the levels of IL-17 and IL-21 and emphysematous lesions as measured by DI and Lm in CS-exposed mice. The lung levels of IL-17 and IL-21 were positively correlated with DI ($r = 0.87$, $P = 0.001$ and $r = 0.707$, $P = 0.022$, resp., Figures 3(a) and 3(b)) and with Lm ($r = 0.747$, $P = 0.013$ and $r = 0.821$, $P = 0.004$, resp., Figures 3(c) and 3(d)). Similarly, the peripheral blood levels of IL-17 and IL-21 were positively correlated with DI ($r = 0.757$, $P = 0.011$ and $r = 0.738$, $P = 0.015$, resp., Figures 3(e) and 3(f)) and with

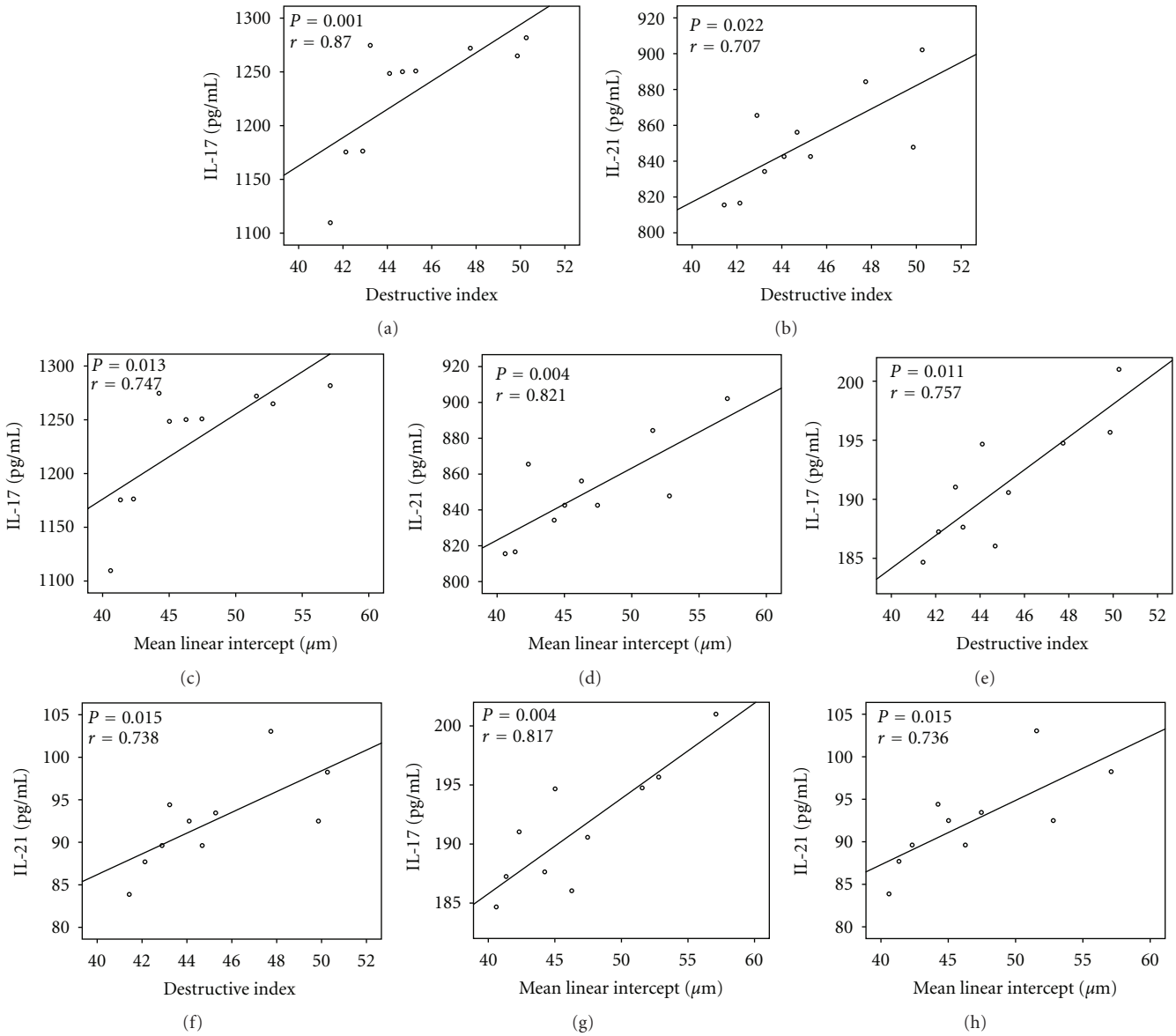

Figure 3: Correlations between (a) the protein levels of IL-17 in lungs and DI, (b) the protein levels of IL-21 in lungs and DI, (c) the protein levels of IL-17 in lungs and Lm, (d) the protein levels of IL-21 in lungs and Lm, (e) the protein levels of IL-17 in peripheral blood and DI, (f) the protein levels of IL-21 in peripheral blood and DI, (g) the protein levels of IL-17 in peripheral blood and Lm, and (h) the protein levels of IL-21 in peripheral blood and Lm. Data were determined by Pearson's rank correlation coefficients.

Lm ($r = 0.817$, $P = 0.004$ and $r = 0.736$, $P = 0.015$, resp., Figures 3(g) and 3(h)).

3.3. The Frequencies of Th17, IL-21$^+$Th17, and CD8$^+$IL-21R$^+$ T Cells Were Increased in CS-Exposed Mice. We analyzed the frequencies of Th17, IL-21$^+$Th17, and CD8$^+$IL-21R$^+$ T cells in the peripheral blood and the lungs by using flow cytometry. The frequencies of Th17 and IL-21$^+$Th17 cells in the peripheral blood of CS-exposed mice were significantly increased compared to air-exposed littermates ($P < 0.05$, Figures 4(a) and 4(b)). Also after chronic CS-exposure for 6 months, a significant increase in CD8$^+$IL-21R$^+$ T cells was observed in the peripheral blood from CS-exposed mice compared with those in air-exposed littermates ($P < 0.001$, Figure 4(c)). In addition, the frequencies of Th17, IL-21$^+$Th17, and CD8$^+$IL-21R$^+$ T cells in the lungs of CS-exposed mice were significantly higher than those of the controls ($P < 0.05$, Figures 5(a), 5(b), and 5(c)).

3.4. Correlation between Frequencies of Th17, IL-21$^+$Th17, and CD8$^+$IL-21R$^+$ T Cells and Emphysematous Lesions in CS-Exposed Mice. The frequencies of peripheral blood Th17, IL-21$^+$Th17, and CD8$^+$IL-21R$^+$ T cells were positively correlated with DI ($r = 0.892$, $P = 0.001$; $r = 0.777$, $P = 0.008$

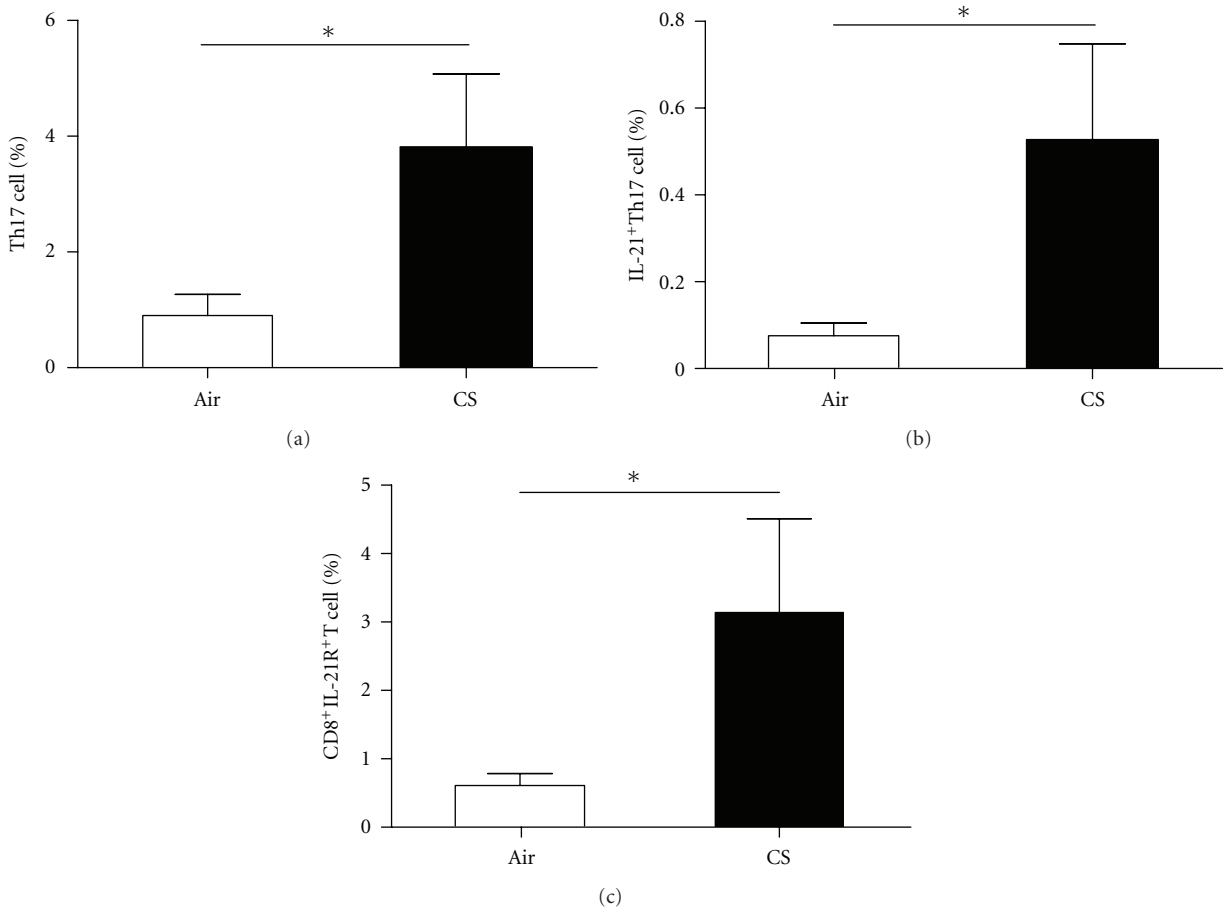

Figure 4: The frequency of $CD4^+IL\text{-}17^+$(Th17), cells, IL-21^+Th17 cells and $CD8^+IL\text{-}21R^+$ T cells in peripheral blood was measured by using flow cytometry. The frequency (%) of $CD4^+IL\text{-}17^+$(Th17) cells (a), IL-21^+Th17 cells (b), and $CD8^+IL\text{-}21R^+$ T cells (c) increased in peripheral blood of CS-exposed mice compared with air-exposed mice. Results are expressed as % (mean ± SD). $n = 10$ animals/group; $^*P < 0.05$.

and $r = 0.697$, $P = 0.025$, resp.) (Figures 6(a), 6(c), and 6(e)) and with Lm ($r = 0.757$, $P = 0.011$; $r = 0.789$, $P = 0.007$ and $r = 0.716$, $P = 0.020$, resp.) (Figures 6(b), 6(d), and 6(f)) in CS-exposed mice. In addition, IL-21^+Th17 cells were correlated positively with $CD8^+IL\text{-}21R^+$ T cells ($r = 0.648$, $P = 0.005$) (Figure 6(g)).

The frequencies of lung Th17, IL-21^+Th17, and $CD8^+IL\text{-}21R^+$ T cells were significantly and positively correlated with DI ($r = 0.861$, $P = 0.001$; $r = 0.700$, $P = 0.024$ and $r = 0.818$, $P = 0.004$, resp.) (Figures 7(a), 7(c), and 7(e)) and with Lm ($r = 0.865$, $P = 0.001$; $r = 0.785$, $P = 0.007$ and $r = 0.885$, $P = 0.001$, resp.) (Figures 7(b), 7(d), and 7(f)) in CS-exposed mice. In addition, IL-21^+Th17 cells were correlated positively with $CD8^+IL\text{-}21R^+$ T cells ($r = 0.73$, $P = 0.017$) (Figure 7(g)). Our findings indicated that Th17 cells might be able to regulate $CD8^+$T cells via IL-21/IL-21R system.

3.5. IL-21 Upregulates Perforin and Granzyme B Expression in $CD8^+$ T Cells. To evaluate the contribution of IL-21 to the function of $CD8^+$ T cells in CS-exposed mice, we next cultured $CD8^+$ T cells *in vitro* with rIL-21 to analyze cytokine production. We isolated $CD8^+$ T cells by MACS from CS-exposed mice and from air-exposed mice. The purified $CD8^+$ T cells were cultured in the presence of PHA (10 ng/mL) and rIL-21 (50 ng/mL) or PHA (10 ng/mL) alone for 3 days. Results showed that administration of rIL-21 and PHA significantly upregulated the expression of perforin and granzyme B in $CD8^+$ T cells; this effect was greater in CS-exposed mice than in air-exposed mice (Figure 8(a)). Notably, there were more perforin+ cells (88.29 ± 11.03%) than granzyme B+ cells (69.47 ± 5.31%, $P < 0.01$) in $CD8^+$ T cells of CS-exposed mice (Figure 8(a)). In parallel, the protein levels of perforin and granzyme B in the culture supernatants significantly increased following 3 days of culture with IL-21 and PHA; this effect was also greater in CS-exposed mice than in air-exposed mice (Figure 8(b)). The protein levels of perforin in the culture supernatants of CS-exposed mice (24.47 ± 2.61 ng/L) were significantly increased more than the levels of granzyme B (1.92 ± 0.21 ng/L, $P < 0.001$) (Figure 8(b)). These data suggest that $IL\text{-}21R^+CD8^+$ T cells have the capacity to secrete perforin and granzymes and further support the potential relevance of Th17 cells in emphysema.

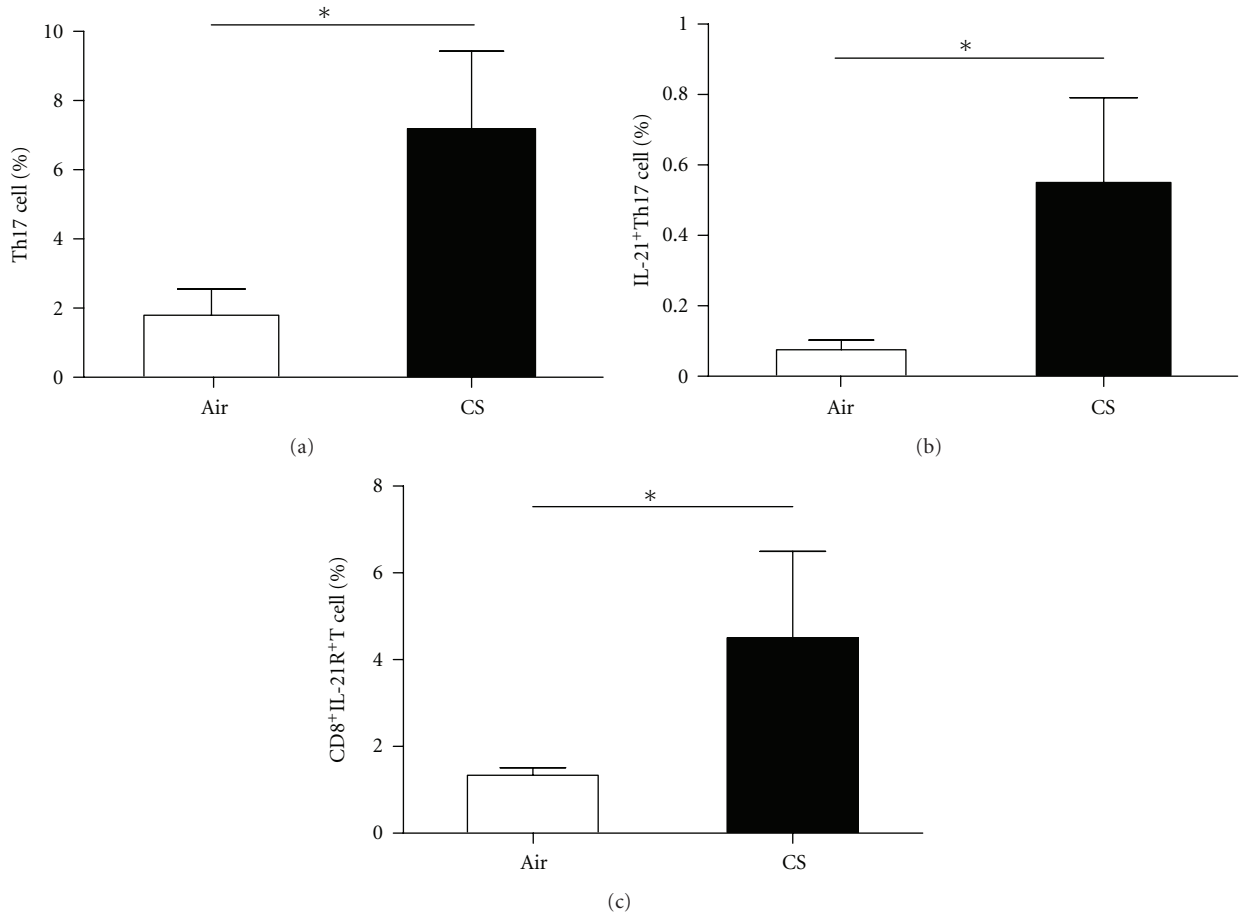

Figure 5: The frequencies of $CD4^+IL\text{-}17^+$(Th17) cells, $IL\text{-}21^+$Th17 cells, and $CD8^+IL\text{-}21R^+$ T cells in lungs were measured by using flow cytometry. The frequencies (%) of $CD4^+IL\text{-}17^+$(Th17) cells (a), $IL\text{-}21^+$Th17 cells (b), and $CD8^+IL\text{-}21R^+$ T cells (c) increased in lungs of CS-exposed mice compared with air-exposed mice. Results are expressed as % (mean ± SD). $n = 10$ animals/group; $^*P < 0.05$.

4. Discussion

Emphysema is considered a T-cell-mediated autoimmune disease, but its etiology and pathology have not been elucidated. In this study, we employed a murine model of cigarette smoke-induced lung emphysema to investigate the capacity of Th17 cells to participate in emphysema pathogenesis. We found that the numbers of Th17, $IL\text{-}21^+$Th17, and $CD8^+IL\text{-}21R^+$ T cells and the levels of IL-17 and IL-21 in the peripheral blood and lungs of CS-exposed mice were much higher than those of air-exposed mice and correlated with emphysematous lesions. Additionally, the number of $IL\text{-}21^+$Th17 cells positively correlated with the number of $CD8^+$ $IL\text{-}21R^+$ T cells. Furthermore, IL-21 significantly augmented the secretion of perforin and granzyme B in $CD8^+$ T cells from CS-exposed mice *in vitro*. These data indirectly demonstrate that IL-21 produced by Th17 cells can act on $CD8^+$ T cells to promote cytotoxic function.

$CD4^+$ T cells are known as important key cells in immunoregulation, whereas $CD8^+$ T cells have cytotoxic function in COPD [29]. Classically, naive $CD4^+$ T cells have been thought to differentiate into two main lineages, Th1 and Th2 cells on the basis of their cytokines secretion and immune regulatory function [30]. Regulatory T cells represent only a small subset of $CD4^+$ T cells in the peripheral circulation and are responsible for the balance of immune responses, which is essential for health [31]. Th17 cell changes the classical Th1/Th2 paradigm of Th cell differentiation [32]. Early studies have suggested that infiltrating $CD4^+$ T cells in COPD exhibit a Th1 phenotype [29]. Our previous study showed that decreased regulatory T cells were found in lungs of emphysema group [33]. More recently, we have reported that increased Th17 cells could be found in lungs of smoke-exposed mice, and these Th17 cells might be due to Th17 differentiation stimulated by lung proinflammatory cytokines and to recruitment of Th17 cells via CCR6/CCL20 [34]. In the current study, we also demonstrated that increased Th17 cells were present in peripheral blood and lungs of CS-exposed mice. More important, the increased frequency of Th17 cells positively correlated with emphysematous lesions. These findings are in agreement with the *in vivo* data of Shan et al. [17], who demonstrated that Th17 cells were present in lungs from patients with emphysema, and Harrison et al. [35] also found that Th17 cells presented in the BALF from smoke-exposed

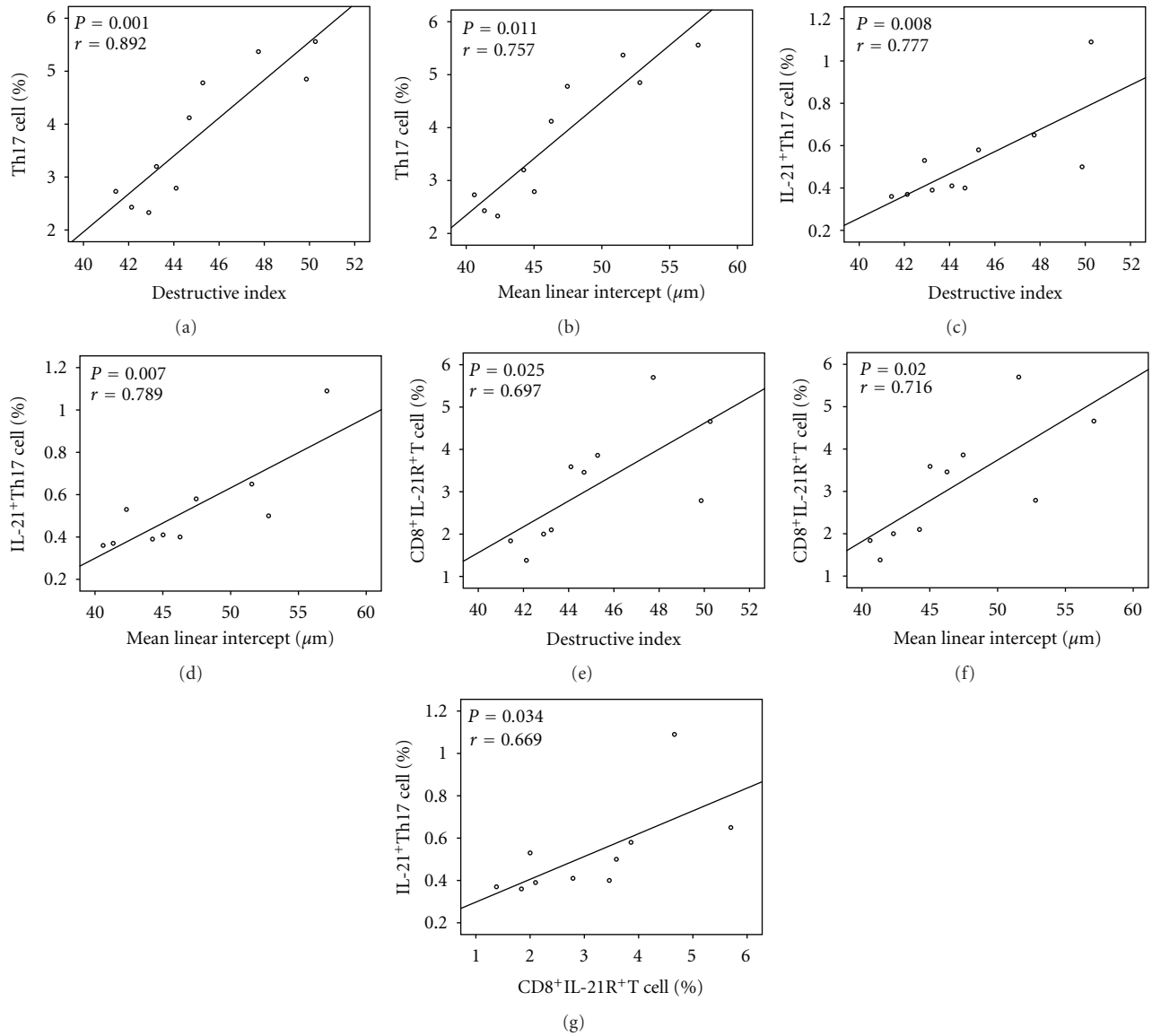

Figure 6: Correlations between (a) the frequency of $CD4^+IL\text{-}17^+Th17$ cells in peripheral blood and DI, (b) the frequency of $CD4^+IL\text{-}17^+Th17$ cells in peripheral blood and Lm, (c) the frequency of $IL\text{-}21^+Th17$ cells in peripheral blood and DI, (d) the frequency of $IL\text{-}21^+Th17$ cells in peripheral blood and Lm, (e) the frequency of $CD8^+IL\text{-}21R^+$ T cells in peripheral blood and DI, (f) the frequency of $CD8^+IL\text{-}21R^+$ T cells in peripheral blood and Lm, and (g) the frequency of $CD8^+IL\text{-}21R^+$ T cells and the frequency of $IL\text{-}21^+Th17$ cells in peripheral blood. Data were determined by Pearson's rank correlation coefficients.

mice. Thus, these studies indicated that Th17 cells may have a relevant role in the local and system inflammatory process of COPD.

Th17 cells secrete not only IL-17A but also IL-17F, IL-21, and IL-22, these cytokines most likely induce tissue inflammation [36]. Recent studies have been showed that IL-17A and IL-17F could stimulate chemokine production and promote neutrophil and macrophage recruitment to the lung [37, 38]. But additional roles for this and other Th17-derived cytokines in COPD remain largely unexplored. IL-21 might act in a positive feedback loop, preserving and/or amplifying generation of Th17 cells [39, 40], and serve to recruit Th17 cells into the inflamed tissue [41]. Moreover, Leonard and Spolski [42] indicated that IL-21 could significantly increase lymphocytes survival and cytolytic potential. Given these findings, it is perhaps not surprising that exaggerated IL-17 and IL-21 responses are implicated in the pathogenesis of COPD. In the present study, significantly elevated levels of IL-17 and IL-21 were found in the peripheral blood and lungs from CS-exposed mice. In addition, a positive correlation between levels of IL-17 and IL-21 and emphysematous lesions was found in CS-exposed mice.

Although these findings suggest that the enhanced Th17 cells and IL-21 production in emphysema are biologically relevant, the mechanism for Th17 cells in emphysema pathogenesis remains unidentified. Several studies have shown that

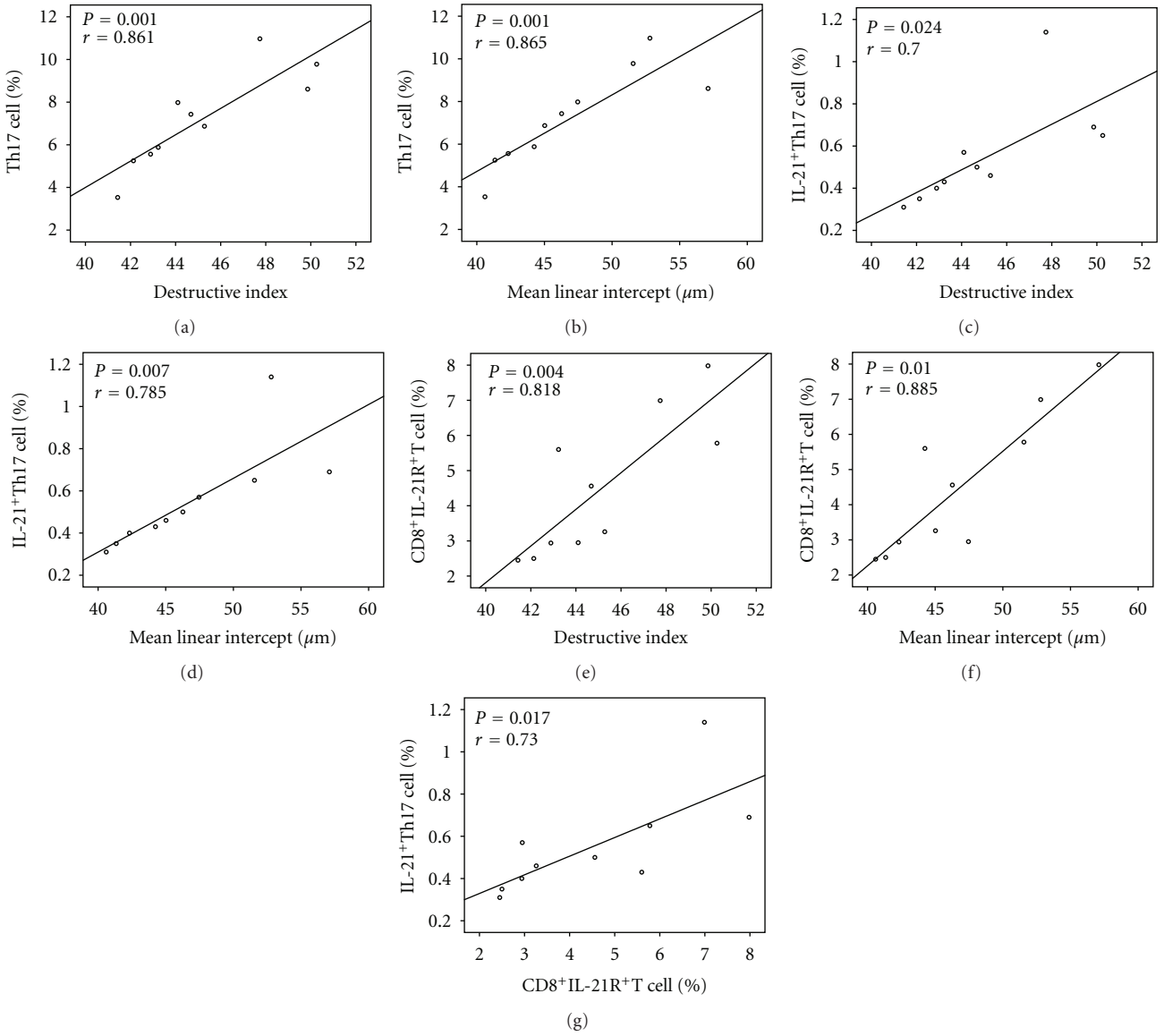

Figure 7: Correlations between (a) the frequency of $CD4^+IL\text{-}17^+Th17$ cells in lungs and DI, (b) the frequency of $CD4^+IL\text{-}17^+Th17$ cells in lungs and Lm, (c) the frequency of $IL\text{-}21^+Th17$ cells in lungs and DI, (d) the frequency of $IL\text{-}21^+Th17$ cells in lungs and Lm, (e) the frequency of $CD8^+IL\text{-}21R^+$ T cells in lungs and DI, (f) the frequency of $CD8^+IL\text{-}21R^+$ T cells in lungs and Lm, and (g) the frequency of $CD8^+IL\text{-}21R^+$ T cells and the frequency of $IL\text{-}21^+$ Th17 cells in lungs. Data were determined by Pearson's rank correlation coeficients.

the number of $CD8^+$ T cells found in the lungs of patients with COPD correlates with disease severity [43], and lung $CD8^+$ T cells may directly cause cytotoxicity contributing to emphysema by inducing apoptosis through secretion of perforin, granzyme, and by Fas/Fas ligand (FasL) interactions [44]. Further, it has been demonstrated that $CD4^+$ T cells are essential in the promotion of functional $CD8^+$ T-cell memory after an acute infection [45]. Therefore, it is likely that Th17 cells may participate in COPD immunoregulation via generation of $CD8^+$ cytotoxic T cells.

IL-21 is an effector cytokine that is made predominantly by Th17 cells [36]. It mediates its effects through a class I cytokine family receptor IL-21R, which specifically binds IL-21 [20]. In the current study, we demonstrated that the number of $IL\text{-}21^+Th17$ and $CD8^+IL\text{-}21R^+$ T cells was significantly increased in the lungs and peripheral blood of CS-exposed mice. More importantly, a significantly positive correlation between the number of $IL\text{-}21^+Th17$ and $CD8^+IL\text{-}21R^+$ T cells and emphysematous lesions was found in CS-exposed mice, which indicated the importance of $IL\text{-}21^+Th17$ and $CD8^+IL\text{-}21R^+$ T cells in the development of emphysema. In addition, a significant correlation existed between $IL\text{-}21^+Th17$ and $CD8^+IL\text{-}21R^+$ T cells in the lungs and peripheral blood of CS-exposed mice. This suggests that Th17 cells could produce IL-21 to mediate inflammatory response through IL-21R, which is expressed on $CD8^+$ T cells. Thus, we presume that IL-21 released from Th17 cells might play a more important role in

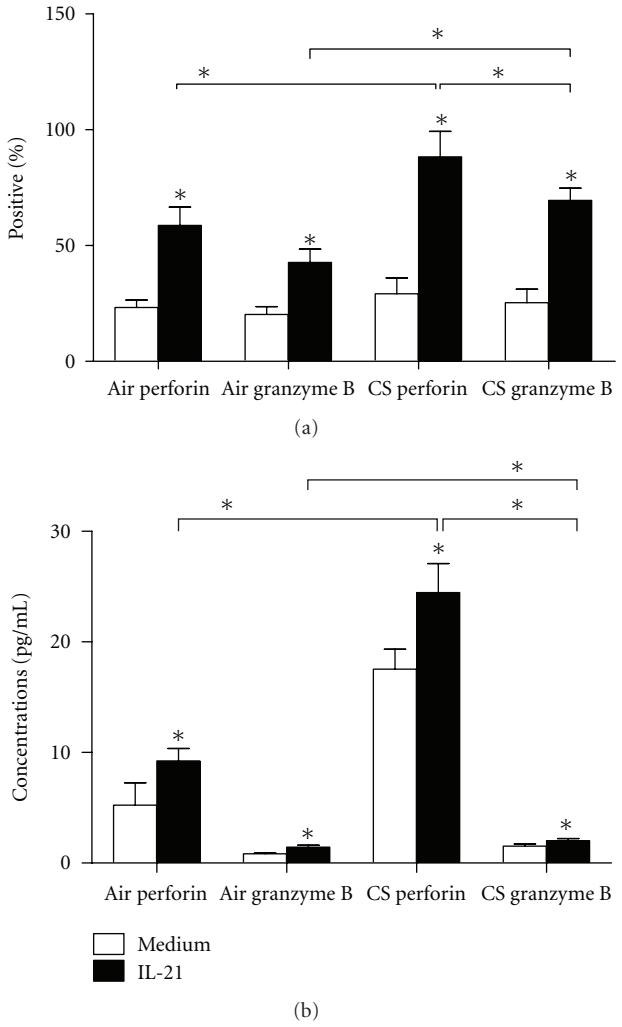

Figure 8: IL-21 upregulates perforin and granzyme B expression and protein levels in $CD8^+$ T cells. The purified $CD8^+$ T cells were cultured in the presence of PHA (10 ng/mL) and rIL-21(50 ng/mL) or PHA (10 ng/mL) alone for 3 days and analyzed for (a) perforin and granzyme B expression in $CD8^+$ T cells of air-exposed and CS-exposed mice ($n = 10$). (b) Perforin and granzyme B concentrations in $CD8^+$ T cells of air-exposed and CS-exposed mice ($n = 10$). Significance was determined by independent-samples t-test.

the immunopathology of emphysema through its actions on $CD8^+$ T cells.

To further address this issue, we examined the capacity of rIL-21 to promote $CD8^+$ T cells functions *in vitro* and found that coculture of IL-21 and $CD8^+$ T cells results in cells producing increased amounts of perforin and granzyme-B upon chronic smoke expose. Interestingly, the $CD8^+$ $perforin^+$ cells were proportionally higher than $CD8^+$ granzyme B^+ cells, and the perforin levels were also much higher than the granzyme B levels. Perforin can form pores in the target cells' membranes, while granzymes, as serine proteases, enter the cytoplasm of the target cells, altering their function and/or activating cell death [46]. These observations indirectly demonstrate that IL-21 produced by Th17 cells can promote $CD8^+$ T cells to induce apoptosis and tissue damage via the granzyme-B/perforin-mediated pathway. However, the precise mechanism for the effect of Th17 cells on the development of smoke-induced emphysema undoubtedly needed successive studies.

5. Summary

Our data showed that the frequencies of Th17, IL-21^+Th17, and $CD8^+$IL-$21R^+$ T cells and the levels of IL-17 and IL-21 in the peripheral blood and lungs of CS-exposed mice were significantly increased compared to controls and correlated with emphysematous lesions. Furthermore, expression of perforin and granzyme B by $CD8^+$ T cells was increased by *in vitro* stimulation with IL-21. Our findings support the concept that Th17 cells and related cytokine IL-21 were involved in the pathogenesis of COPD. However, to the best of our knowledge, although in the *in vitro* experiments IL-21 promotes $CD8^+$ T cell cytotoxic responses, whether or not IL-21 derived from the Th17 cells contributes to the enhanced $CD8^+$ T cells function response remains unclear. Further research should be done by using adoptive transfer of Th17 cells and IL-21-null (or IL-21 deficient) mice.

Authors' Contribution

Min-Chao Duan designed and performed research, analyzed data, and wrote the paper; Xiao-Ning Zhong designed research and reviewed the paper; Ying Huang participated in setting up the model for emphysema induction in mice, performed research, and analyzed data; Hai-Juan Tang participated in setting up the model for emphysema induction in mice and performed research. All authors read and approved the final paper. Min-Chao Duan and Ying Huang contributed equally to this work.

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Clinical Study

IL-8, IL-10, TGF-β, and GCSF Levels Were Increased in Severe Persistent Allergic Asthma Patients with the Anti-IgE Treatment

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Background. Allergic asthma is showed an increase in Th2-cytokine and IgE levels and an accumulation activation of Th2 cells, eosinophils and mast cells. However, recent studies focused on cell-based mechanisms for the pathogenesis of allergic asthma. *Objectives*. In this study, we compare the anti-IgE treatment modality in the dynamics of immune system cytokine levels in severe persistent asthma (SPA) patients who had no other any allergic disease, newly diagnosed allergic asthma patients and healthy volunteers. *Study Design*. The study population consisted of 14 SPA patients, 14 newly diagnosed allergic asthma patients and 14 healthy volunteers included as controls. Cytokine levels were measured. Total and specific IgE levels of anti-IgE monoclonal antibody treated patients, serum high-sensitivity C-reactive protein (hsCRP) levels, FEV1/FVC rates and asthma control test (ACT) were measured for the clinical follow-up. *Results*. We observed that SPA patients presented increasing levels of IL-8, IL-10, TGF-β and GCSF during the anti-IgE treatment in period of sampling times at 4 months and 18 months. However this increase was not correlated neither with serum hsCRP levels nor FEV1/FVC rates. *Conclusions*. Our study gives a different perspective for the SPA and anti-IgE immunotherapy efficacy at the cell cytokine-linked step.

1. Introduction

Asthma is the most common serious chronic lung disease that affects people of all ages with evidence for a growing prevalence in industrialized as well as in developing countries [1, 2]. With airway hyper-responsiveness being the physiological hallmark of asthma, it is also characterized by chronic inflammation of the respiratory tract, allergen-specific IgE production, infiltration of eosinophils, the recruitment of T cells into the airways, and alterations in the fine balance between type 1 helper T lymphocytes (Th1) and type 2 helper T lymphocytes (Th2) responses towards Th2 bias [3, 4].

Th2 cells secrete a panel of cytokines with several overlapping functions including Interleukin-4 (IL-4), IL-5, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF). By mediating differentiation of the Th2 subpopulation and eosinophils, as well as modulating B-cell proliferation and IgE switching, the Th2 cytokines are thought to play a prominent role in asthma [5, 6]. The sentinel Th1 cytokine, interferon gamma (IFNγ), and IL-12 reciprocally stimulate their production and function during cell-mediated immunity and development of naïve T lymphocytes into Th1 cells. Evidence suggests a contributory role of Th1 cells and their cytokines in asthmatic inflammation and airway hyper-responsiveness [7, 8]. The T cell subset of regulatory T cells (Treg) acts by expressing immunosuppressive cytokines, such as IL-10, of which impaired production has been reported in asthmatic patients [9]. Moreover, the lymphocyte lineage Th17 is increased in inflamed airways and characterized by the production of IL-17 [10, 11]. This proinflammatory cytokine is capable of causing the release of other proinflammatory cytokines, such as IL-8, tumor necrosis factor alpha (TNFα), and GM-CSF,

which have been associated with asthma in murine models, in humans or with disease severity [12–18].

Asthma—probably the most heterogeneous lung disease—classification is based on severity and there is no universally accepted utility in diagnosis of asthma and certain subtypes. Current Global Initiative for Asthma (GINA) guidelines emphasize the need to evaluate asthma control to guide asthma management decisions. The Asthma Control Test (ACT) questionnaire—a simple, self-administered, and rapidly completed assessment tool—is also appropriate to assess the patients and has an advantage of can be applied at all levels of healthcare. Symptoms in the most severe form of asthma, also called "severe persistent allergic asthma", are thought to be precipitated by allergens. In addition to allergens, environmental factors or infectious pathogens often trigger epithelial stress and altered innate immunity that induce different types of inflammation, thereby resulting in the heterogeneous forms of asthma.

Most recent treatment modality—anti-IgE therapy—was developed for severe allergic asthma. Anti-IgE therapy affects by lowering free IgE and leading to downregulation of high-affinity IgE receptors on circulating basophils and mast cells. So that the early and late phase responses to inhaled allergens will be attenuated [19–22].

This study surveyed the levels of chosen serum IL-8, IL-17, TGF-β, and GCSF of the allergic asthma patients treated with anti-IgE therapy to investigate their roles in the pathogenesis of disease perpetuation, and anti-IgE therapy's impact on them.

2. Materials and Methods

2.1. Patients Samples. Twenty eight allergic asthma—allergic rhinitis patients were included in the study and divided into two groups according to the severity. In the first group there were 14 patients of 5 male and 9 female, whom were suffering from severe persistent allergic asthma—allergic rhinitis and underwent anti-IgE therapy for 18 months within the product label (omalizumab) every 2 weeks. Assessment of clinical changes and adverse effects were evaluated at each bimonthly patient visit including vital signs, full physical examination, details of any allergy incidents, total and specific IgE levels, serum high-sensitivity C-reactive protein levels, pulmonary function test (FEV1/FVC rates), and asthma control test (ACT) (Quality Metric Incorp.). A spirometry was performed at each visit (once or twice a month depending on the patient's visit schedule). Reference values for the Mediterranean population were used [23]. Need for the steroid therapy and doses they were using were given in Tables 1 and 2. Blood samples were taken during these followups first in the time of diagnosis (Group IA), 4 months after the anti-IgE therapy (Group IB), and at the 18th month of treatment during the remission (Group IC).

The other patients group included the newly diagnosed allergic asthma-allergic rhinitis patients (non-severe) (Group II).

The healthy volunteers (group III, $n = 14$) had no history of allergy/atopy, family atopy, cardiac and pulmonary diseases or smoking.

The study was approved by the local ethics committee, and written consent was obtained from all patients and healthy volunteers.

2.2. Treatment Control. Patients were asked to describe their asthma treatment at each outpatient visit, and the total monthly oral corticosteroid dose was recorded. In the case of exacerbation, patients were asked to come to the hospital, if possible to the outpatient center at our pulmonary service during business hours rather than the emergency room (ER) in order to facilitate treatment control. Nonetheless, data for patients who came to the ER and discharge treatment were recovered, since the clinical histories at the hospital are computerized.

2.3. Skin Prick Test (SPT). Skin prick tests on the forearm were performed in all patients using standardized latex extract containing high ammonia natural rubber latex, and a full set of 35 common and 35 food allergens. In addition, venom SPT was performed on one patient based on the subject's clinical history. SPTs were performed by skilled nursing personnel. Positive tests were counted as wheals of 3 mm in diameter after 20 minutes. Tests were compared with positive histamine controls and negative saline controls. Commercial extracts used were manufactured by Allergopharma (Germany). No intradermal tests were performed.

2.4. Treatment Protocol. Best Standard Care (BSC) following the recommendations of the GINA included inhaled corticosteroids (fluticasone 500 mg bid), inhaled long-acting beta-agonists (LABA) (salmeterol 50 mg bid), and oral methyl-prednisolone. Prior to starting omalizumab treatment, patients underwent a run-in period of at least 18 months. The protocol followed for decreasing oral steroid administration was as follows; the daily dose was decreased by 2 mg/day; if the patient remained stable, at the end of the two weeks the daily dose was decreased by a further 2 mg for the following weeks. Steroid dose was then increased to the previous level and the process was repeated.

2.5. Experimental Procedures. Concentrations of IL-8, IL-10, IL-17, TNF-α, TGF-β, and GCSF in the serum samples were quantified using ELISA kits. The assays were performed according to the recommendations of the manufacturer using standard curve for every cytokine. The results were reported as means of duplicate measurements.

Total and specific IgE levels were enumerated by fluoroenzyme immunoassay (ImmunoCAP—FEIA) using an ImmunoCAP (Pharmacia, Uppsala, Sweden) kit. Values above 100 kU/L and 0.35 kU/L for total and specific IgE levels were considered abnormal.

Serum hs-CRP levels were measured using a hs-CRP assay (Behring Latex-Enhanced using the Behring Nephelometer BN-100; Behring Diagnostics, Westwood, MA, USA). The sensitivity of the assay ranged 0.04–5.0 mg/L.

2.6. Statistical Analysis. All the data were analyzed by using student t-test with the statistical package for the Social

Table 1: Demographics of severe persistent asthma patients (Group I).

Patient	Age (y) and Sex	Prick Test Positivity	Number of Injection	Injection Dose of Omalizumab	Inhalant Steroid Doses Pre-omalizumab	Oral Steroid Doses Pre-omalizumab
1	62 male	Grass, tree, mold, mite	30	375 mg q. 2 weeks	600 μg	6 mg
2	39 male	Grass, mite, cockroach	28	225 mg q. 2 weeks	500 μg	8 mg
3	50 male	Wheat, Mite, tree	31	300 mg q. 2 weeks	400 μg	0
4	19 female	Mold, mite, dog epithelia	27	225 mg q. 2 weeks	500 μg	0
5	18 female	Grass, wheat, tree, mold, mite, cockroach, kiwi and orange, cat epithelia	29	300 mg q. 2 weeks	500 μg	0
6	57 female	Grass, tree, mite	33	300 mg q. 2 weeks	600 μg	6 mg
7	50 female	Grass, wheat, tree, mold, mite, cockroach, tomato, eggplant, strawberry, dog and cat epithelia	34	300 mg q. 2 weeks	800 μg	8 mg
8	34 female	Grass, tree, mite	29	300 mg q. 2 weeks	600 μg	6 mg
9	42 female	Grass, wheat, tree, mold, mite, cockroach, honeybee, dog and cat epithelia	31	300 mg q. 2 weeks	1200 μg	12 mg
10	59 male	Grass, wheat, tree, mold, mite, cockroach, shrimp, perch, egg and latex	32	300 mg q. 2 weeks	1000 μg	10 mg
11	49 female	Grass, tree, mite, dog epithelia	28	300 mg q. 2 weeks	800 μg	8 mg
12	37 female	Mold, mite, cockroach	34	300 mg q. 2 weeks	400 μg	0
13	52 male	Mold, mite, dog epithelia	21	300 mg q. 2 weeks	600 μg	6 mg
14	48 female	Grass, wheat, tree, mite, cockroach	23	225 mg q. 2 weeks	400 μg	0

Table 2: Demographics of controlled allergic asthma patients (Group II) and control group (Group III).

Patient	Age (y) and Gender Group II	Inhalant Steroid Doses Group II	Prick Test Positivity of Group II	Age (y) and Gender Group III
1	58 male	200 μg	Grass, mite, cockroach	58 male
2	42 male	200 μg	Grass, mite, cockroach	39 male
3	54 male	100 μg	Wheat, mite, grass	55 male
4	22 female	100 μg	Mold, mite	21 female
5	20 female	200 μg	Grass, wheat, tree, mold, cat epithelia	19 female
6	62 female	100 μg	Grass, tree, mite	58 female
7	49 female	100 μg	Grass, wheat, mold, mite, dog and cat epithelia	50 female
8	40 female	200 μg	Grass, cockroach, mite	38 female
9	43 female	100 μg	Grass, tree, mold, mite, cat epithelia	43 female
10	59 male	100 μg	Grass, tree, mold, mite	61 male
11	49 female	100 μg	Mite, dog epithelia, tree	50 female
12	39 female	100 μg	Mold, mite, cockroach	42 female
13	52 male	200 μg	Mold, mite, cockroach, dog and cat epithelia	53 male
14	48 female	100 μg	Grass, wheat, tree, mite, cockroach	50 female

Sciences 13.0 software for Windows (SPSS Inc., Chicago, III). A P value less than 0.05 was considered to be statistically significant. GraphPad Prism version 5 (La Jolla, CA, USA) were used to plot the data and perform correlation analyses. All correlation analyses used Spearman's Rho tests.

3. Results

Main demographic and clinical characteristics of study participants were summarized in Tables 1 and 2. Clinical data from the patients during the treatment with anti-IgE,

indicated the beneficial effects on symptoms and perceived quality of life without any exacerbation, as well as a reduction in unscheduled healthcare visits.

In Table 2, healthy and asthmatic patients are compared as well as healthy and severe persistent asthma patients. Except of IL-8, IL-10, TGF-β, and GCSF in the anti-IgE treated severe persistent asthma patients group, none of the cytokine concentrations in serum differ significantly between healthy and severe persistent asthma diseased patients (Figures 1, 2, 3, 4, and 5—only the significant data with P values were given as in figures). These values were also increased during the anti-IgE therapy and this difference is significant between the fourth/eighteenth month of anti-IgE therapy and control group and newly diagnosed allergic asthma patients. In contrast, the levels of IL-8, TGF-β, and GCSF did not differ between newly diagnosed allergic asthma patients, non-treated severe persistent asthma patients and control. Moreover, the mean serum IL-10 levels were lower than the control group in newly diagnosed allergic asthma patients and non-treated severe persistent asthma patients. Furthermore, IL-17 levels did not change between any groups and are not associated with any clinical parameters of asthma.

Results of serum cytokine measurements of the severe persistent asthma patients and distinct clinical parameters were then further analyzed. However, this increase was not correlated neither with any of the clinical follow-up markers and serum hsCRP levels. Even though, ACT score of the patients and serum hsCRP levels together with FEV1/FVC status were significantly different between two patient groups (see Figures 6(a), 6(b), 6(c), and 6(d)).

Prick tests were in all patients in Group I and Group II were detected in mite and grass allergy. These results correlated with specific IgE. The study subjects' baseline characteristics are shown in Tables 1 and 2. The mean IgE levels were as follow: (Group IA: 551.99 IU/mL; Group II: 144.25 IU/mL and Group III: 38.62 IU/mL).

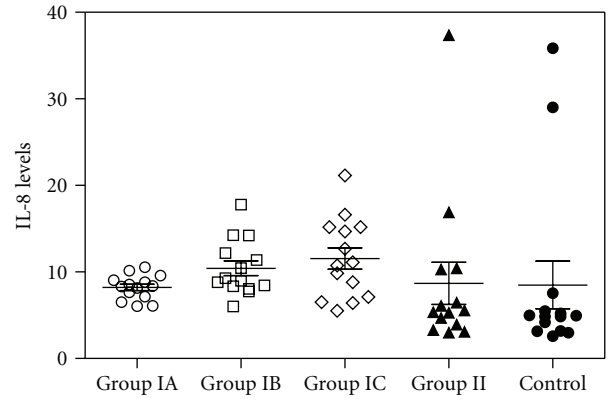

Figure 1: Plot graphic of serum IL-8 concentrations in all groups. The numbers of samples of the all groups are 14 for each. Group IA: severe persistent asthma patients before the treatment. Group IB: 4 months after the anti-IgE therapy, severe persistent asthma patients. Group IC: 18 months after the anti-IgE therapy, severe persistent asthma patients. Group II: newly diagnosed controlled allergic asthma patients. Group III: healthy individuals as control. P values were as below: Group IA versus IB: $P = 0.02$, Group IA versus IC: $P = 0.019$, Group IA versus II: $P = 0.42$, Group IA versus Control: $P = 0.46$, Group IB versus IC: $P = 0.27$, Group IB versus Control: $P = 0.25$, Group IC versus Control: $P = 0.16$, and Group II versus Control: $P = 0.48$.

4. Discussion

Multiple pathophysiological effects have been associated with imbalanced T cell activation and the presence or absence of distinct immune mediators in asthma patients [18]. Thus there has developed an increased interest in the role of cytokines and chemokines for diagnosis and therapy. The present study aimed at understanding the pattern of expression of several circulating cytokines in asthmatic individuals. To assess the potential value of these cytokines as biomarker for asthma, or certain asthma phenotypes, or prediction of anti-IgE therapy efficacy, we compared serum levels of these asthma associated mediators in two groups: patients with severe persistent asthma treated with anti-IgE therapy, and newly diagnosed allergic asthma patients. Anti-IgE therapy with omalizumab reduces serum levels of free IgE and downregulates expression of IgE receptors (Fc epsilonRI) on mast cells and basophils. In the airways of patients with mild allergic asthma, omalizumab reduces Fc epsilon RI+ and IgE+ cells and causes a profound reduction in tissue eosinophilia, together with reductions in submucosal T-cell and B-cell numbers. Omalizumab decreases Fc epsilonRI expression on circulating dendritic cells, which might lead to a reduction in allergen presentation, T(h)2 cell activation, and proliferation. And our result of no difference between anti-IgE treated patients and the control group emphasize the fundamental importance of anti-inflammatory effects of omalizumab and IgE in allergic inflammation. A number of authors have studied serum cytokines in asthmatic individuals [24–28]. However, no study focused on the relation between these cytokine levels and anti-IgE therapy.

Distinct type of asthma is related to neutrophilic inflammation. However, there is still a multicellular process leads to multicellular inflammation in the pathogenesis of asthma [29]. IL-8 takes role in the activation of neutrophils and is a potent chemoattractant of neutrophils during the airway inflammation [30]. There is also growing evidence that IL-17 is involved in the pathogenesis of asthma. IL-17 orchestrates the neutrophilic influx into the airways and also enhances T-helper 2 (Th2) cell-mediated eosinophilic airway inflammation in asthma [31, 32]. Moreover eosinophils are also a central feature in asthma and are very prominent cells. And GM-CSF promotes eosinophil activation and survival [33]. Eosinophils are also thought to be an important source of the potent pro-fibrotic cytokine TGF-β, although numerous other cells types including platelets, fibroblasts, smooth muscle and epithelial cells can also produce TGF-β. However, the precise role of eosinophil-derived TGF-β in airway remodeling is complicated and related to both eosinophils and mast cells [34–36].

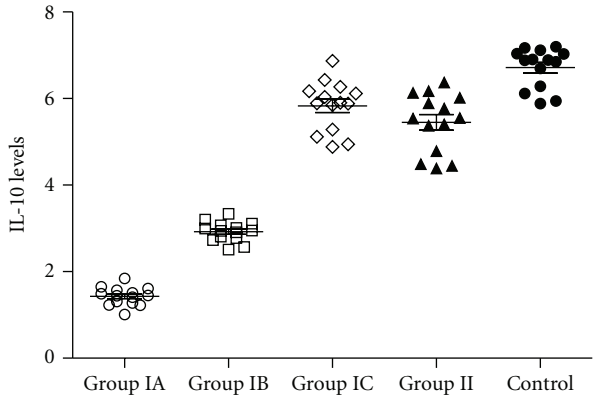

Figure 2: Serum IL-10 levels of all study groups. The numbers of samples of the all groups are 14 for each. Group IA: severe persistent asthma patients before the treatment. Group IB: 4 months after the anti-IgE therapy, severe persistent asthma patients. Group IC: 18 months after the anti-IgE therapy, severe persistent asthma patients. Group II: newly diagnosed controlled allergic asthma patients. Group III: healthy individuals as control. P values were as below: Group IA versus IB: $P < 0.0001$, Group IA versus IC: $P < 0.0001$, Group IA versus II: $P < 0.0001$, Group IA versus Control: $P < 0.0001$, Group IB versus IC: $P = 0.0024$, Group IB versus Control: $P < 0.0001$, Group IC versus Control: $P = 0.0018$, and Group II versus Control: $P < 0.0001$.

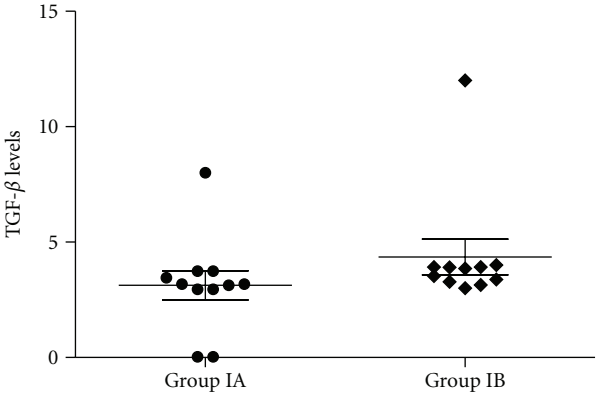

Figure 3: TGF-β levels of severe persistent asthma patients before and 4 months after the anti-IgE therapy ($P = 0.013$).

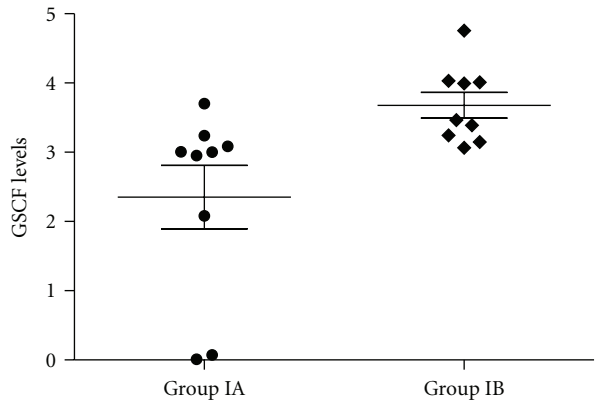

Figure 4: The concentration of GSCF in severe persistent asthma patients in group IA and IB ($P = 0.009$).

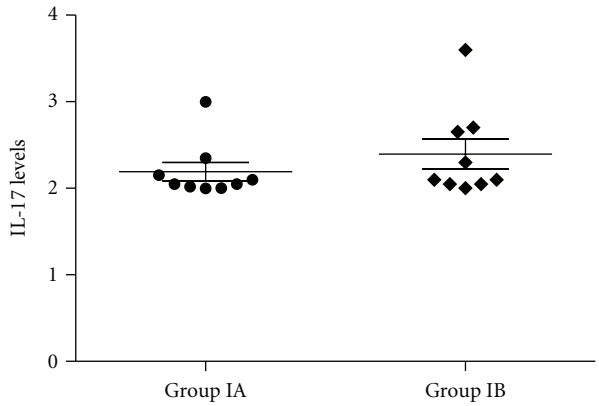

Figure 5: Serum IL-17 levels of severe persistent asthma patients were shown in dot-plot graph ($P = 0.17$).

Researchers therefore should keep in mind that the change in cytokine levels in the context of asthma, inflammation, and within different treatment modalities, and discuss the therapeutic potential of various strategies targeting cytokines for asthma that might have been applied as a therapeutic approach.

In our study for this purpose we evaluated the cytokine levels of different T cell sub-types. However, no differences were observed in IL-8 levels between healthy and diseased individuals before anti-IgE therapy. IL-10 levels were higher in treated patients and healthy individuals than the newly diagnosed patients as it was previously reported that inhaled corticosteroid therapy restores the reduced IL-10 release [37] IL-17 levels did not change during the anti-IgE therapy in severe persistent asthma patients. In contrast, IL-8, IL-10, TGF-β, and GSCF patterns showed a statistically significant difference in patients before/after therapy, suggesting a value in monitoring circulating cytokine levels in severe persistent asthma patients receiving anti-IgE therapy that also indicates that anti-IgE therapy provides clinical benefits. In our study, the levels of TNF-α were also investigated because of its important role in the bronchus allergic inflammation. However, there was no significant difference in the level between groups (data not shown).

It has been suggested that asthma is not necessarily associated with changes of serum cytokines [1]. However, this controversy may be in part at least explained by the heterogeneity of the overall asthmatic patient population. Asthma patients referred to our clinic in this study were divided into two subgroups; group I patients with severe persistent asthma for periods ranging from 3 to 7 years, and group II subjects who were diagnosed as allergic asthma with a history ranging from 6 to 27 years. Group I patients had been receiving anti-IgE therapy while group II received inhalant steroids therapy and had been classified as controlled allergic asthma subjects for 1 to 3 years. According to our previous experiences of anti-IgE therapy in clinical use, its indications and our studies on, the clinical

Marker	Group IA	Group IB	Group IC	Group II	Group III (Control)
ACT Score	10.32 ± 4.16	22.48 ± 2.9	23.02 ± 1.8	24.8 ± 0.8	25
hsCRP level	3.6 ± 0.22	2.9 ± 0.16	2.68 ± 0.09	2.92 ± 0.18	2.74 ± 0.08
FEV1	58	86	92	92	110
FVC	52	82	96	89	112

(a)

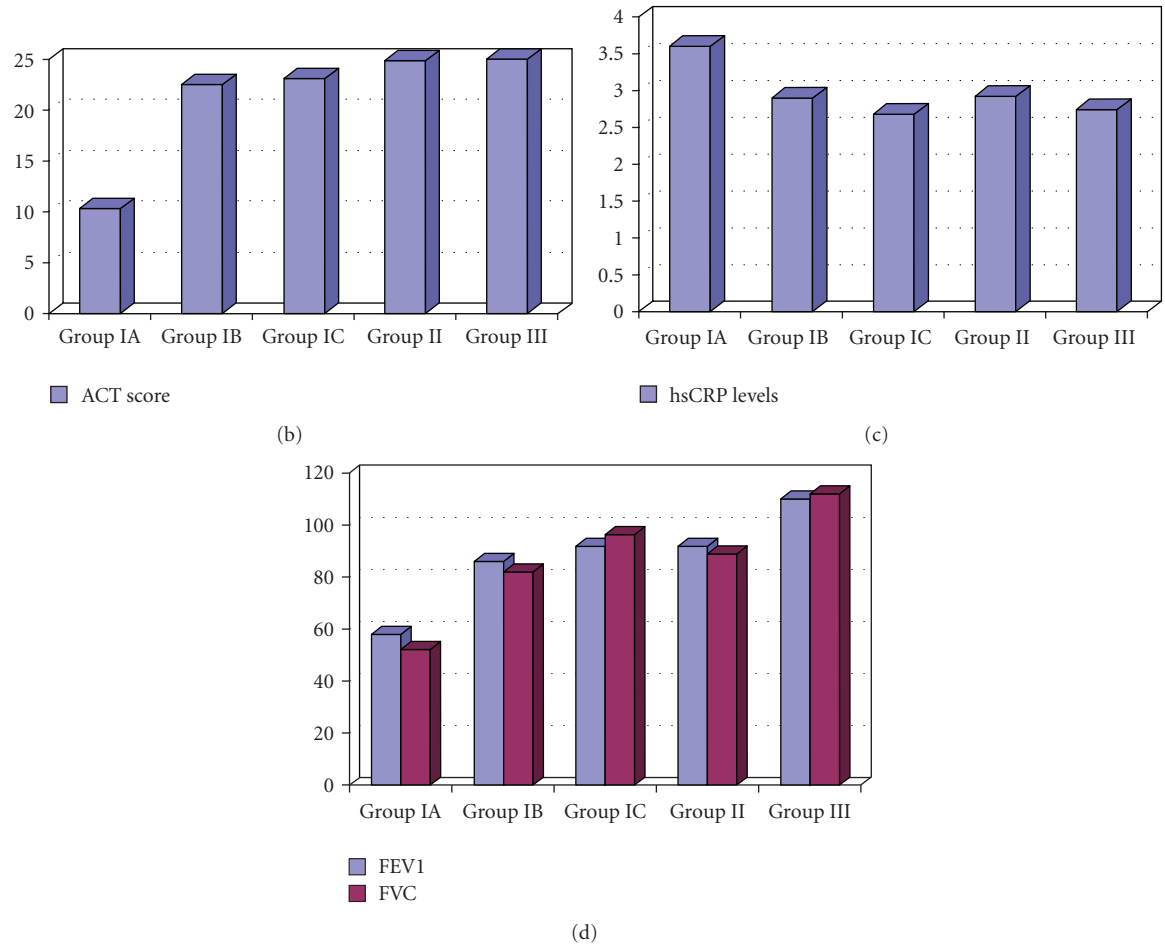

Figure 6: Clinical follow-up markers; FEV1/FVC rate, serum hsCRP levels, and ACT score of groups. ACT score was significantly increased similar to the controls after anti-IgE therapy (Group IA versus IB: $P = 0.005$). HsCRP levels were significantly decreased to the levels of controls after the treatment (Group IA versus IB: $P = 0.04$ and Group IA versus IC: $P = 0.02$). FEV1 and FVC values also significantly increased by the anti-IgE treatment depending on the time of therapy (FEV1: Group IA versus IB: $P = 0.02$ and Group IA versus IC: $P = 0.01$ and FVC: Group IA versus IB: $P = 0.01$ and Group IA versus IC: $P = 0.008$). Values are presented as mean ± standard deviation (SD). Additional bar graphs used to compare data are given.

effect begins at the third month of treatment [38, 39]. And no other exacerbations had seen on the patients after then. So that might be in the relation of alterations in cytokine expressions profiles and clinical symptoms during the omalizumab treatment.

We also evaluated serum cytokine levels in relation to clinical parameters, including total and specific IgE, asthma onset, pulmonary function tests, hsCRP level, and ACT. There was no clear pattern in the expression levels of circulating cytokines and clinical parameters of asthma. In this regard, our results are in accord with previous studies that indicate that serum cytokine levels reflecting activity of Th1, Th2, and Th17 cells and clinical symptoms are independent of one another [24, 28, 40–42]. Note that the hsCRP levels and FEV1/FVC rates were different from healthy individuals in both group I and group II patients, reflecting the clinical manifestations of asthma.

IL-8 is a pro-neutrophilic chemokine that is secreted by various cell types. It is thought to play an important role in asthma, with levels correlated with the severity of disease [14, 16, 18]. We found IL-8 levels increased along with those of IL-10 the immune regulatory and anti-inflammatory cytokine and TGF-β and GSCF in severe persistent asthma patients who were receiving anti-IgE

therapy. Anti-IgE (omalizumab) treatment attenuates both the early- and late-phase responses to inhaled allergens in patients with asthma [19]. Further anti-inflammatory effects, including changes in interleukin levels, have been observed and postulated to contribute to the clinical efficacy of omalizumab treatment [20, 21]. Other studies in severe persistent allergic asthma patients receiving omalizumab therapy have focused on modulation of serum soluble TNF-related apoptosis-inducing ligand, total antioxidant capacity, hydrogen peroxide, malondialdehyde and total nitric oxide concentrations, and ceruloplasmin oxidase activity measurements, as markers of the efficacy of anti-IgE treatment modality [43–46]. Our data add IL-8, IL-10, TGF-β, and GCSF to this list.

Both local and systemic inflammation is associated with pathogenesis in asthma [47, 48]. To assess systemic inflammation, we monitored serum levels of CRP in patients. Because of possible confounding effects on CRP levels, subjects with kidney disease, heart disease, liver disease, diabetes mellitus, cancer, obesity, smoking history, and autoimmune disease were excluded from our study. No correlation was observed between levels of any of the cytokines measured, clinical outcome, and serum hsCRP concentrations.

In conclusion, the present study documents evidence for altered patterns in serum cytokines in severe persistent asthma patients following anti-IgE therapy. However, the basal serum cytokine profiles excluding the IL-10, patterns were not different between healthy and asthmatic individuals, regardless of whether the latter were newly diagnosed allergic asthma or non-treated severe persistent asthmatic patients. We believe this study provides a novel perspective on the mechanism of action of anti-IgE immunotherapy in severe persistent asthma patients and inflammatory mediators in defining clinical benefits.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contributions

A. D. Yalcin and A. Bisgin contributed equally to this paper. A. D. Yalcin and A. Bisgin conceived and designed the study. Clinical followup: A. D. Yalcin. A. Bisgin analyzed the data. Contribution of reagents/materials: R. M. Gorczynski and A. Bisgin. Writing of the paper: A. Bisgin and A. D. Yalcin.

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Research Article

4-1BB/4-1BBL Interaction Promotes Obesity-Induced Adipose Inflammation by Triggering Bidirectional Inflammatory Signaling in Adipocytes/Macrophages

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Obesity-induced adipose inflammation is characterized by recruitment of macrophages to adipose tissue and release of inflammatory cytokines. 4-1BB, a costimulatory receptor, modulates inflammatory processes through interaction with its ligand 4-1BBL on immune cell surfaces. In this study, we examined whether a 4-1BB/4-1BBL interaction between adipocytes and macrophages participates in obesity-induced adipose inflammation. We found that 4-1BB was expressed on adipocytes and was upregulated by obesity-related factors, which also enhanced 4-1BBL expression on macrophages. 4-1BB and/or 4-1BBL agonists, respectively, activated inflammatory signaling molecules (MAPK/IκBα and MAPK/Akt) in adipocytes and macrophages and enhanced the release of inflammatory cytokines (MCP-1, TNF-α, and IL-6). Moreover, disruption of the 4-1BB/4-1BBL interaction decreased the release of inflammatory cytokines from contact cocultured adipocytes/macrophages. These findings indicate that 4-1BB/4-1BBL-mediated bidirectional signaling in adipocytes/macrophages promotes adipose inflammation. 4-1BB and 4-1BBL may be useful targets for protection against obesity-induced adipose inflammation.

1. Introduction

Obesity-induced inflammation is considered to be a potential cause of metabolic disorders such as insulin resistance, type 2 diabetes, and cardiovascular diseases [1–3]. Adipose tissue actively participates in obesity-induced inflammation through recruitment of macrophages and T cells and release of inflammatory cytokines (monocyte chemotactic protein-1, MCP-1; tumor necrosis factor alpha, TNF-α; interleukin-6, IL-6) which modulate adipocyte differentiation, metabolism, and local/systemic inflammatory responses, causing undesirable metabolic imbalances [2, 4]. Interestingly, recent studies have shown that direct contact coculture of adipocytes and macrophages results in markedly elevated release of inflammatory cytokines [5–7], indicating that interaction between cell surface molecules on these cells is important for promoting their inflammatory responses.

4-1BB (also known as CD137 and TNFRSF9) is a classic example of a costimulatory molecule, and a well-known inflammatory receptor that is expressed by activated T cells at sites of inflammation [8]. Stimulation of 4-1BB on T cells leads to cell expansion, cytokine production, and development of cytolytic effector functions [9]. 4-1BB ligand (4-1BBL, also known as CD137L and TNFSF9) is highly expressed by most immune and many nonimmune cells and can receive and transmit reverse signals into cells such as macrophages [10]. Accumulating evidence shows that bidirectional cell surface 4-1BB/4-1BBL interactions in immune cells are critical in initiating and modulating

various inflammatory responses (e.g., rheumatoid arthritis, autoimmune myocarditis, and hematological malignancies) [11–13]. Moreover, 4-1BB/4-1BBL-mediated interactions also occur between immune and nonimmune cells, again influencing inflammatory responses. For example, interaction between 4-1BB and 4-1BBL on endothelial cells and macrophages is involved in vascular inflammation [14, 15], and interaction between the two molecules on epithelial cells and natural killer cells is involved in renal ischemia-reperfusion injury [16]. We previously showed that expression of 4-1BB and 4-1BBL was upregulated in adipose tissue that was inflamed due to obesity, and that ablation of 4-1BB reduced adipose inflammation [17]. Hence, we hypothesized that interaction between 4-1BB and 4-1BBL on adipose cells and immune cells such as macrophages plays a role in adipose inflammation in obesity.

In this study, we show for the first time that 4-1BB is expressed on adipocytes and is upregulated by obesity-related factors, and we demonstrate that 4-1BB/4-1BBL-mediated bidirectional signaling in adipocytes/macrophages plays a crucial role in initiating and promoting the obesity-induced adipose inflammatory cascade.

2. Materials and Methods

2.1. Animals. C57BL/6 mice (male, 8 weeks old) (Orient Ltd., Busan, Korea) were fed a high-fat diet (HFD, 60% of calories from fat (Research Diets Inc., New Brunswick, NJ, USA); obese mice) or a low fat diet (LFD; 10% of calories from fat (Research Diets); nonobese mice) for 9 weeks. All animal experiments were approved by the animal ethics committee of the University of Ulsan and conformed to National Institutes of Health guidelines.

2.2. Antibodies. Nude mice were primed with pristane and injected intraperitoneally with a subcloned hybridoma producing an agonistic monoclonal antibody (Ab) against 4-1BB (3E1) to induce ascite formation [18]. The monoclonal Ab was purified from the ascites fluid by affinity column chromatography with protein G-Sepharose (Sigma-Aldrich). Recombinant 4-1BB Fc (r4-1BB Fc) was purchased from Adipogen (Seoul, Korea). Antagonistic monoclonal Ab against 4-1BBL (TKS-1) was purchased from e-Bioscience (San Diego, CA, USA). Rat immunoglobulin G (Rat IgG) and human IgG1 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as control.

2.3. Cell Cultures and Treatments. The murine macrophage cell line Raw264.7 was obtained from the Korean Cell Line Bank (KCLB40071, Seoul, Korea). This cell line was maintained in RPMI1640 (Gibco BRL, NY, USA) containing 10% (vol/vol) FBS (fetal bovine serum) (Gibco BRL, NY, USA) and incubated at 37°C in humidified 5% CO_2. 3T3-L1 preadipocytes were maintained in DMEM (Dulbecco's Modified Eagle Medium) high glucose (Gibco BRL, NY, USA) containing 10% FBS. Confluent 3T3-L1 preadipocytes (day 0) were incubated in DMEM containing 10 μg/mL insulin (Sigma-Aldrich), 0.25 μM DEX (dexamethasone, Sigma-Aldrich), 0.5 mM IBMX (3-isobutyl-1-methyl-xanthine, Sigma-Aldrich), and 10% FBS for 2 days. Briefly, 3T3-L1 cells were differentiated into mature adipocytes by incubation in DMEM with 10% FBS and 5 μg/mL insulin for 2 days. Mature adipocytes were maintained in this medium, and the culture medium was replaced with fresh medium every 2 days. Free fatty acid (FFA, palmitic acid mixture, Sigma-Aldrich) was dissolved in ethanol containing bovine serum albumin (BSA, 25 μM) and conjugated with BSA at a 10 : 1 molar ratio before use. 3T3-L1 adipocytes (3×10^5 cells/well) or Raw264.7 macrophages (3×10^5 cells/well) in 24-well plates, were treated with obesity-related factors (palmitic acid : pal, lipopolysaccharide : LPS) for 24 h or 4 h, respectively. To stimulate 4-1BB on adipocytes, 3T3-L1 adipocytes on 24-well plates were incubated with agonistic 4-1BB Ab (3E1, 1 μg/mL) or rat IgG for 48 h in serum-free medium. To immobilize r4-1BB Fc or human IgG1 on culture plates, r4-1BB Fc or human IgG1 were incubated in 24-well plates at 37°C for 1 h in a CO_2 incubator and the wells were rinsed with phosphate buffered saline (PBS). The plates were then incubated with RPMI (10% FBS) at 37°C for 1 h in a CO_2 incubator and the wells were rinsed with PBS. Raw264.7 macrophages were incubated at 5×10^5 cells/well in 24-well flat-bottomed plates precoated with 100 ng/mL r4-1BB Fc or human IgG1 for 24 h.

2.4. Isolation of Stromal Vascular Cells from Adipose Tissue. To isolate the stromal vascular fraction (SVF) of adipose tissue, epididymial fat pads of C57BL/6 mice (male, 8 weeks old) were minced and digested for 30 minutes at 37°C with type 2 collagenase (1 mg/mL; Sigma-Aldrich) in DMEM (pH 7.4). The resulting suspensions were centrifuged at 500 g for 5 minutes. The pellets were resuspended in erythrocyte lysis buffer, and the suspensions incubated at room temperature for 3 minutes, then centrifuged at 500 g for 5 minutes. After washing in DMEM, the suspensions were passed through sterile 100 μm nylon meshes (SPL Lifescience, Pocheon, Korea). The filtrated cells were transferred to 100 mm^2 dishes contained DMEM supplemented with 10% FBS and 0.4% Fungizone and maintained in an incubator at 37°C in 5% CO_2. Cells were allowed to attach and floating cells were removed by aspiration and the culture media was replenished for every day. The SVF cells were collected after 2 day. The SVF cells were plated at 5×10^5 cell/well in 24-well plates. The confluent SVF-derived preadipocytes were differentiated into adipocytes by treatment with DMEM containing 10 μg/mL insulin (Sigma-Aldrich), 0.25 μM DEX (Sigma-Aldrich), 0.5 mM IBMX (Sigma-Aldrich), and 10% FBS for 2 days. Mature adipocytes were maintained in the culture medium that was replaced with fresh medium every 2 days. To detect 4-1BB and 4-1BBL expression on SVF-derived adipocytes exposed to obese factors, these cells incubated with palmitic acid 250 μM, LPS 100 ng/mL for 24 h.

2.5. Isolation of Peritoneal Macrophages. C57BL/6 mice (male, 8 weeks old) were intraperitoneally injected with 3 ml

Table 1: Sequences of mouse primers used for qRT-PCR analysis.

Gene	Forward primer sequence	Reverse primer sequence
4-1BB	CTCTGTGCTCAAATGGATCAGGAA	TGTGGACATCGGCAGCTACAA
4-1BBL	CCTGTGTTCGCCAAGCTACTG	CGGGACTGTCTACCACCAACTC
MCP-1	GCATCCACGTGTTGGCTCA	CTCCAGCCTACTCATTGGGATCA
TNF-α	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
36B4	TGTGTGTCTGCAGATCGGGTAC	CTTTGGCGGGATTAGTCGAAG
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

of 3% thioglycollate broth (Difco, Detroit, MI, USA) 4 days before being killed. Peritoneal macrophages were collected by centrifugation in MEM media (Minimum Essential Medium, Gibco) and the resulting pellet was washed and resuspended in culture medium MEM with 10% FBS. The peritoneal macrophages were purified by adherence to tissue culture plates for 2 hours [19]. To detect 4-1BB and 4-1BBL expression on peritoneal macrophages exposed to obese factors, these cells incubated with palmitic acid 250 μM, LPS 100 ng/mL for 4 h.

2.6. Coculture of Adipocytes and Macrophages. 3T3-L1 adipocytes were cultured and differentiated for 6 days. Coculture of adipocytes and macrophages was performed by two methods: direct contact coculture and transwell coculture. In the direct contact system, Raw264.7 macrophages (3×10^5 cells: 50% macrophages, 3×10^4 cells: 10% macrophages) or peritoneal macrophages (3×10^5 cells) were placed in 24-well plates containing 3T3-L1 adipocytes (3×10^5 cells). The cells were cultured for 24 h in contact with each other and harvested. As a control, adipocytes and macrophages were also cultured separately, with cell numbers per well equal to those in the contact system and mixed after harvest. In the trans-well system, cells were cocultured using transwell inserts with a 0.4 μm porous membrane (Corning, NY, USA) to separate adipocytes (3×10^5 cells, lower well) from macrophages (3×10^5 cells, upper well). After incubation for 4 h, 8 h, and 12 h, the supernatants were harvested.

2.7. Measurement of Cytokine Levels. Cytokine levels in culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA). The assays were conducted using OptEIA mouse TNFα, a mouse MCP-1 set (BD Bioscience Pharmingen, CA, USA) and a mouse IL-6 and adiponectin set (R&D Systems, Minneapolis, MN, USA), and IL-10 kit (R&D Systems, Minneapolis, MN, USA). Values for cytokine levels were derived from standard curves using the curve-fitting program SOFTmax (Molecular Devices, Sunnyvale, CA, USA).

2.8. Quantitative Real-Time PCR (qRT-PCR). Total RNA extracted from cultured cells was reverse transcribed to generate cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR amplification of the cDNA was performed in duplicate with a SYBR premix Ex Taq kit (TaKaRa Bio Inc., Foster, CA, USA) using a Thermal Cycler Dice (TaKaRa Bio Inc., Japan). All reactions were performed by the same procedure: initial denaturation at 95°C for 10 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. All values for genes of interest were normalized to values for housekeeping genes (36B4 for adipocytes; β-actin for macrophages and cocultures). Mouse primer sequences used are shown in Table 1.

Data were analyzed using Thermal Cycler Dice Real Time System Software (Takara Bio, Inc.). Relative standard curves were generated by plotting the cycle threshold (Ct) values. Base on the Ct values obtained from each sample, the relative amounts of target genes were calculated using the standards curves with software provided by Takara Thermal Cycler Dice Real Time System.

2.9. Separation of Adipocytes and Macrophages. Cocultured 3T3-L1 adipocytes and Raw264.7 macrophages in equal numbers (as described earlier) were separated following the manufacturer's protocol, using the CD11b MicroBeads system (MACS; Miltenyi Biotec, Sunnyvale, CA, USA). Briefly, cocultured cells were collected, washed twice with buffer (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin-BSA), and incubated with CD11b microbeads for 15 min at 4°C. Washed and resuspended cells were applied to MACS column, which retained $CD11b^+$ cells and allowed negative cells (adipocytes) to pass through. The column was then removed from the separator and placed on a suitable collection tube. Appropriate amounts of column buffer were pipetted onto the column to flush out positive cells (macrophages) using a plunger supplier with the column. This method resulted in 90% to 95% pure $CD11b^+$ cells, as evaluated by flow cytometry.

2.10. Flow Cytometry (FACS) Analysis. 3T3-L1 adipocytes and Raw264.7 macrophages treated with obesity-related factors (as described earlier) were gently trypsinized, washed twice in PBS, and incubated with Fcγ receptor-blocking antibodies (24G2) for 10 minutes on ice, then stained with phycoerythrin (PE) conjugated anti-4-1BB (eBioscience, San Diego, CA, USA), anti-4-1BBL (eBioscience), or anti-Rat $IgG2_{a,k}$ (eBioscience), Golden Syrian Hamster IgG (eBioscience) and anti-CD11b (eBioscience) as a control to define the gate for adipocytes/macrophages. The cells were then washed with FACS buffer and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) with CellQuest software

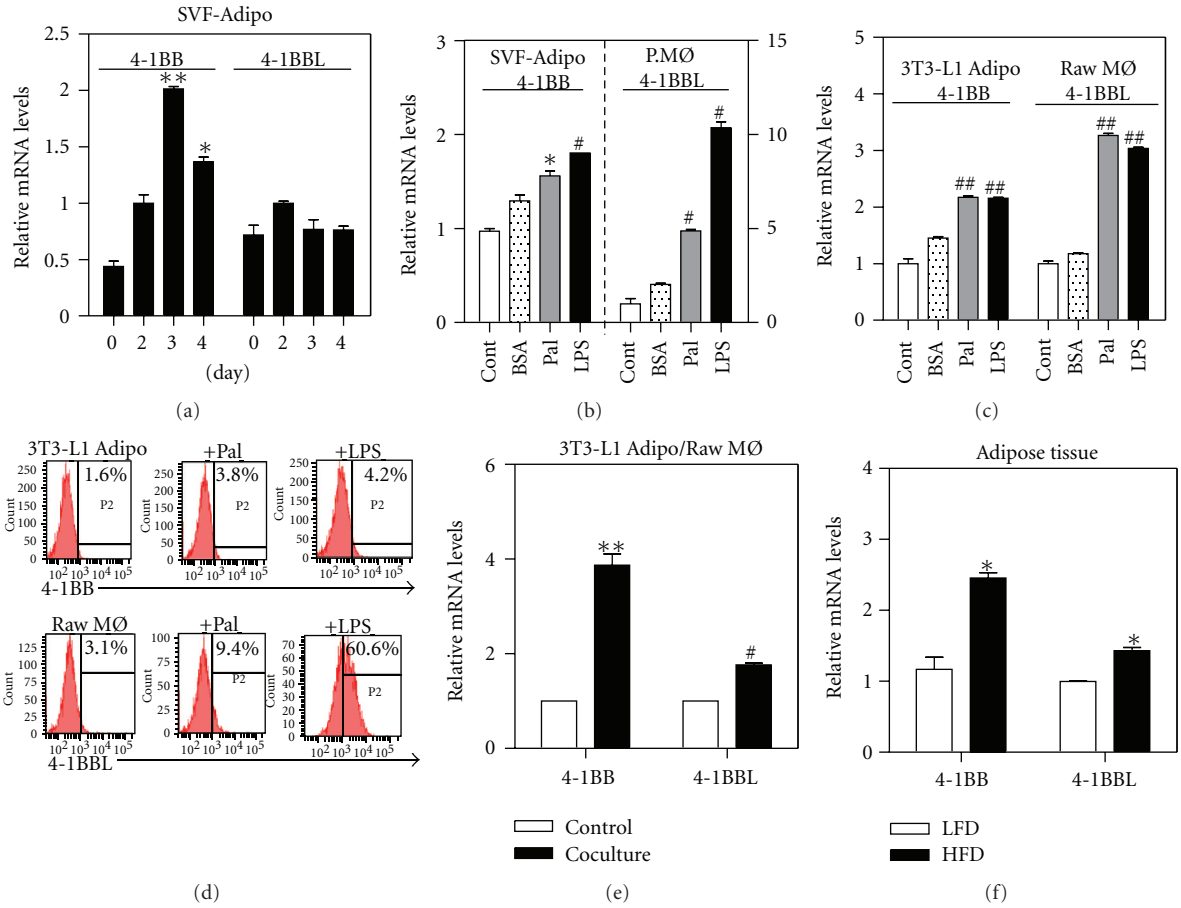

Figure 1: 4-1BB and 4-1BBL expression is upregulated by obesity-related factors in adipocytes and macrophages. 4-1BB and 4-1BBL mRNA expression in SVF-derived adipocytes (a) during adipogenesis. SVF-derived confluent preadipocytes (day 0) were differentiated into adipocytes (days 2–4), as described in Section 2. 4-1BB and 4-1BBL mRNA levels in SVF-derived adipocytes and peritoneal macrophages treated with obesity-related factor (250 μM Pal, 100 ng/mL LPS) for 24 and 4 h (b). 4-1BB/4-1BBL mRNA (c) and protein expression (d) in 3T3-L1 adipocytes and Raw264.7 macrophages treated with obesity-related factor (250 μM Pal, 100 ng/mL LPS) for 24 h and 4 h. mRNA was measured by qRT-PCR, and protein levels were detected by FACS. 4-1BB and 4-1BBL mRNA levels in 3T3-L1 adipocytes/Raw264.7 macrophages cocultured for 24 h (e), control indicates mixed adipocytes/macrophages, which were cultured separately for 24 h and mixed after harvest, and in the epididymal adipose tissue (f) of mice fed a high-fat diet (HFD) or low-fat diet (LFD) ($n = 4$ mice per group). Levels of mRNA were estimated by qRT-PCR. Pal, palmitic acid; Adipo, adipocytes; P.MØ, peritoneal macrophages; Raw MØ, Raw264.7 macrophages. Data are the mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$; $^{\#\#}P < 0.001$ (compared with control).

(BD Biosciences). Number in the graphs indicates the percentages of positive cells.

2.11. Western Blot Analysis. 3T3-L1 adipocytes were plated at 1×10^6 cells/well in 6-well plates and incubated with 3E1 (1 μg/mL) or rat IgG for 3 h. Raw264.7 macrophages were plated at 1×10^6 cells/well in 6-well plates coated with r4-1BB Fc or human IgG for 1 h. The 3E1-treated adipocytes and r4-1BB Fc-treated macrophages were rinsed with PBS, resuspended by scraping in lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 0.5% deoxycholate, 1% IGEPAL, and protease inhibitors cocktail), and centrifuged at 3000 rpm for 5 minutes. Samples containing 10–30 μg of total protein were subjected to western blot analysis using polyclonal antibodies to phosphorylated IKK (I kappa B kinase alpha/beta; p-IKK α/β, Ser180/Ser181), total IKKβ, p-p38 MAPK (mitogen-activated-protein kinase), p-JNK (c-Jun amino-terminal kinase), total JNK, p-Akt (protein kinase B; Ser473), total Akt (Cell Signaling, Danvers, MA, USA), and IκBα (inhibitor of nuclear factor-κB alpha; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma).

2.12. Statistical Analysis. Results are presented as means ± SEM of three independent performed in duplicate. Statistical comparisons were performed using Student's t-test or ANOVA with Duncan's multiple-range test. Differences were considered to be significant at $P < 0.05$.

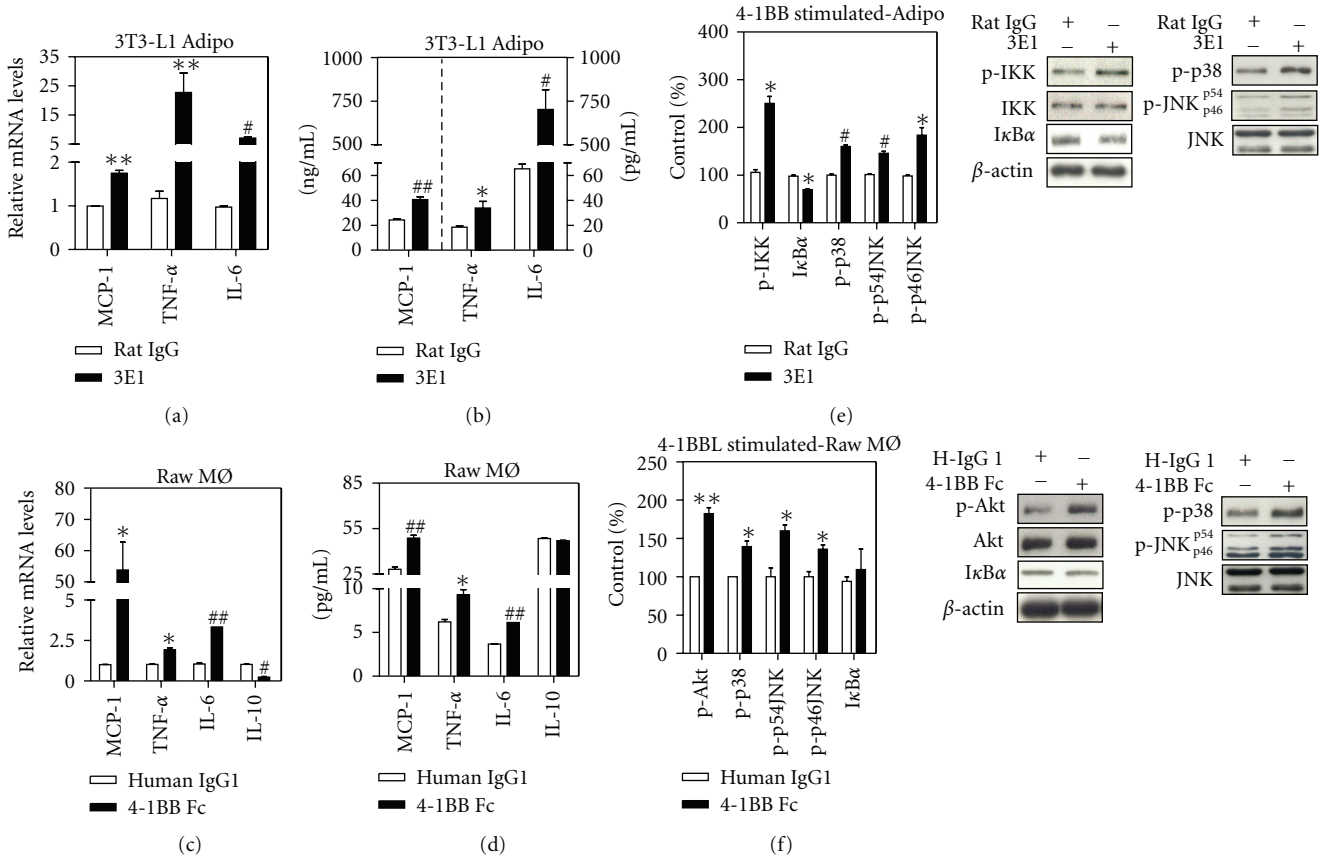

Figure 2: 4-1BB or 4-1BBL stimulation enhances release of various inflammatory cytokines from adipocytes or macrophages. 3T3-L1 adipocytes and Raw264.7 macrophages were treated with 1 μg/mL 3E1 and 100 ng/mL r4-1BB Fc, respectively, for 48–24 h. MCP-1, TNF-α, IL-6, and IL-10 transcripts and proteins were then measured in the 3T3-L1 adipocytes (a-b) and Raw264.7 macrophages (c-d). The phosphorylation of IKKα/β (p-IKKα/β)/IKKβ, IκBα, p-p38, p-JNK/JNK, pAkt/Akt and β-actin were measured by western blotting. 3T3-L1 adipocytes were incubated with 1μg/mL 3E1 for 3 h (e), and Raw264.7 macrophages with 100 ng/mL r4-1BB Fc for 1 h (f). Results of densitometry were showed as percentage of control. Data are the mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$; $^{\#\#}P < 0.001$ (compared with control).

3. Results

3.1. Expression of 4-1BB and 4-1BBL in Adipocytes/Macrophages and Adipose Tissue. We first measured expression of 4-1BB during adipogenesis at the mRNA level using qRT-PCR. We found that levels of 4-1BB transcripts were greater in SVF-derived adipocytes after differentiation (Figure 1(a)). Importantly, obesity-related substances such as palmitic acid and LPS significantly upregulated levels of 4-1BB transcripts in SVF-derived adipocytes and 4-1BBL transcripts in peritoneal macrophages (Figure 1(b)). The upregulation of the transcripts is confirmed in 3T3-L1 adipocytes and/or Raw264.7 macrophages (Figure 1(c)). FACS analysis also revealed that 4-1BB protein on 3T3-L1 adipocytes and 4-1BBL protein on Raw264.7 macrophages (Figure 1(d)) were increased by these obesity-related factors. In addition, 4-1BB and 4-1BBL transcripts increased in cocultured adipocytes/macrophages (Figure 1(e)), as well as in the epididymal adipose tissue of obese mice fed an HFD (Figure 1(f)).

3.2. Release of Inflammatory Cytokines and Activation of Inflammatory Signaling Molecules by 4-1BB and 4-1BBL Stimulation in Adipocytes and Macrophages, Respectively. To examine whether 4-1BB on adipocytes or 4-1BBL on macrophages provide an inflammatory signal, we treated each cell type with agonists that specifically stimulate these molecules; 3T3-L1 adipocytes were treated with an agonistic 4-1BB antibody (3E1) for 48 h, and Raw264.7 macrophages with r4-1BB-Fc for 24 h and we then measured levels of inflammatory cytokines in the respective cells. Both 4-1BB stimulation of adipocytes and 4-1BBL stimulation of macrophages markedly increased the production of proinflammatory cytokines such as MCP-1, TNF-α, and IL-6 at the mRNA (Figures 2(a), and 2(c)) and protein levels (Figures 2(b) and 2(d)). Adiponectin secretion from adipocytes was not altered by 4-1BB stimulation (data not shown). 4-1BBL stimulation of macrophages decreased the transcripts of IL-10 (Figure 2(c)), but no change was observed in IL-10 protein release (Figure 2(d)).

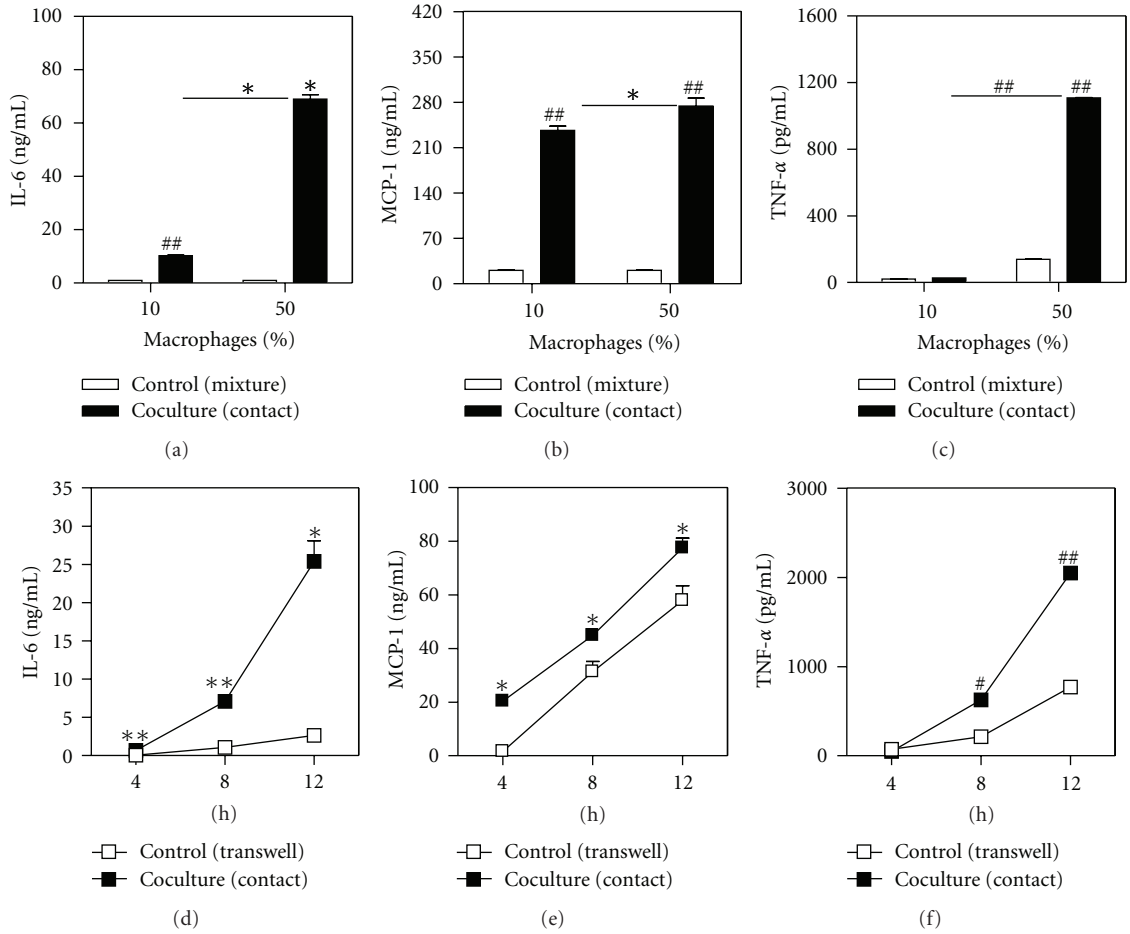

Figure 3: Release of inflammatory cytokines is enhanced in cocultures of 3T3-L1 adipocytes and Raw264.7 macrophages. Release of cytokines (IL-6, MCP-1, and TNF-α) from 3T3-L1 adipocytes cocultured with Raw264.7 macrophages (10% or 50%) for 24 h (a–c). Control indicates mixed adipocytes/macrophages, which were cultured separately for 24 h and mixed after harvest. Release of cytokines (IL-6, MCP-1, and TNF-α) from the contact coculture with 50% Raw264.7 macrophages or transwell system for indicated times (4 h, 8 h, and 12 h) (d–f). Cell-free supernatants were collected, and concentrations of these inflammatory cytokines were determined by ELISA. Data are the mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$; $^{\#\#}P < 0.001$ (compared with control).

To understand the molecular mechanisms by which 4-1BB and/or 4-1BBL activate inflammatory signaling in adipocytes and/or macrophages, we examined the effects of 4-1BB/4-1BBL stimulation on intracellular signaling molecules. Stimulation of 4-1BB on adipocytes increased the phosphorylation of p38 MAPK and JNK as well as the phosphorylation of IKK, the upstream molecule of NF-κB, and induced IκBα degradation (Figure 2(e)), while stimulation of 4-1BBL increased phosphorylation of Akt and p38 MAPK, and JNK (Figure 2(f)), but had no effect on degradation of IκBα protein (Figure 2(f)) and phosphorylation of IKK (data not shown). Consistent with previous reports [20, 21], 4-1BBL signaling not only activated p38 MAPK but also induced Akt activation in macrophages, leading increased inflammatory cytokines expression.

3.3. Release of Inflammatory Cytokines in a Contact Coculture System. Because 4-1BB/4-1BBL stimulation enhanced the release of inflammatory cytokines from adipocytes and/or macrophages, respectively, we investigated whether cell-cell interaction via surface molecules, presumably 4-1BB/4-1BBL, has a role in initiating and triggering inflammatory responses. We first cocultured 3T3-L1 adipocytes and Raw264.7 macrophages in a direct contact system and found that the production of inflammatory cytokines IL-6, MCP-1, and TNF-α was correlated with the number of macrophages in the culture (Figures 3(a)–3(c)) and increased with time (Figures 3(d)–3(f)).

3.4. Effect of Disruption of the Interaction between 4-1BB and 4-1BBL on Release of Inflammatory Cytokines in a Contact Coculture System. To test whether the 4-1BB/4-1BBL-mediated interaction between adipocytes and macrophages participates in the inflammatory response in the contact cocultured 3T3-L1 adipocytes/Raw264.7 macrophages, we blocked the interaction using a neutralizing antibody (TKS-1). The neutralizing monoclonal antibody reacts specifically with mouse 4-1BBL, by which 4-1BBL cannot bind to 4-1BB

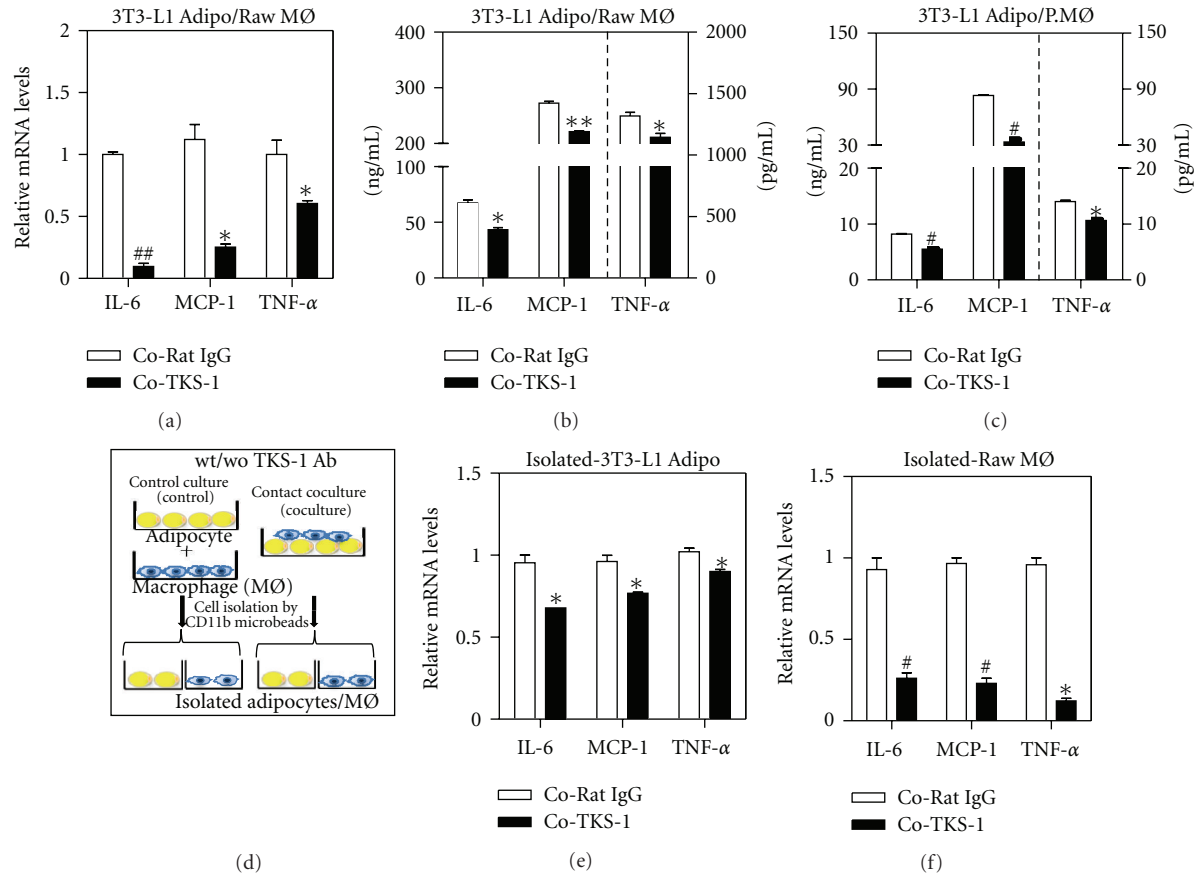

Figure 4: Disruption of the 4-1BB/4-1BBL interaction suppresses expression of inflammatory cytokines in direct contact cocultures. Raw264.7 macrophages were seeded onto 3T3-L1 adipocytes with/without pretreated with neutralizing anti-4-1BBL antibody (TKS-1) or Rat IgG (5 μg/mL) in serum-free medium for 24 h. Total RNAs were isolated and analyzed the level of IL-6, MCP-1, and TNF-α by qRT-PCR (a). The protein levels of IL-6, MCP-1, and TNF-α in cell-free supernatants were collected from 3T3-L1 adipocytes and Raw264.7 macrophages coculture (b) and from cocultures of 3T3-L1 adipocytes and peritoneal macrophages (c). Illustration of coculture system with adipocytes/macrophages (d). After 24 h coculture of Raw264.7 macrophages and 3T3-L1 adipocytes, these cells were separated using the CD11b MicroBead system. Levels of inflammatory cytokine mRNAs were detected in the adipocytes (e) and macrophages (f). Co, coculture. Data are the mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$; $^{\#\#}P < 0.001$ (compared with rat IgG-treated cells).

receptor and can interrupt the interaction between 4-1BBL and 4-1BB. Hence, both 4-1BB-mediated signal in adipocytes and 4-1BBL-mediated signal in macrophages can be blunted by TKS-1 treatment. We found that treatment with TKS-1 significantly reduced levels of IL-6, MCP-1, and TNF-α mRNA in the contact cocultured adipocytes/macrophages (Figure 4(a)). The reduction in the expression of these inflammatory cytokines was confirmed at the protein level (Figure 4(b)). Moreover, we also found that disruption of the interaction between 4-1BB and 4-1BBL reduced the release of inflammatory cytokines from peritoneal macrophages cocultured with adipocytes (Figure 4(c)). To examine the relative contributions of 4-1BB and 4-1BB signaling to inflammatory gene expression in the cocultured adipocytes/macrophages, we separated the macrophages from the adipocytes and measured levels of inflammatory cytokine transcripts in the two types of cell (Figure 4(d)). The neutralizing antibody significantly reduced the increase in levels of IL-6, MCP-1, and TNF-α mRNAs in the adipocytes as well as in the macrophages (Figures 4(e) and 4(f)).

4. Discussion

Obesity-induced adipose inflammation is characterized by recruitment of macrophages into adipose tissue, and the macrophages are an important source of inflammatory responses. Cell-cell contact between adipocytes and macrophages is considered to be important for triggering inflammatory pathways in adipose tissue [5, 6], although it is unclear which molecules are involved. Recent studies have shown that the engagement of co-stimulatory receptor 4-1BB with its ligand 4-1BBL through cell-cell contact modulates various inflammatory responses [13, 14, 16]. In previous work, we found that 4-1BB deficiency

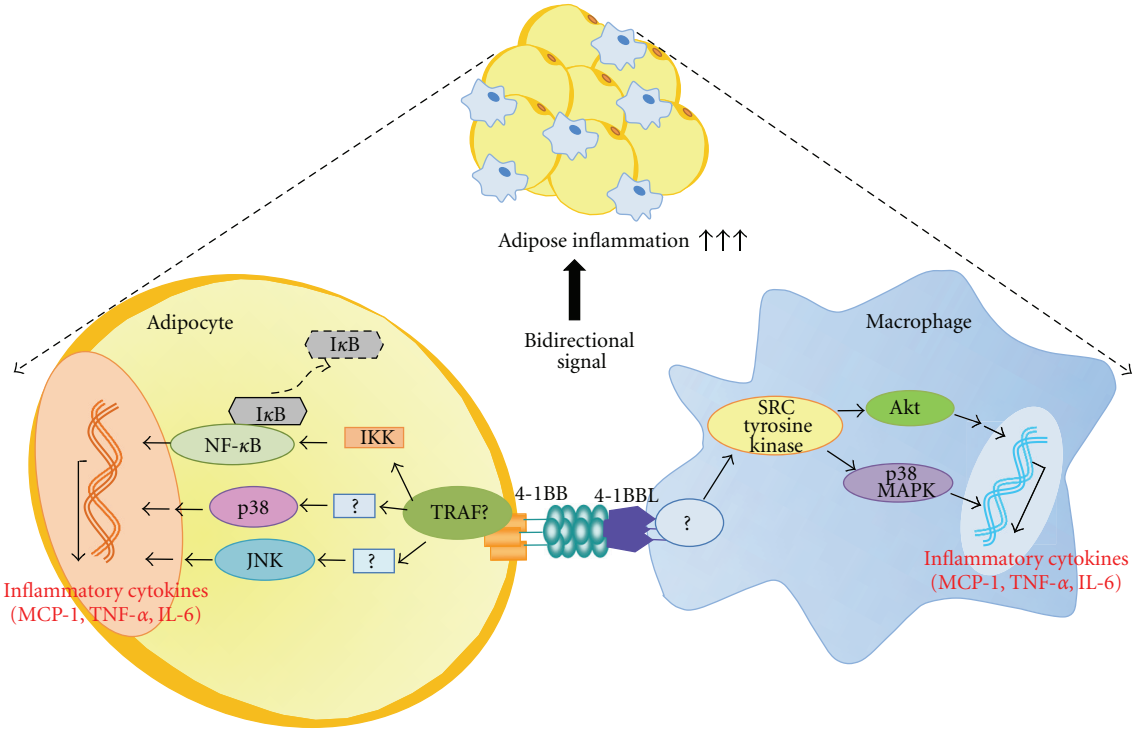

Figure 5: Schematic representation of the bidirectional signal transduction induced by 4-1BB/4-1BBL-mediated interaction between adipocytes and macrophages. The release of inflammatory cytokines (MCP-1, TNF-α, and IL-6) in response to the bidirectional signaling appears to participate in adipose inflammation.

reduced adipose inflammation by decreasing macrophage recruitment and the release of inflammatory cytokines [17]. Based on these findings, we hypothesized that 4-1BB/4-1BBL-mediated cell-cell interaction between adipose cells and macrophages might be important in the onset and/or maintenance of obesity-induced adipose inflammation. Interestingly, 4-1BB transcripts in adipocytes and 4-1BBL transcripts in macrophages were markedly upregulated by obesity-related factors (e.g., FFA and LPS), and their expression was also strongly increased in contact cocultured adipocytes/macrophages. Moreover, the upregulation of these molecules was accompanied by enhanced release of inflammatory cytokines from the cells. These findings together with the upregulation in obese adipose tissue and the reduction of adipose inflammation in 4-1BB-deficient obese mice [17] suggest that 4-1BB and 4-1BBL participate in the onset and/or promotion of adipocytes/macrophage-induced inflammatory responses.

In order to see whether 4-1BB on adipocytes or 4-1BBL on macrophages was responsible for the inflammatory signals that triggered inflammatory responses, we stimulated the cells with agonists which bind specifically to either 4-1BB or 4-1BBL. We found, for the first time, that stimulation of 4-1BB on adipocytes markedly increased the release of inflammatory cytokines MCP-1, TNF-α, and IL-6. Stimulation of 4-1BBL-mediated reverse signaling, which is known to activate macrophages [22], also increased levels of inflammatory cytokines. Recent evidence indicates that 4-1BB signaling results in activation of the MAPK/NF-κB pathway, which is TNF receptor-associated factor (TRAF)-2-dependent [23] in lymphocytes [24]. In adipocytes, we found that stimulation of 4-1BB activated p38 MAPK, JNK, IKK and induced IκBα protein degradation. On the other hand, stimulation of 4-1BBL led to activation of inflammatory signaling molecules such as Akt and p38 MAPK in macrophages, which is consistent with previous studies [10, 20, 21]. More importantly, we found that treatment with a 4-1BBL neutralizing antibody reduced release of inflammatory cytokines in cocultures at both the mRNA and protein level. These findings together suggest that the 4-1BB/4-1BBL-mediated interaction between adipocytes and macrophages triggers bidirectional inflammatory signaling and is a potent inducer of inflammatory responses in obese adipose tissue (Figure 5).

Interestingly, disruption of the 4-1BB/4-1BBL interaction did not completely suppress release of inflammatory cytokines from cocultured adipocytes and macrophages. This may be due to the presence of other cell surface molecules which participate in cell-cell interactions and mediate inflammatory responses. Indeed, adipocytes and macrophages express many inflammatory receptors and ligands on their surfaces [6, 25, 26]. For example, CD40 and herpes virus entry mediator (HVEM), which are expressed on adipocytes, are considered to be mediators of contact-dependent signaling of macrophages, and ablation of these receptors reduces obesity-induced inflammatory responses [26–30]. Thus it is conceivable that other receptors and ligands in addition to 4-1BB and 4-1BBL are also involved

in the interaction between adipocytes and macrophages that leads to the initiation and maintenance of inflammatory responses.

In conclusion, we have demonstrated for the first time that the contact-dependent interaction between adipocytes and macrophages mediated by 4-1BB and 4-1BBL, which generates bidirectional signals, plays a crucial role in the release of adipose inflammatory cytokines from these cells. 4-1BB and 4-1BBL, along with other molecules involved in cell-cell interaction between adipocytes and macrophages, may be valuable targets for preventing obesity-induced adipose inflammation.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Clinical Study

Association between Polymorphisms in Interleukin-17A and -17F Genes and Chronic Periodontal Disease

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Objective. Interleukin-17 (IL-17) is a cytokine that induces neutrophil recruitment and the release of inflammatory mediators in several inflammatory conditions; nevertheless, the involvement of IL-17 gene polymorphisms in chronic periodontitis (CP) has not been addressed yet. Our aim was to evaluate the association between periodontal status and the polymorphisms IL-17A G197A and IL-17F C7488T in subjects with CP along with their impact on levels of inflammatory mediators. *Material and Methods*. Genomic DNA was obtained from 30 CP patients and 30 healthy controls (HCs). IL-17A G197A and IL-17F C7488T polymorphisms were determined using PCR-RFLP. Serum and periodontal tissues were collected and processed for ELISA, myeloperoxidase (MPO), and/or microscopic analysis. *Results*. The frequencies of genotypes in the CP group were significantly different from those of HC. Odds ratio indicated that increased risks for CP were associated with the -197A allele, not with the -7488T allele. In addition, the -197A allele was correlated with worse clinical parameters, higher MPO activity, and increased expression of inflammatory mediators (IL-17A and IL-8) than the other genotypes. *Conclusions*. These results indicate that the IL-17A -197A allele is associated with increased risk for CP, likely because this genotype relates to the enhanced inflammation in periodontal tissues.

1. Introduction

Periodontitis is an inflammatory disease that affects the tooth-supporting tissues. It is considered one of the most significant causes of tooth loss in humans and may associate with systemic diseases, such as diabetes and arthritis [1–4]. Pathogens of the subgingival bacterial biofilm are essential for the initiation and progression of periodontitis. Nevertheless, disease results from the host inflammatory reaction that primarily mediates tissue damage [2, 5, 6]. For many years, the pathogenesis of periodontitis was classically viewed as involving an immunological Th1/Th2 paradigm. According to this view, the tissue destructive Th1 cells and cytokines would arise in the early period of the disease, while the tissue protective Th2 cells and cytokines would arise in the late phase [2, 6, 7]. However, in several clinical contexts, the Th1/Th2 balance/imbalance is not sufficient to explain the progression and/or remission of periodontitis observed in patients [7].

In 2005, the Th17 subset of $CD4^+$ T cells was identified [8] and added greater complexity to Th function. Th17 cells are generally considered to be proinflammatory, in particular

through the production of the cytokines interleukin-17A (IL-17A) and IL-17F. These cells and cytokines have been associated with the pathogenesis of an extensive list of autoimmune and inflammatory diseases, including rheumatoid arthritis, inflammatory bowel diseases, psoriasis [9–11], and periodontitis [12–15]. The majority of studies have reported increased IL-17 levels associated with the development of chronic periodontitis (CP) [14–18]. In experimental models, Th17 is suggested to play a role in the development of disease [17]. In humans, elevated levels of IL-17 have been reported in patients with CP, but it is not clear why such elevated levels are found in these patients [14, 15, 17]. The study of genetic polymorphisms in CP has received increasing attention lately as they describe the contribution of genes to disease progression [18].

Allelic variants of cytokine genes are typically related to either higher or lower production of these molecules [18, 19]. In this regard, it is reasonable to hypothesize that genetic variation affecting the expression or activity of IL-17 may influence the susceptibility and severity of periodontitis. IL-17A and IL-17F genes are mapped on the same chromosome at position 6p12 [20], and the polymorphisms of IL-17A G197A (rs2275913) and IL-17F C7488T (rs763780) have recently been associated with higher susceptibility to rheumatoid arthritis [21] and ulcerative colitis [22]. Nevertheless, the possible involvement of IL17 gene polymorphisms in CP has not been evaluated yet.

The purpose of the current study was to determine whether the IL-17A G197A and IL-17F C7488T polymorphisms were associated with increased susceptibility to periodontitis. We studied the association between each single nucleotide polymorphism (SNP) and the clinicopathological features of CP and local and systemic production of inflammatory mediators.

2. Material and Methods

2.1. Subjects and Sample Collection. Gingival tissue samples were obtained from periodontal tissues resected during periodontal surgery from 30 patients with CP who attended the Periodontal Clinic, School of Dentistry, Universidade Federal de Minas Gerais (UFMG). All patients had a previous history of CP and were diagnosed according to previously described criteria, including >35 years of age, no marked familial aggregation, and variable distribution of periodontal destruction [23, 24]. The inclusion criteria were subjects with attachment loss >5 mm at more than one tooth, more than three sites of probing depth >6 mm, and lesions distributed at more than two teeth in each quadrant; these subjects were diagnosed with CP. Exclusion criteria were aggressive periodontitis, use of antibiotic, anti-inflammatory and/or immunosuppressive medications in the 6 months preceding the research, and/or any systemic diseases (i.e., immunologic and autoimmune disorders, diabetes mellitus). Thirty periodontally healthy patients subjected to fully impacted third molar extraction, age- and gender-matched to the CP group, comprised the healthy control group (HC). In the current study, the individuals have not been stratified in ethnic groups based on skin color, race, or geographic origin due to the significant miscegenation among Brazilian population [25, 26].

Both groups of patients, CP and HC, were also subjected to periodontal examination including determination of probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BOP), and tooth mobility. The BOP was considered positive if bleeding occurred within 30 seconds after probing [27]. Measurements were performed full mouth at 6 sites per tooth (mesiobuccal, mid buccal, distobuccal, mesiolingual, mid lingual, and distolingual). At the time of the examination, a peripheral blood sample was taken from each patient and processed for polymorphism determination and serum obtainment.

Written informed consent was obtained from all patients. This study protocol was approved by the local Institutional Ethics Committee (324/08).

2.2. Inflammatory Infiltrate Evaluation. Gingival tissue samples were also fixed in 10% buffered formalin, embedded in paraffin wax, and cut longitudinally (3 μm). The sections were deparaffinized, rehydrated, and stained with H&E for the evaluation of the inflammatory infiltration. Inflammatory cells were counted in four fields in two independent sections (total evaluated area: ~1 mm^2), using light microscope (Axioskop 40 ZEISS; Carl Zeiss, Gottingen, Germany) at 400x magnification. Data were expressed as total of inflammatory cells/field.

2.3. ELISA. The concentrations of the cytokines IL-17A, IL-17F, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and the chemokines CXCL10 and IL-8 were measured in gingival tissues and serum by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN, USA).

The assay was performed according to the manufacturer's instructions. In brief, tissue samples have been weighed, mechanically homogenized in buffer solution (0.4 mM NaCl, 10 mM $NaPO_4$, pH 7.4) containing inhibitors of proteases (0.1 mM phenylmethylsulfonyl—PMSF fluoride—0.1 mM benzethonium chloride, 10 mM EDTA and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4 (normalization: 1000 μL of solution for 100 mg of wet tissue), and centrifuged (10,000 rpm, 10 min. 4°C).

Each cytokine was detected by an anticytokine horseradish peroxidase-labelled monoclonal antibody. The OPD (*o*-phenylenediamine dihydrochloride, Sigma-Aldrich, Saint Louis, MO, USA) peroxidase substrate kit was used to determine the amount of horseradish peroxidase bound to each well. The reaction was stopped by the addition of 1 M sulfuric acid (H_2SO_4). The plates were read at 492 nm. The data were determined using a standard curve prepared for each assay and expressed as picograms of cytokine/chemokine per 100 mg of tissue or mL of serum.

2.4. Myeloperoxidase. Gingival tissue samples were also used for determination of myeloperoxidase (MPO) activity, a neutrophil enzyme marker, as previously described [28].

After processing for ELISA, the remaining tissue pellets were subjected to hypotonic lysis: 0.2% NaCl solution for 30 s followed by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose. After further centrifugation, the pellets were resuspended in 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa-1,6-bisdecyltrimethylammonium bromide (HTAB, Sigma-Aldrich). The suspensions were freeze thawed three times and finally centrifuged at 10,000 rpm for 10 min at 4°C. MPO activity in 25 μL of the resulting supernatant was assayed by adding 25 μL of 3,3′-5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) prepared in dimethylsulfoxide (DMSO, Merck, NJ, USA, 1.6 mM), and 100 μL of H_2O_2 (0.002%, v/v) was diluted in phosphate buffer (pH 5.4) containing 0.5% HTAB. The assay was performed in a 96-well microplate incubated for 5 min at 37°C. The reaction was stopped by adding 100 μL of 4 M H_2SO_4 and quantified colorimetrically at 450 nm in a spectrophotometer.

2.5. DNA Isolation and Genotyping Analysis. Total genomic DNA was extracted from blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. Quality, integrity and quantity of DNA were analyzed by Nanodrop spectrophotometer (Thermo Scientific-GE). Single nucleotide polymorphisms (SNPs) of the IL-17A (rs2275913) and IL-17F (rs763780) genotyping were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Table 1).

The PCR amplification was performed in a total volume of 25 μL mixture containing 100 ng genomic DNA, 1.0 μM of each primer 20 μL of Premix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Premix buffer contained 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1% Triton X-100, 1.5 mM MgCl2, deoxynucleoside triphosphates, and 1.25 units of Taq DNA polymerase.

PCR products were digested overnight at 37°C with XagI (Fermentas) for IL-17A G197A. The products of IL-17A G197A and IL-17F C7488T were viewed in a 6.5% polyacrylamide gel electrophoresis stained with silver.

2.6. Data Analysis. Data were expressed as mean ± standard deviation (SD). All data were analyzed using SPSS 17 (SPSS Inc., Chicago, IL, USA). Chi-square test analysis was used to test for deviation of genotype frequencies from Hardy-Weinberg equilibrium.

The levels of cytokines in periodontal tissues and the frequency of gene polymorphisms were compared by the Student's t-test and chi-square test. Odds ratios were calculated for the minor allele at each SNP. P values < 0.05 were considered statistically significant.

3. Results

3.1. Differences between Healthy Controls (HCs) and Chronic Periodontitis (CP) Subjects. The demographic characteristics of the studied population are presented in Table 2. The age and gender were not significantly different between groups.

Table 1: Primer sequences for each gene.

	Primers
IL-17A	Sense 5′-AACAAGTAAGAATGAAAAGAGGACATGGT-3′
	anti-sense 5′-CCCCCAATGAGGTCATAGAAGAATC-3′
IL-17F	Sense 5′-GTTCCCATCCAGCAAGAGAC-3′
	anti-sense 5′-AGCTGGGAATGCAAACAAAC-3′

The frequency of smoker subjects in the studied sample was 3.3% in HC ($n = 1$) and 20% in CP ($n = 6$) groups. These patients did not present significant differences in the clinical parameters and production of inflammatory mediators when compared with nonsmokers ($P > 0.05$; data not shown). In this regard, data from smoker and nonsmoker subjects were grouped and presented together.

The clinical features PD, CAL, BOP, and tooth mobility (not shown) were significantly higher in the group CP than those in the group HC ($P < 0.0001$) (Table 2).

Besides clinical features, we also evaluated the levels of the cytokines IL-17A, IL-17F, IFN-γ and TNF-α, and the chemokines IL-8 and CXCL10 in periodontal tissues and/or serum of HC and CP subjects. Overall, levels of inflammatory mediators were increased in tissue and serum of CP patients when compared to the HC group, with the exception of IL-17F in serum ($P > 0.05$) (Figures 1(a)–1(c)). Levels of IFN-γ (HC: 45 ± 23 pg/100 mg tissue; CP: 131 ± 98 pg/100 mg tissue; $P < 0.0001$), TNF-α (HC: 21 ± 9 pg/100 mg tissue; CP: 117 ± 49 pg/100 mg tissue; $P < 0.0001$), and CXCL10 (HC: 15 ± 4 pg/100 mg tissue; CP: 34 ± 27 pg/100 mg tissue; $P = 0.003$) in periodontal tissues were also greater in CP patients than those in controls. Moreover, the inflammatory infiltrate in the gingival tissue, characterized by mixed polymorpho- and mononuclear cells, with a predominance of mononuclear leukocytes, was significantly higher in the CP than in the HC group (HC: 18 ± 8 inflammatory cells/field; CP: 88 ± 20 inflammatory cells/field; $P < 0.0001$). MPO activity was also significantly greater in CP than in HC subjects ($P < 0.0001$) (Figure 1(d)).

Frequencies of polymorphisms (IL-17A G197A and IL-17F C7488T genotypes) were investigated in blood samples of HC and CP subjects (Table 3). The frequency of these genotypes agreed with the Hardy-Weinberg equilibrium ($P > 0.05$). The mean ages of the control group (AA: 44.2; AG: 47.8; GG: 45.2 years old) and patients with CP (AA: 40.0; AG: 42.0; GG: 40.8 years old) versus genotype did not present statistical differences ($P > 0.05$). The IL-17A genotypes of the CP group (GG 20%; GA 30% and AA 50%) were significantly different from the frequencies observed in the HC group (GG 59.26%; GA 14.81% and AA 25.92%) ($\chi^2 = 9.307$; $P = 0.01$). The overall A carrier subjects (GA or AA) were associated with increased risk for periodontal disease when compared with GG carriers (OR 3.00, 95% CI: 1.34–6.67, $P = 0.001$). In contrast, the distribution of the IL-17F C7488T polymorphism was similar among the groups ($\chi^2 = 0.954$; $P = 0.62$) (Table 3).

3.2. Association between the IL-17A G197A and IL-17F C7488T Polymorphisms and Clinical Periodontal Parameters.

Table 2: Demographic and clinical features of the studied subjects.

	HC ($n = 30$)	CP ($n = 30$)	P value
Age (SD; range)	40.5 (8.1; 26–52)	45.5 (8.7; 37–61)	0.97
Gender (% F)	60.86	50.00	0.44
Ethnic origin	Brazilian mixed population		
PD (SD)	2.50 (0.8)	4.52 (0.19)*	<0.0001
CAL (SD)	2.65 (0.15)	5.74 (0.17)*	<0.0001
BOP (SD)	2.0 (0.4)	31.17 (4.02)*	<0.0001

HC: healthy controls, CP: chronic periodontitis, SD: standard deviation, PD: probing depth, CAL: clinical attachment loss, BOP: and bleeding on probing.
*Significantly higher than control (χ^2 test or Student's t-test).

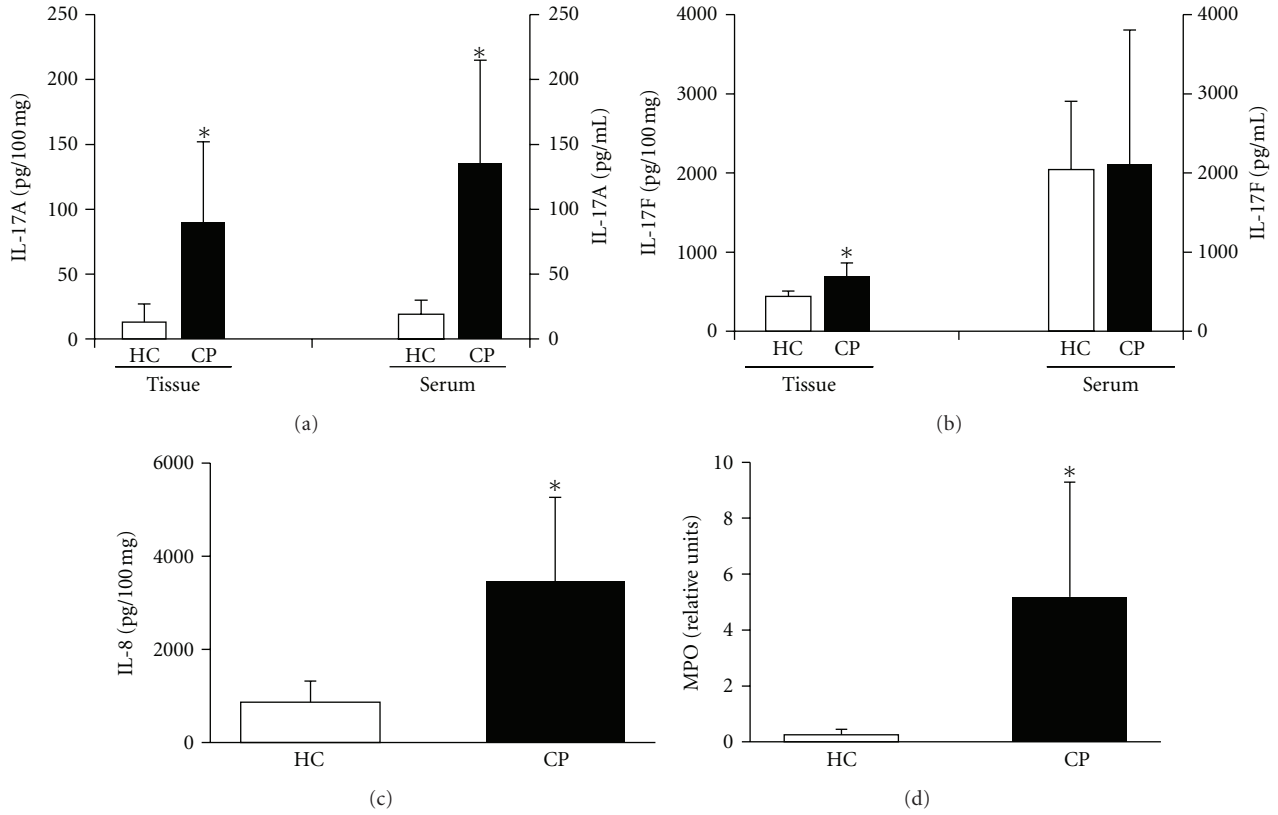

Figure 1: Levels of (a) IL-17A and (b) IL-17F in the gingival tissue and serum samples from CP and HC subjects. (c) Levels of IL-8 and (d) MPO activity in gingival tissue samples from CP and HC subjects. *Statistically significant difference at $P < 0.05$. HC: healthy control, CP: chronic periodontitis, and MPO: myeloperoxidase.

In view of the results indicating higher frequency of AG/AA alleles among the IL-17A G197A genotypes of patients with periodontitis, we further investigated whether some of these polymorphisms were associated with worse clinical periodontal parameters. As shown in Table 4, the intragroup comparison of the three IL-17A G197A genotypes indicated that PD and CAL, but not BOP, were significantly higher in AG and AA subjects than in patients with the GG genotype. Indeed, there was a significant correlation between the levels of IL-17A and PD in subjects with the genotype AA (data not show). In contrast, the IL-17F C7488T genotypes did not affect the clinical features of periodontally affected patients (Table 4).

3.3. Association between the IL-17A G197A and IL-17F C7488T Polymorphisms and Inflammatory Features. The association between IL-17 gene polymorphisms and the presence of inflammatory mediators in periodontal tissues and serum was also investigated. As shown in Figure 2(a), the levels of IL-17A in periodontal tissues from cases were not different when comparing the IL-17A G197A genotypes to each other. However, the serum levels of IL-17A were higher in subjects with the allele A than in subjects with the alleles GG (Figure 2(b)). In patients without CP, there were no differences in the levels of IL-17A in the gingival tissue or serum among the genotypes (gingival tissue: AA: 14 ± 9 pg/100 mg tissue; AG: 17 ± 17 pg/100 mg tissue; GG: 8

Table 3: Genotypes of IL-17 polymorphisms in patients with chronic periodontitis (CP) and healthy controls (HCs).

Genotype	HC (%)	CP (%)	*P* value	OR (95% CI)
IL-17A G197A				
AG/AA	40.73	80.00*	0.001	3.00 (1.34–6.67)
AA	25.92	50.00*	0.002	3.03 (1.34–6.86)
AG	14.81	30.00*	0.014	2.94 (1.24–7.00)
GG	59.26	20.00*	0.001	1
IL-17F C7488T				
CT/TT	73.26	63.4	0.310	1.30 (0.80–2.15)
CT	16.66	20.0	0.350	0.47 (0.09–2.30)
TT	56.66	43.4	0.400	0.58 (0.18–2.04)
CC	23.33	36.6	0.450	1

OR: odds ratio and CI: confidence interval.
*Significantly different from control $P < 0.05$ (χ^2 test).

Table 4: Association between IL-17A G197A and IL-17F C7488T polymorphisms and clinicopathological features of chronic periodontitis.

Genotype	*n*	PD (mm)	*P* value	CAL (mm)	*P* value	BOP (%)	*P* value
IL-17A G197A							
AA/AG	24	4.82*	0.012	6.00*	0.004	33.40	0.31
AA	15	4.58*	0.005	5.77*	0.01	39.45	0.14
AG	9	5.29*	0.007	6.429*	0.004	22.14	1.00
GG	6	3.30		4.70		23.75	
IL-17F C7488T							
CT/TT	19	4.86	0.11	5.571	0.14	25.89	0.27
TT	6	4.91	0.20	5.63	0.16	25.24	0.25
CT	13	4.67	0.18	6.67	0.59	28.30	0.71
CC	11	5.25		6.25		37.33	

PD: probing depth, CAL: clinical attachment loss, and BOP: bleeding on probing.
*Significantly different from GG or CC genotype ($P < 0.05$, Student's *t*-test).

± 3 pg/100 mg tissue; $P > 0.05$; serum: AA: 26 ± 10 pg/100 mg tissue; AG: 14 ± 1 pg/100 mg tissue; GG: 17 ± 10 pg/100 mg tissue; $P > 0.05$). Interestingly, the levels of the chemokine IL-8 were increased in tissues from CP patients with the allele A (Figure 2(c)), while the levels of CXCL10 were higher only in AG carriers (AA: 35 ± 29 pg/100 mg tissue; AG: 60 ± 7 pg/100 mg tissue; GG: 15 ± 2 pg/100 mg tissue; $P = 0.004$), as also occurred for MPO activity (Figure 2(d)). The histological findings indicated a mixed polymorpho- and mononuclear inflammatory infiltrate equally distributed among the polymorphisms groups (AA: 95 ± 11 inflammatory cells/field; AG: 87 ± 25 inflammatory cells/field; GG: 97 ± 5 inflammatory cells/field; $P > 0.05$).

The levels of IL-17F were not different, neither in periodontal tissues nor in serum, among the IL-17F C7488T genotypes (Figures 3(a) and 3(b)). The same occurred with levels of IL-8 and MPO activity, which were not different among the groups (Figures 3(c) and 3(d)). In regard to inflammatory infiltration, there were no differences among the three genotypes (CC: 102 ± 11 inflammatory cells/field; CT: 115 ± 23 inflammatory cells/field; TT: 82 ± 24 inflammatory cells/field; $P > 0.05$).

4. Discussion

Several experimental and clinical studies have shown that IL-17 levels are elevated in diseased human periodontal tissues and may play a destructive role in experimental models of periodontal disease [3, 14–17, 29, 30]. In the current study, we investigated the involvement of IL-17 genes polymorphisms in CP. Our results confirm that IL-17 levels are elevated in periodontal tissues of CP patients. More importantly, we show for the first time that polymorphisms of IL-17A, specially the SNP involving the allele A, are associated with the clinical and inflammatory parameters of disease. There are increased levels of IL-17A in the serum of allele A carriers, and this is accompanied by an increase of IL-8 and MPO activity in periodontal tissues.

The high levels of IL-17 in gingival crevicular fluid and periodontal tissues of patients with CP have been shown to associate with periodontal tissue damage [14, 17], but also seem to be relevant to control excessive microbial replication and, hence, disease [31]. Our study showed that the clinical parameters of CP were associated with increased levels of IL-17A and IL-17F in gingival tissues, in agreement with

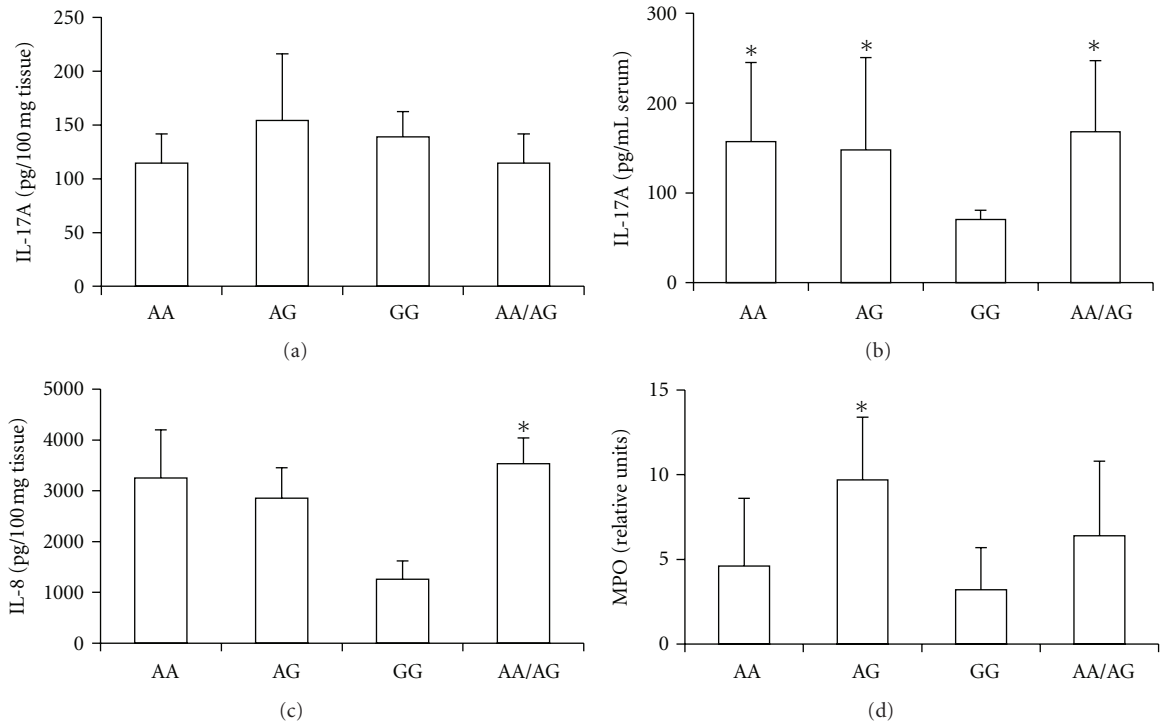

Figure 2: Levels of inflammatory mediators in chronic periodontitis patients according to each IL-17A G197A genotype. (a) Levels of IL-17A in gingival tissues and (b) serum. (c) Levels of IL-8 and (d) MPO (myeloperoxidase) activity in gingival tissue samples. *Statistically significant difference ($P < 0.05$) comparing with the genotype GG.

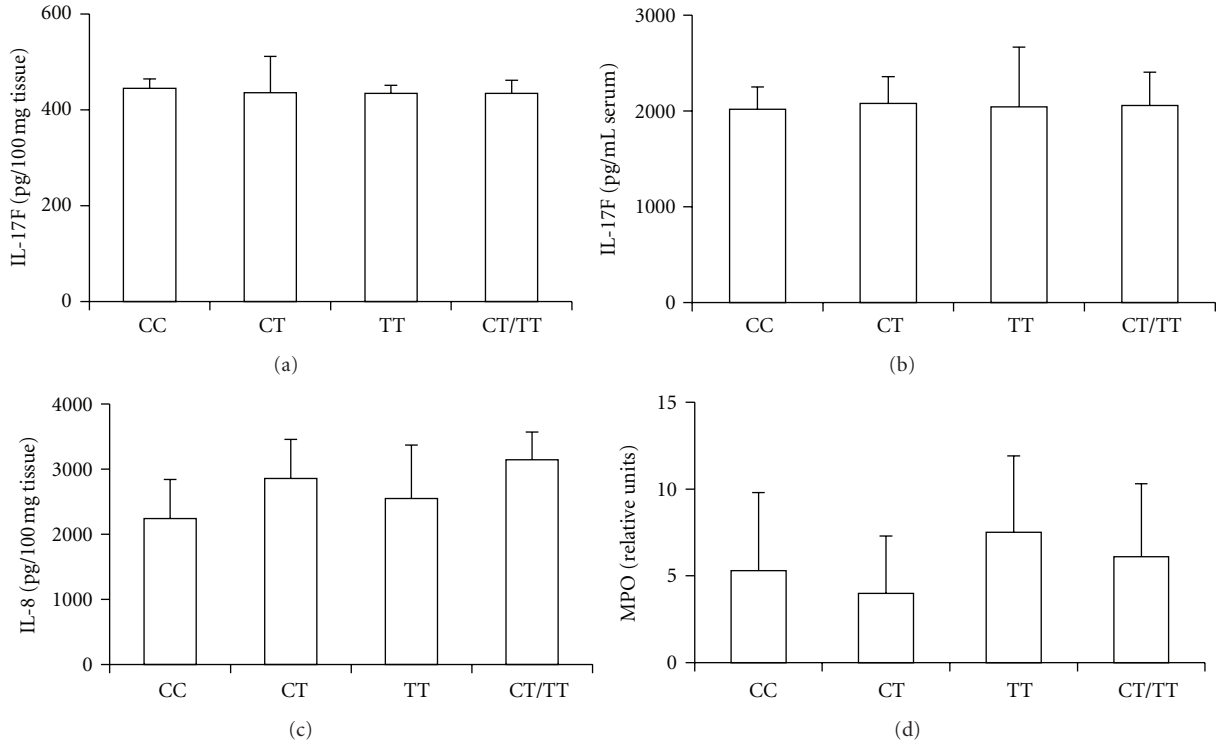

Figure 3: Levels of inflammatory mediators in chronic periodontitis patients according to each IL-17F C7488T genotype. (a) Levels of IL-17F in gingival tissues and (b) serum. (c) Levels of IL-8 and (d) MPO (myeloperoxidase) activity in gingival tissue samples.

previous reports [14, 17, 30]. In contrast, only the levels of IL-17A, not IL-17F, were enhanced in the serum of CP patients. This is in line with studies showing increased serum concentrations of IL-17A, mainly for patients with aggressive periodontitis [1, 32]. As CP is marked by recurrent phases of remission and activation, IL-17 may be related to the destructive period of the disease given that this cytokine has already been shown to be overexpressed in active periodontal sites [33]. Along with the high levels of IL-17 in tissue and serum, the MPO activity and IL-8 levels were higher in periodontal tissues of CP patients than in healthy subjects, as well as the presence of neutrophils in the diseased gingiva. Despite the nonmechanistic nature of the current data, IL-17 has already been shown to present a prominent role in the activation and recruitment of neutrophils to inflammatory sites [16], and MPO and IL-8 have been reported to be correlated with worse clinical status of periodontitis [34, 35]. These findings suggest an active inflammatory scenario, with increased expression of IL-17 in CP patients and the probable involvement of IL-17 in the increased influx of neutrophils to periodontal affected sites.

After detecting high levels of IL-17 in CP, we analyzed the frequency of IL-17 polymorphisms in CP and HC subjects. The investigation of gene polymorphisms in CP has long been conducted, likely because they present a role in immune responses, tissue destructive mechanisms, and metabolic processes [36]. Several research groups have studied the association between CP and polymorphisms of candidate genes, including pro- and anti-inflammatory cytokines, such as IL-1β, IL-4, IL-6, IL-10, and TNF-α [36–40]. Most studies recognized the proinflammatory gene cluster polymorphisms, especially TNF-α and IL-1, as some of the best candidates associated with the induction and severity of CP [36, 41]. In the current investigation, the IL-17F C7488T polymorphism was not different in healthy subjects and CP patients. Despite some evidence suggesting that IL-17F may play a role in periodontal bone destruction [14] and also in the stimulation of some cytokines and chemokines, including IL-6, IFN-γ, and CXCL10 in inflammatory conditions [42], our data have shown that neither the clinical parameters nor the levels of inflammatory mediators in periodontal tissues were influenced by the different IL-17F C7488T genotypes. In contrast, when evaluating IL-17A, we detected a significant difference in the distribution of genotypes for the polymorphism IL-17A G197A comparing subjects with and without periodontitis. CP patients presented increased frequencies of AA and AG genotypes, and the presence of the allele A significantly increased the risk for CP. These findings are in line with previous studies demonstrating the relationship between IL-17 polymorphisms, especially the allelic polymorphic A, and chronic inflammatory diseases, including rheumatoid arthritis [21], Behçet's disease [43], ulcerative colitis [22], and gastric and breast cancer [44, 45]. They are also in line with the previously described role of polymorphisms of proinflammatory cytokine genes during CP [36, 41]. Nevertheless, it seems reasonable to remember that gene mutations alone are neither sufficient nor necessary to explain disease phenotype, although they may contribute significantly to environment and life-style parameters in the outcome of CP.

In the current study, we show that IL-17A G197A allele A carriers presented higher serum levels of IL-17A, worse clinical periodontal parameters, and increased neutrophil activity (MPO activity and IL-8 levels) when compared with the GG genotype. Although not mechanistically conclusive, these findings seem to be in line with the hypothesis of a neutrophil-mediated tissue injury associated with increased levels of IL-17A during CP, which has recently been suggested as a target mechanism for tissue destruction in experimental conditions of periodontitis associated with old age [16, 46]. Th17 lymphocytes have already been shown to be present and play a significant role in CP [47]. IL-17A can directly or indirectly (via production of chemokines) chemoattract neutrophils [48] and enhance the activity of proteolytic enzymes such as neutrophil protease and myeloperoxidase [31]. Moreover, IL-17A can stimulate the expression of bone resorption mediators, such as RANKL [49] and induce the direct differentiation of bone resorptive cells [50] and the production and release of a large range of inflammatory mediators [51], such as TNF-α, IFN-γ, and the chemokines CXCL10 and IL-8, all detected here. Indeed, a recent study showed that IL-17 can enhance CXCL10 production *in vitro* by TNF-α- and IFN-γ-stimulated human gingival fibroblasts [52] and may induce IL-8 production by gingival fibroblasts [53]. In line with these biological functions, the increased production of IL-17 seems to be predictive of tissue destruction in inflammatory conditions, such as in rheumatoid arthritis [54]. Altogether, these data point that IL-17A G197A polymorphism, especially carriers of the allele A, might be associated with increased expression of IL-17A, recruitment of neutrophils, and worse clinical conditions in CP patients.

In conclusion, this is the first study to show that the IL-17A G197A polymorphism is related to CP in a convenient sample of Brazilian patients. Although this is a relatively small sample, the presence of the allele A in IL-17A-197 polymorphism was associated with worse clinical and inflammatory periodontal parameters. It is not simple to determine in humans the mechanisms underlying the greater risk of disease in carriers of the IL-17A-197 allele A. However, our study suggests that the latter polymorphism may contribute to disease by regulating IL-17A production and, probably, the consequent release of inflammatory and bone destructive mediators. It is, therefore, suggested that IL-17A might be an interesting target for development of new therapies for periodontal disease, an assertion that needs testing in further cohorts and clinical trials.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Simulating Sleep Apnea by Exposure to Intermittent Hypoxia Induces Inflammation in the Lung and Liver

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Sleep apnea is a breathing disorder that results from momentary and cyclic collapse of the upper airway, leading to intermittent hypoxia (IH). IH can lead to the formation of free radicals that increase oxidative stress, and this mechanism may explain the association between central sleep apnea and nonalcoholic steatohepatitis. We assessed the level of inflammation in the lung and liver tissue from animals subjected to intermittent hypoxia and simulated sleep apnea. A total of 12 C57BL/6 mice were divided into two groups and then exposed to IH ($n = 6$) or a simulated IH (SIH) ($n = 6$) for 35 days. We observed an increase in oxidative damage and other changes to endogenous antioxidant enzymes in mice exposed to IH. Specifically, the expression of multiple transcription factors, including hypoxia inducible factor (HIF-1α), nuclear factor kappa B (NF-κB), and tumor necrosis factor (TNF-α), inducible NO synthase (iNOS), vascular endothelial growth factor (VEGF), and cleaved caspase 3 were shown to be increased in the IH group. Overall, we found that exposure to intermittent hypoxia for 35 days by simulating sleep apnea leads to oxidative stress, inflammation, and increased activity of caspase 3 in the liver and lung.

1. Introduction

Obstructive sleep apnea (OSA) consists of sleep-disordered breathing. Cyclic episodes result in the momentary closure, partial or complete, of the upper airway at the level of the pharynx. The repeated pauses in breathing can lead to intermittent hypoxia (IH) and increased reactive oxygen species (ROS) [1].

The increase of ROS in OSA is likely due to the repeated oxygen depletion followed by the hyperoxia that develops to restore oxygen pressure (PO_2). A similar phenomenon is observed in ischemia followed by reperfusion [2–5]. In ischemia/reperfusion, xanthine oxidase generates free radicals in the presence of oxygen, contributing to oxidative stress [6–8].

OSA is associated with chronic liver diseases, such as nonalcoholic steatohepatitis (NASH) [9–16]. Savransky and colleagues demonstrated that IH can act as a "second hit" to liver disease by amplifying the tissue damage induced by a high dose of paracetamol [17, 18]. The injury mechanism, triggered by OSA, appears to be related to the formation of peroxynitrite, depletion of glutathione, and apoptosis of hepatocytes [18].

In OSA, inflammatory factors, such as nuclear factor kappa B (NF-κB), are activated at a systemic level [17, 19]. NF-κB is a master regulator of the inflammatory process, by inhibiting its inhibitor IKK-b, and its activation leads to the increased expression of tumor necrosis factor (TNF-α), interleukins 1 and 6, and inducible nitric oxide synthase (iNOS). Alternatively, these factors can be activated by

hypoxia inducible factor (HIF-1α) [20–23], which results in apoptosis [24].

Several studies have shown that OSA is associated with inflammation, NASH, oxidative stress, and apoptosis. This is the first experimental study that evaluated the inflammatory process in the lung and liver with intermittent hypoxia, suggesting that there is a recruitment of inflammatory mediators recognized during ischemia and reperfusion. Here, we investigate the molecular mechanism involved in the lung and liver injury in an animal model of OSA.

2. Methods

The experiments were approved and completed according to the Research and Ethics Committee of the Research and Postgraduation at the Hospital de Clínicas de Porto Alegre, Brazil.

A total of 12 C57BL/6 mice (8–11 weeks old) were housed in plastic boxes (30 × 19 × 13 cm) at the Animal Experimentation Unit of the Hospital de Clínicas de Porto Alegre. The mice were kept on a 12-hour light/dark cycle (lights on from 7 AM to 7 PM) at 22 ± 4°C and given free access to food (Purina-Nutripal, Porto Alegre, RS, Brazil) and water.

The mice were randomly divided into two experimental groups ($n = 6$ per group). The groups consisted of mice exposed to intermittent hypoxia for 35 days (IH group) and mice that underwent a simulation of the IH procedure (SIH group).

The mice were placed in intermittent hypoxia chambers 8 hours a day (9 AM to 5 PM) for 5 weeks (Figure 1). The animals were exposed to a gas mixture consisting of 90% nitrogen and 10% carbon dioxide for 30 seconds. The gas mixture reduces the oxygen fraction in the chambers by 6 ± 1%. In sequence, the gas release is then blocked and fans are triggered to restore ambient air for the remaining 30 seconds. The SIH group was housed in a cage and subjected to the same adjacent fan activity as the IH group but no gas was introduced into the cage [25].

After 35 days, the animals were deeply anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg) and the liver and lungs were removed. The organs were immediately frozen in liquid nitrogen and kept at −80°C for subsequent analysis. The animals were euthanized by exsanguination under deep anesthesia [26, 27].

The organs were cut and divided for biochemical and protein analyses. For analysis of oxidative stress, 100 mg of tissue was added to 0.9 mL of buffer (140 mM KCl, 20 mM phosphate, pH 7.4) and homogenized with a micropestle in microtubes. After centrifugation at 2150.4 g for 10 minutes in a refrigerated centrifuge (4°C), the supernatant was discarded and the pellet was stored at −80°C for further analysis. For western blotting, a nuclear extraction protocol was used. Briefly, 100 mg of tissue was added to 0.6 mL of lysis buffer (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 mL NaCl, 25 mM NaF, 1 mM sodium orthovanadate, and a protease inhibitor cocktail) and homogenized with a micropestle in microtubes. After centrifugation at 15,000 g for 10 minutes at 4°C, the supernatant was discarded and the pellet was stored at −80°C for further analysis.

2.1. Oxidative Stress

2.1.1. Proteins. The protein concentration in the homogenate was measured spectrophotometrically at 595 nm using the Bradford method. The values are expressed in mg/mL [28] and were used in the calculations for the TBARS and antioxidant enzymes.

2.1.2. Assessment of Lipid Peroxidation. The TBARS technique consists of heating the homogenate with thiobarbituric acid to produce a colored product that is subsequently measured at 535 nm using a spectrophotometer. The change in color is due to the presence of malondialdehyde and other substances produced from lipid peroxidation in the biological material.

Briefly, 0.25 mL of 10% trichloroacetic acid (TCA), 0.10 mL of homogenate, 0.067 mL of 0.67% thiobarbituric acid (TBA), and 0.033 mL of distilled water were added to a tube, stirred, and then heated at 100°C. After the tubes cooled, 0.20 mL of n-butyl alcohol was added to extract the pigment. The tubes were then stirred and centrifuged for 10 minutes at 1110 g. A 0.20 mL aliquot of the supernatant was added to a 96-well plate. The absorbance of the samples was quantified on a spectrophotometer at 535 nm. The TBARS concentration was expressed in nmol per mg protein [29].

2.1.3. Determination of Superoxide Dismutase (SOD). The technique used to measure SOD was based on the level of inhibition caused by the reaction of the enzyme with O^{-2}. We used adrenaline in an alkaline medium to produce adrenochrome and O^{-2} [30].

In a 96-well plate, we measured SOD activity in the reaction medium (50 mM glycine-NaOH, pH 10) and three samples containing different concentrations of homogenate. After addition of 10.5 μL epinephrine (60 mM, pH 2.0), the reaction was monitored for 2 min at 480 nm. The enzymatic activity was expressed in units SOD/mg protein.

2.1.4. Determination of Catalase (CAT). Catalase enhances the decomposition of hydrogen peroxide into water and oxygen. The rate of decomposition of hydrogen peroxide is directly proportional to enzyme activity and follows pseudo-first-order kinetics with respect to hydrogen peroxide.

The decrease in absorption at 240 nm was determined after adding 7 μL of 300 mM H_2O_2 to the reaction medium (50 mM phosphate regulator). The catalase concentration was expressed as pmol/mg protein [31].

2.2. Western Blots. A total of 50 mg of protein was added to a buffer (60% glycerol, 2 M Tris, SDS, and 10% Pyrroline 0.5%) and incubated for four minutes at 100°C. After electrophoresis was performed [32] on a 9–12% polyacrylamide gel, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane [33]. The membrane was washed with

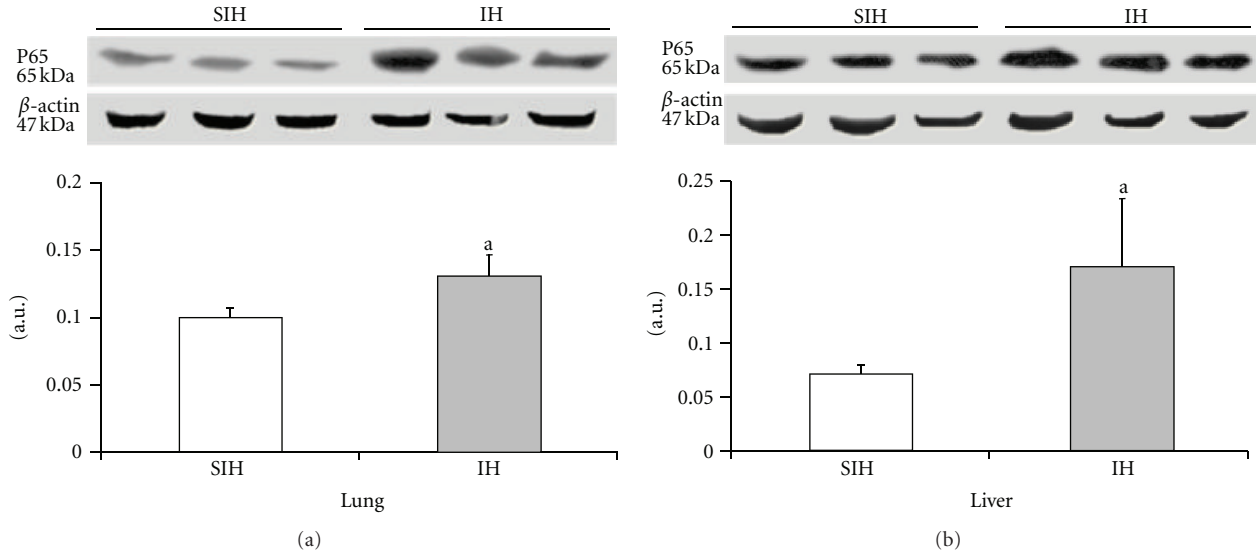

Figure 1: The effect of intermittent hypoxia on the expression of phosphorylated NF-κB in the liver ((a), $P = 0.0247$) and lung ((b), $P = 0.0033$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

PBS contained 0.5% Tween 20 and then incubated in a blocking solution (5% skim milk powder and 0.5% Tween 20 in cold PBS) for 30 minutes. After washing, the membrane was incubated overnight at 4°C with the primary antibody. Next, the membrane was washed and incubated in the secondary antibody (HRP) for two hours at room temperature. After another wash, the protein was visualized using chemiluminescent detection (Chemiluminescent HRP Substrate), film, and a transiluminator (L-Pix Chemi molecular imaging—Loccus Biotechnology). β-actin was used as a loading control. The results were quantified using LabImage 1D (Loccus Biotechnology) and are expressed as arbitrary units.

2.3. Statistical Analysis. For analyzing the result, the Student's t-test was performed using SPSS version 18.0 (Statistical Package for Social Science). The results are represented as the mean ± standard error of the mean. The statistical significance level was set as $P < 0.05$.

3. Results

Lipid peroxidation, a marker of oxidative damage, was significantly increased in the lung (14%) and liver (29%) of the IH group when compared with the SIH group (Table 1).

The activity of endogenous SOD was significantly lower in lung tissue (56%) and higher in liver tissue (87%) from IH animals when compared with the control group (Table 1). The activity of CAT was significantly higher in both organs (32% in the lung and 184% in the liver) from the IH group when compared with the SIH group (Table 1).

The activated (phosphorylated) p65 subunit of NF-κB was increased by 30% in the lung and 39% in the liver of IH mice when compared with SIH mice.

The expression of HIF-1α and TNF-α was significantly increased in the IH group when compared with the SIH group (Figures 2 and 3). In the lung tissue, HIF-1α increased by 96% and TNF-α increased by 38%. In the hepatic tissue, HIF-1α was increased by 19% and TNF-α was increased by 48%.

The expression of iNOS and VEGF was significantly higher in the IH group when compared with controls (Figures 4 and 5). There was a 35% increase in iNOS and a 22% increase in VEGF in the lung tissue. The liver showed a 79% increase in iNOS levels and a 71% increase in VEGF. Cleaved caspase 3 was increased by 237% in the lung and 182% in the liver of IH animals when compared to the SIH group (Figure 6).

4. Discussion

Animal models that use intermittent hypoxia can help elucidate the mechanism of damage to various systems caused by sleep apnea. Independent of body mass index, the respiratory disturbance index is directly related to the degree of liver damage and is recognized as a risk factor for nonalcoholic fatty liver disease (NAFLD) [10, 34]. It has been proposed that the development of NASH is produced in two phases consisting first of the accumulation of triglyceride, which is attributed to insulin resistance and obesity, and then the presence of inflammation and fibrosis [35], which is correlated with oxidative stress and hepatic lipid peroxidation [36, 37].

Our research group has described [38] oxidative damage to membrane lipids measured by TBARS and changes in endogenous antioxidant enzymes in the liver tissue that indicate the role of oxidative stress in our model system. These data are in agreement with the results described in other model systems [38–41]. Oxidative stress occurs through xanthine oxidase by producing the superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide [42, 43]; it is suggested

Table 1: The effect of intermittent hypoxia on hepatic lipid peroxidation as shown by the TBARS assay and liver antioxidant enzyme activity.

		SIH	IH	P value
Liver	$TBARS_{(nmol/mg\ prot)}$	2.90 ± 0.23	3.76 ± 0.15	0.0389
	$SOD_{(USOD/mg\ prot)}$	3.13 ± 0.53	5.86 ± 0.70	0.0118
	$CAT_{(nmol/mg\ prot)}$	0.82 ± 0.17	2.33 ± 0.09	0.0015
Lung	$TBARS_{(nmol/mg\ prot)}$	4.57 ± 0.10	5.22 ± 0.10	0.0116
	$SOD_{(USOD/mg\ prot)}$	7.27 ± 0.99	4.64 ± 0.22	0.0272
	$CAT_{(nmol/mg\ prot)}$	2.62 ± 0.18	3.48 ± 0.13	0.0042

Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.
SIH: sham intermittent hypoxia group; IH: intermittent hypoxia.
SOD: superoxide dismutase; CAT: catalase.

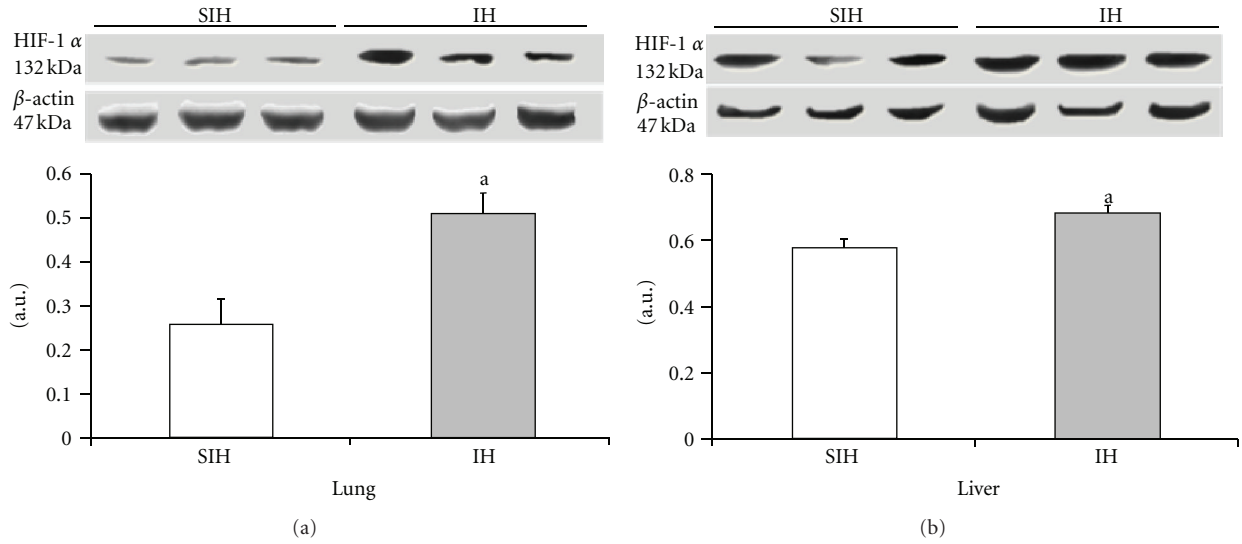

Figure 2: The effect of intermittent hypoxia on the expression of HIF-1α in the liver ((a), $P = 0.0227$) and lung ((b), $P = 0.0086$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

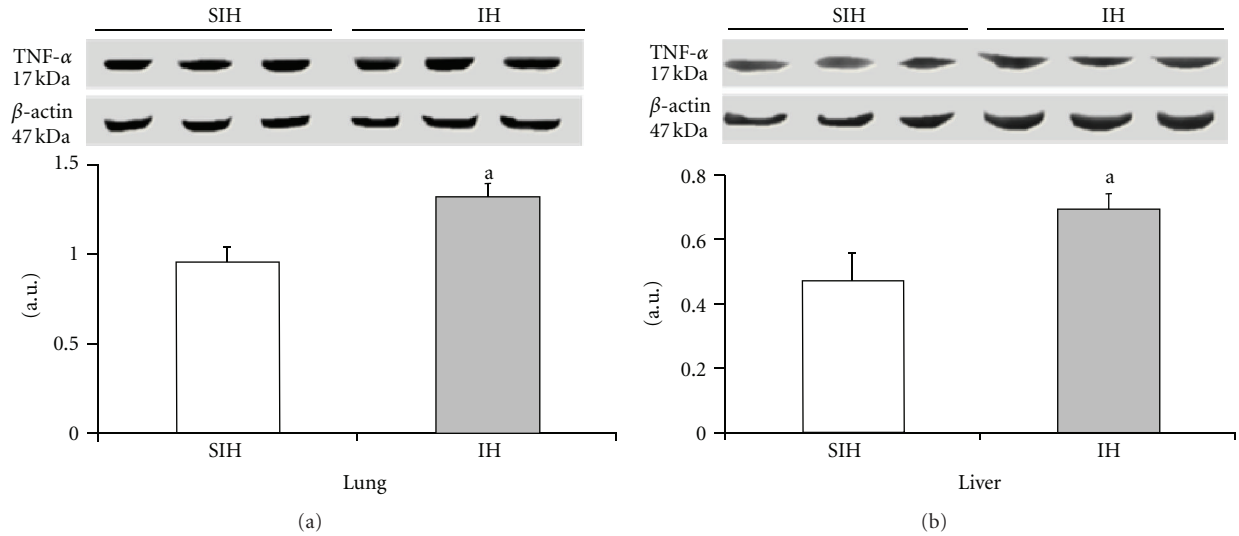

Figure 3: The effect of intermittent hypoxia on the expression of TNF-α in the liver ((a), $P = 0.0382$) and lung ((b), $P = 0.0171$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

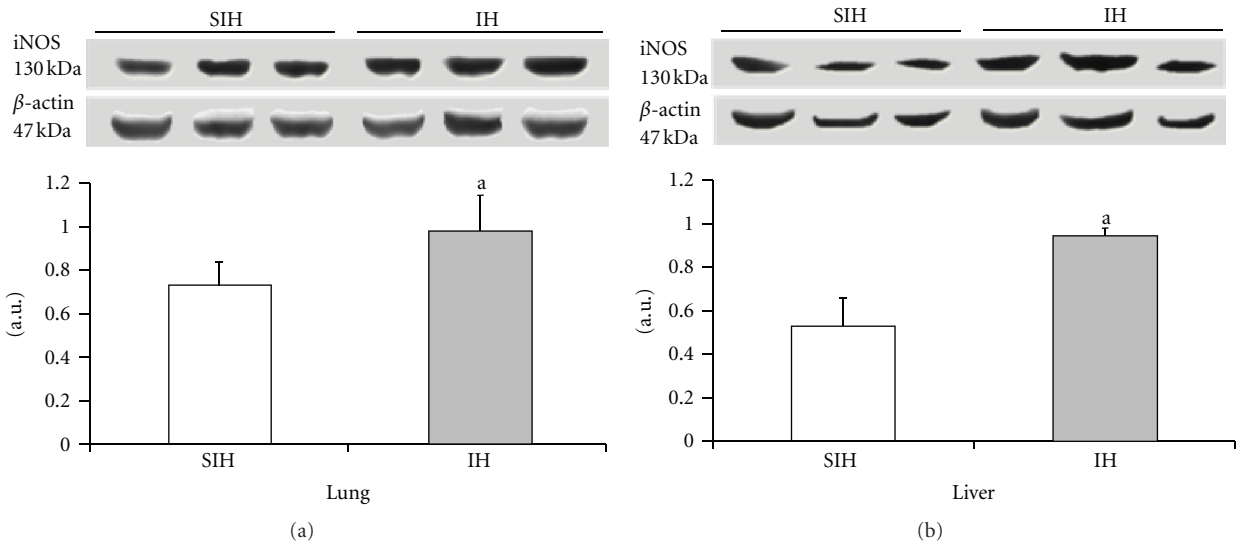

Figure 4: The effect of intermittent hypoxia on the expression of iNOS in the liver ((a), $P = 0.0091$) and lung ((b), $P = 0.0107$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

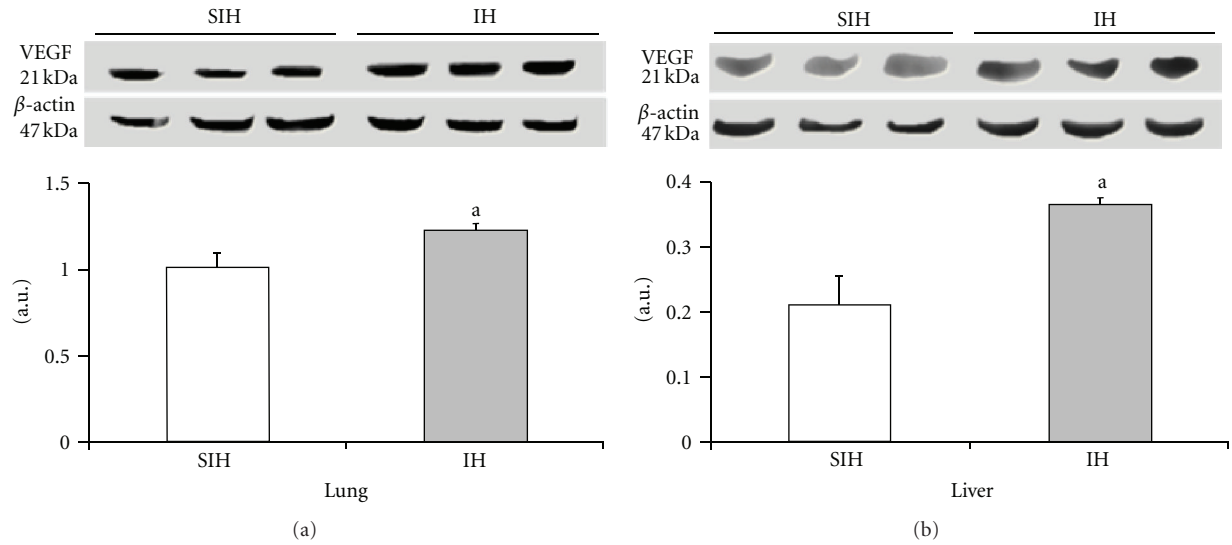

Figure 5: The effect of intermittent hypoxia on the expression of VEGF in the liver ((a), $P = 0.0062$) and lung ((b), $P = 0.0184$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

that the $O_2^{-\bullet}$ and H_2O_2, formed by the activity of xanthine oxidase, act independently on the activity of SOD and CAT [44]. Nitrosative stress includes the formation of nitric oxide (NO) that binds $O_2^{-\bullet}$ to form the radical peroxynitrite [38, 42, 45, 46].

HIF-1α regulates the concentration of oxygen, and it can be the initiator of inflammation in intermittent hypoxia [47, 48] or stimulated by oxidative stress [49]. This protein is correlated with chronic alcohol use and the presence of NAFLD [50]. HIF-1α also stimulates macrophages, increases the production of VEGF and iNOS [51, 52], reduces apoptosis [24, 53], and stimulates cell proliferation [54].

It is suggested that inflammatory activity is dependent on NF-κB [55], indicating that NF-κB can regulate HIF-1α transcription [56]. Although indirect, inhibition of IKK experimentally prevents the activation of NF-κB and was found to prevent the development of steatosis and NASH [57]. In the present study, we observed an increase in the expression of HIF-1α in the liver and the lung of mice exposed to hypoxia.

The stimulation of TNF-α leads to phosphorylation of IκB, which results in activation of NF-κB. Activation of NF-κB causes it to translocate to the nucleus and promote the transcription of numerous proinflammatory genes [58].

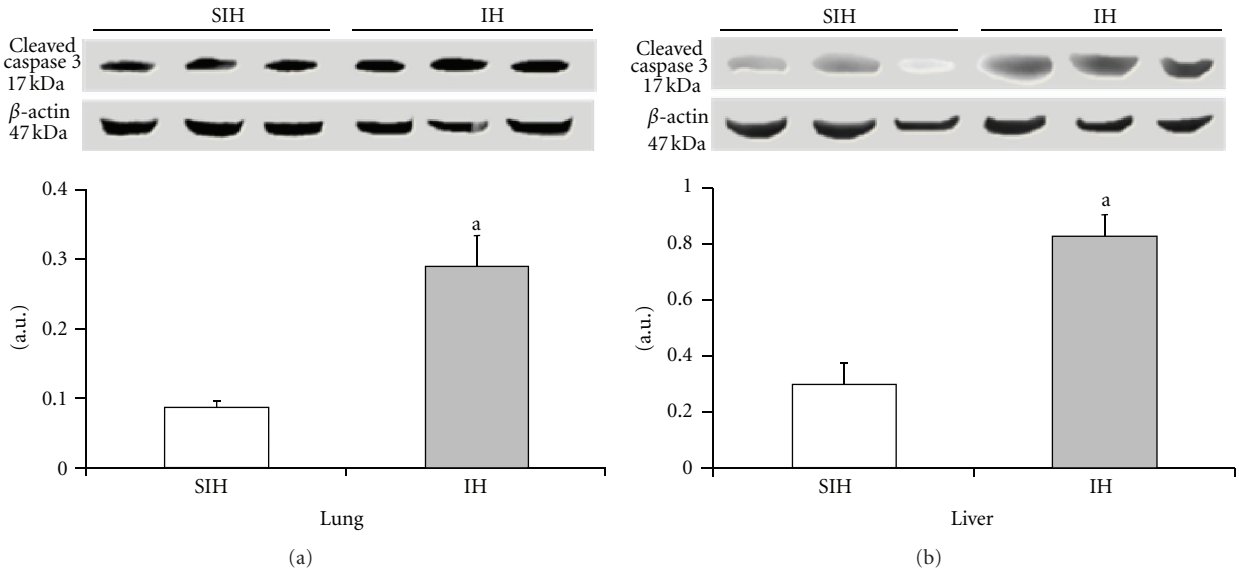

Figure 6: The effect of intermittent hypoxia on the expression of cleaved caspase 3 in the liver ((a), $P = 0.0022$) and lung ((b), $P = 0.0003$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

Here, we showed that TNF-α and NF-κB were increased in animals exposed to intermittent hypoxia.

VEGF is essential for the initiation of angiogenesis, and it has a strong effect on vascular elements in response to hypoxia [59, 60]. In this study, we found increased expression of VEGF in both organs when mice were subjected to intermittent hypoxia.

In our previous work, we found that there is an increase in nitric oxide metabolites (NO) after exposure to intermittent hypoxia [38]. In the present study, we evaluated an enzyme responsible for NO production, iNOS, and found that the levels of this enzyme were increased in the lung and liver of animals exposed to hypoxia.

Apoptosis in all cells is regulated by caspases. After cleavage, caspases become active and initiate pathways that lead to apoptosis [61]. We found that cleaved caspase 3 expression is increased in the liver and lung of the IH group, demonstrating that there was activation of this apoptotic cascade.

Thus, the data suggest that intermittent hypoxia leads to liver and lung damage that can result from a cascade of signals initiated by oxidative stress, inflammation, and apoptosis.

5. Conclusion

In mice, the cyclic oxygen deprivation observed in sleep apnea induces oxidative stress and activation of HIF-1α, which stimulates a cascade of inflammatory signaling, nitric oxide generation, angiogenesis, and apoptosis in the lung and liver.

Acknowledgments

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Review Article

Muscarinic Receptors and Their Antagonists in COPD: Anti-Inflammatory and Antiremodeling Effects

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Muscarinic receptors are expressed by most cell types and mediate cellular signaling of their natural ligand acetylcholine. Thereby, they control numerous central and peripheral physiological organ responses to neuronal activity. In the human lung, muscarinic receptors are predominantly expressed by smooth muscle cells, epithelial cells, and fibroblasts. Antimuscarinic agents are used for the treatment of chronic obstructive pulmonary disease and to a lesser extent for asthma. They are primarily used as bronchodilators, but it is now accepted that they are also associated with anti-inflammatory, antiproliferative, and antiremodeling effects. Remodeling of the small airways is a major pathology in COPD and impairs lung function through changes of the extracellular matrix. Glycosaminoglycans, particularly hyaluronic acid, and matrix metalloproteases are among extracellular matrix molecules that have been associated with tissue inflammation and remodeling in lung diseases, including chronic obstructive pulmonary disease and asthma. Since muscarinic receptors have been shown to influence the homeostasis of glycosaminoglycans and matrix metalloproteases, these molecules may be proved valuable endpoint targets in clinical studies for the pharmacological exploitation of the anti-inflammatory and antiremodeling effects of muscarinic inhibitors in the treatment of chronic obstructive pulmonary disease and asthma.

1. Muscarinic Receptors

The muscarinic receptors are metabotropic receptors that may be linked to plasma membrane K^+ or Ca^{2+} ion channels [1, 2]. They belong to the superfamily of rhodopsin-like, seven transmembrane domains, single-glycoprotein receptors that are connected by intra- and extracellular loops. Muscarinic receptors initiate intracellular responses via interaction with GTP-binding proteins (G-proteins), although activation of other signaling molecules has been reported [1, 3, 4]. There are five subtypes of muscarinic receptors, referred to as M_1 to M_5, based on the order of their discovery, and according to the nomenclature proposed by Caulfield and Birdsall [5]. Muscarinic receptors are symbolized in the literature as "M_1 mAChR," "M_1-mAChR," "m1AChR," or "mAChR1" for the M_1 receptor. In this paper muscarinic receptor subtypes will be referred to as M_1, M_2, M_3, M_4, and M_5, according to IUPHAR [6] and the MeSH Browser [7] of the National Library of Medicine of the National Institute of Health, USA.

Molecular cloning revealed that the five muscarinic receptors are encoded by separate intronless human genes. The muscarinic receptor gene sequences have significant homologies with other members of this large super-family and across mammalian species. The seven hydrophobic transmembrane domains of the muscarinic receptors are highly conserved with an average of 66% identity. In contrast, their intracellular loops are less conserved, with the third intracellular loop being particularly variable and accommodating the binding domain of receptor subtypes. Between the fifth and the sixth transmembrane regions, muscarinic receptors possess a large intracytoplasmic loop that exhibits high divergence between the different subtypes and is considered to be responsible for the G-protein-coupling selectivity [8–10] The name and gene location of the human M_1 is on chromosome 11q13; M_2 is on

chromosome 7q31-35; M_3 is on chromosome 1q43; M_4 is on chromosome 11q12-112; M_5 is on chromosome 15q26 [8, 9, 11].

2. Intracellular Signaling of Muscarinic Receptors

As mentioned above, muscarinic receptors modulate different intracellular signal transduction pathways by coupling to multiple G proteins, which include stimulation of phospholipases C, A2 and D, cAMP degradation, cGMP production, attenuation of cAMP synthesis, and regulation of several ion channels [3, 10]. This diversity in signaling is more complicated, since a single muscarinic receptor subtype is capable of activating more than one type of G protein in a single cell and, thus, is coupled to more than one effector complements of the cell [3, 10, 12]. Muscarinic receptors can be divided into two groups according to their primary coupling efficiency to G-proteins. The first group of M_2 and M_4 muscarinic receptors couple to the pertusiss-toxin sensitive $G_{i/o}$ type proteins. The second group including M_1, M_3, and M_5 can couple to $G_{q/11}$-type proteins [3, 5]. However, there is also evidence that muscarinic receptors couple to a wide range of signaling pathways, some of which are mediated by other types of G-proteins or other signaling mediators [13, 14]. An overview of known muscarinic receptor signaling is provided in Figure 1.

Studies on animal and human cell lines as well as on tissues demonstrated that muscarinic receptors also act via activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) that is referred to as mitogen-activated protein (MAP) kinase 1 [15]. In human bronchial epithelial cells, it was demonstrated that various muscarinic receptor inhibitors including tiotropium (M_1, M_2, and M_3 antagonist), gallamine (M_2 antagonist), telenzepine (M_1 antagonist), and 4-diphenylacetoxy-N-methylpiperidine methiodide (M_3 antagonist) downregulated acetylcholine-induced leukotriene B_4 release via the activation of ERK1/2 and nuclear factor-kappaB (NFκB) pathways [16]. With respect to the involvement of muscarinic receptors in the regulation of inflammatory response, it has been reported that M_2 and M_3 receptors facilitate cigarette-smoke-extract-induced interleukin (IL)-8 secretion by in human airway smooth muscle cells via a protein kinase C-dependent activation of the inhibitor of IκBα and ERK1/2 [17], which suggests a signaling pathway depicted in Figure 2.

3. Functional Role of Muscarinic Receptor Subtypes in the Lung

Muscarinic receptors are expressed by tissue-forming cells in the airways, predominantly by smooth muscle, epithelium, and fibroblasts. In the human lung, the density of parasympathetic cholinergic innervation is greatest in the proximal airways and diminishes peripherally. The predominant role of acetylcholine released by the parasympathetic system is in the control of distal airway resistance and the release of mucus from submucosal glands, and from goblet cells in the airway epithelium [18]. The distribution of muscarinic receptors in the human airway has been mapped by receptor autoradiography and in situ hybridization throughout the bronchial tree and is mainly restricted to muscarinic M_1, M_2, and M_3 receptors [18–20], though M_4 may also be involved. Acetylcholine released by cholinergic nerves regulates airway smooth muscle tone and mucus secretion [21].

In the human lung M_1 subtype occurs not in the bronchus [20], but has been reported in human bronchial fibroblasts [22] and bronchial epithelial cells [16]. The presence of the M_1 receptor mRNA was described in human peripheral lung tissue [19]. Stimulation of M_1 receptors in the human lung causes bronchoconstriction and plays a modulatory role in electrolyte and water secretion [18, 23].

The presence of M_2 receptors was reported in the human peripheral lung and the bronchus [20, 24]. Western blot analysis revealed the presence of M_2 protein in human bronchial fibroblasts [22], epithelial cells [16], and smooth muscle cells [18]. Muscarinic M_2 receptors are expressed by neurons, where they function as autoreceptors, limiting the release of acetylcholine from both preganglionic and parasympathetic nerve terminals of the lung [18, 21], of the human trachea [25], and of bronchi, but not of bronchioli [26]. Here, M_2 mediated the inhibition of adenylyl cyclase and thereby preventing bronchodilation [27].

The M_3 receptor is the primary muscarinic receptor subtype that mediates contraction of bronchial and tracheal smooth muscle, even though it is expressed in these tissues at considerable lower levels (about 1/4) than M_2 [28]. M_3 receptor is expressed by the smooth muscle cells of the airways [29], by human bronchial fibroblasts [22], and by human bronchial epithelial cells [16], as well as in the human peripheral lung [24]. The receptor predominantly occurs in the bronchus and its density decreases from the segmental to subsegmental bronchus and is abolished in lung parenchyma [20].

Stimulation of M_3 receptors in the human lung, human central and peripheral airway smooth muscle, and in the human isolated bronchus causes bronchoconstriction and mucus secretion from submucosal glands [18, 27, 29–31]. However, activation of M_3 receptors on vascular endothelial cells also induces the synthesis of nitric oxide, which diffuses to adjacent vascular smooth muscle cells and causes vasodilatation [32].

4. The Functional Role of Nonneuronal Muscarinic Receptor Subtypes in the Lung

During the past decade, several investigators have demonstrated that the biosynthesis, release mechanisms, and muscarinic receptors of the cholinergic system are functionally expressed independently of cholinergic innervations. It is concluded from such evidence that acetylcholine is not merely a neurotransmitter and that it transcends the nervous system, which in relation to lung pathophysiology can modify the phenotypic and cell function of airway cells, including epithelial cells (M_1–M_4), pulmonary vessel endothelial cells (M_1–M_5), mesenchymal cells, such as smooth muscle fibers

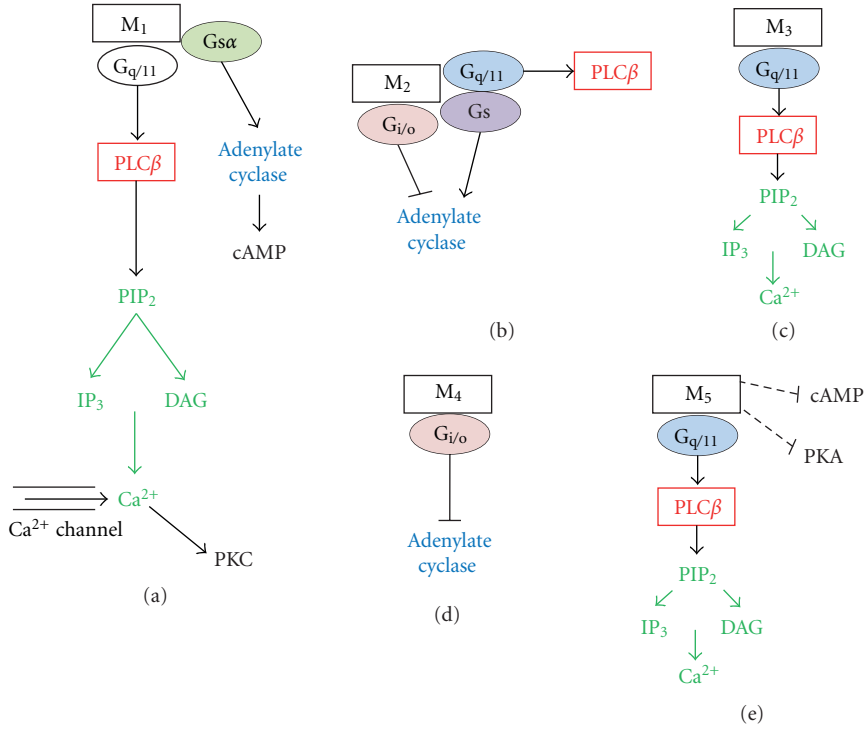

Figure 1: Receptor-specific G-protein coupling and signaling for the five human muscarinic receptors: (a) M_1, (b) M_2, (c) M_3, (d) M_4, and (e) M_5.

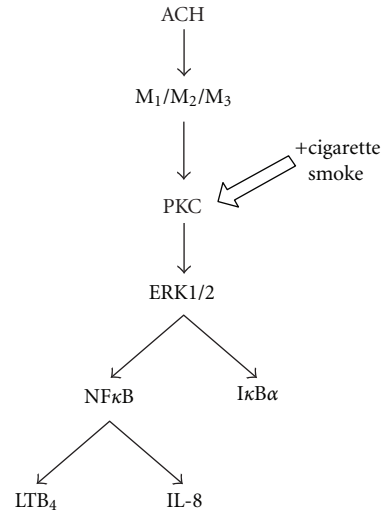

Figure 2: Synergistic effects of acetylcholine (ACH) and cigarette smoke on M_1, M_2, and M_3 receptors. LTB4: leukotriene B4, PKC: protein kinase C, NFκB: nuclear factor kappaB, and IκB: inhibitor of NFκB.

(M_2, M_3) and fibroblasts ($M_2 > M_1 > M_3 > M_4$), and lung-infiltrating immune cells, such as mononuclear leukocytes (M_1–M_5) [33], monocytes, and macrophages (M_1, M_2, and M_3) [34].

The function of nonneuronal acetylcholine released by the airway epithelium may participate in airway smooth-muscle contraction [35], but this remains controversial [36]. Additionally, acetylcholine, either neuronal or nonneuronal, may modulate airway inflammation and tissue remodeling [21]. For example, ensuing cellular effects in the airways following stimulation of M_1 increased proliferation, while M_4 activation increased migration and wound healing in epithelial cells. The stimulation of M_2 increased proliferation of fibroblasts [33].

5. Muscarinic Receptors in Obstructive Pulmonary Diseases

The pathophysiology of pulmonary obstructive diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, is associated with the stimulation of the parasympathetic system, resulting in increased bronchoconstriction and mucus secretion from airway submucosal glands in the human lung. Since the early 70s, it has been established that it is the muscarinic receptor activity of acetylcholine that is involved in the pathophysiology of asthma and COPD. Muscarinic anticholinergic agents proved to be effective in the treatment of asthma and COPD, since the vagal cholinergic tone appears to be a reversible component of airway narrowing [18]. Thus, inhalation of ipratropium bromide, which inhibits M_1, M_2, and M_3, was the first muscarinic inhibitor introduced for the treatment of patients with obstructive pulmonary diseases [37], followed by tiotropium bromide monohydrate that also binds to M_1, M_2, and M_3 and has a longer duration of anticholinergic action [38]. Tiotropium has a considerably slower rate of dissociation from the M_1 and the M_3 receptors than from the M_2 receptor, rendering kinetic selectivity of the drug for M_1

and M_3 receptors [39]. Thus, tiotropium is more effective, since it improves dyspnea and exercise capacity and reduces hyperinflation. It further reduces exacerbations in patients with moderate-to-severe COPD [40].

In addition, there is evidence from animal and human studies of defect expression and/or stimulation of muscarinic receptors in the lungs of asthma and COPD patients. It has been reported that M_2 autoinhibitory receptors do not function normally in airways of some asthmatics [41]. The loss of function of M_2 receptors mediated lung hyperreactivity in antigen-challenged animals and proposed to be an important cause of airway hyperreactivity in asthma [42]. The dysfunction of M_2 autoinhibitory receptors in allergic asthma was proposed to be due to eosinophil-derived major basic protein, which acts as an allosteric antagonist of the M_2 receptor [43], augmenting acetylcholine release, and this may modulate the cellular response associated with airway remodeling [44]. In leukocytes and the bronchi of patients with cystic fibrosis it was shown that the content of acetylcholine is substantially reduced, leading to reduced vesicle storage and transport of nonneuronal acetylcholine [33]. With respect to gene expression of muscarinic receptors, bronchoscopic evaluation of the mucosa in asthma patients revealed an increased expression of M_3 receptor mRNA in severe asthmatics compared to patients with mild-to-moderate asthma and significantly higher levels of M_3 receptor mRNA in patients with brittle asthma [45]. A similar investigation revealed that there are significantly lower levels of the M_3 receptor mRNA in patients with COPD as compared to asthma patients, and that M_3 receptor mRNA gene expression was significantly elevated in COPD patients with bronchial hyperresponsiveness as compared with patients without bronchial hyperresponsiveness [46], indicating that different molecular mechanisms underlie the clinical heterogeneity of bronchoconstriction in severe asthma and COPD.

6. Muscarinic Receptors and Tissue Remodeling in the Lungs

Accumulating evidence over the past decade demonstrated that the pathology of asthma and COPD, in addition to bronchoconstriction, is attributed to inflammation of the airways [18]. The inflammation that occurs in asthma can be described as eosinophilic with an increase in Th2 ($CD4^+$) cells, whereas inflammation that occurs in COPD is mainly neutrophilic with $CD8^+$ T cells predominating [47]. Both neuronal or nonneuronal acetylcholine and muscarinic receptors appear to be involved in inflammation [21].

Pulmonary obstructive diseases are determined by cellular and structural changes of the airways, a process that was associated to chronic airway inflammation. Airway remodeling in asthma and COPD correlates with disease severity [48, 49] and is characterized by mucus gland hypertrophy, goblet cell hyperplasia, and pulmonary vascular remodeling [50]. Specific cellular and structural changes in asthma include basement membrane thickening, subepithelial fibrosis, and thickening of the airway smooth muscle bundle [51], while in COPD specific changes include peribronchial fibrosis and in severe stages of the disease increased airway smooth muscle mass [48]. Acetylcholine, neuronal or nonneuronal and muscarinic receptors appear to play an essential regulatory role in airway remodeling [21, 52, 53]. Recent studies in human-volunteering asthma patients, however, demonstrated that cholinergic stimuli and allergen can induce a very fast remodeling of the airway epithelium and the underlying mesenchymal cells within 8 days [53]. Interestingly, all features of remodeling were prevented by an inhaled beta2-agonist, leading the authors to postulate that relaxation of the bronchi prevented remodeling [53]. Based on our earlier studies, we suggest a more direct inhibitory effect of the beta2-agonist on various extracellular matrix genes [54].

Airway epithelial cells contribute to airway remodeling by hypersecretion of mucous and proliferation, while airway mesenchymal cells contribute by means of proliferation, expression of contractile protein, and the release of components such as mediators, extracellular matrix protein deposition, and matrix metalloproteinase (MMP) secretion [21, 55].

The hypersecretion of mucous by airway epithelial cells contributes to airway obstruction in chronic airway diseases [56]. In vitro and in vivo studies on animal models of asthma and COPD demonstrate the important role of acetylcholine in the regulation of mucus secretion [21]. Using human bronchus and cultured epithelial cells it was shown that the expression of MUC5AC is increased in asthma and COPD patients [57] and can be induced by carbachol and cigarette smoke extract while being inhibited by aclidinium, a long-acting muscarinic antagonist, or atropine [58]. Animals studies show that tiotropium inhibits increased MUC5AC expression and mucus gland hypertrophy in a guinea pig model of COPD [59], as well as the allergen-induced mucus gland hypertrophy and MUC5AC-positive goblet cell number [60]. Tiotropium also reduced the neutrophil elastase-induced goblet cell metaplasia in mice [61]. Acetylcholine may also regulate the proliferative and profibrotic response of airway epithelial cells, either through the induction of mechanical strain or by an autocrine/paracrine mechanism required for the repair of the damaged airway epithelium [21]. Epithelial cell proliferation and the expression of transforming growth factor (TGF)-β (profibrotic cytokine) were increased in bronchial biopsy specimens of patients with mild asthma following repeated challenge with methacholine or house dust mite allergen [53]. Animal studies indicated that acetylcholine induces proliferation of epithelial cells in the rat trachea, mediated by muscarinic M_1 receptors [62] and of airway epithelial cells in monkeys [63].

In the human lung, the stimulation of the M_2 receptor induced cell proliferation of fibroblasts [44, 64] and acetylcholine enhanced cell proliferation in cells isolated from COPD patients, as compared to healthy nonsmokers, through a process involving ERK1/2 and NFκB phosphorylation [65]. Airway smooth muscle thickening is a characteristic pathology of asthma, and to a lesser extent of COPD. Accumulating evidence suggests that stimulation of muscarinic receptors is involved in the proliferation and maturation of airway smooth muscle cells [21].

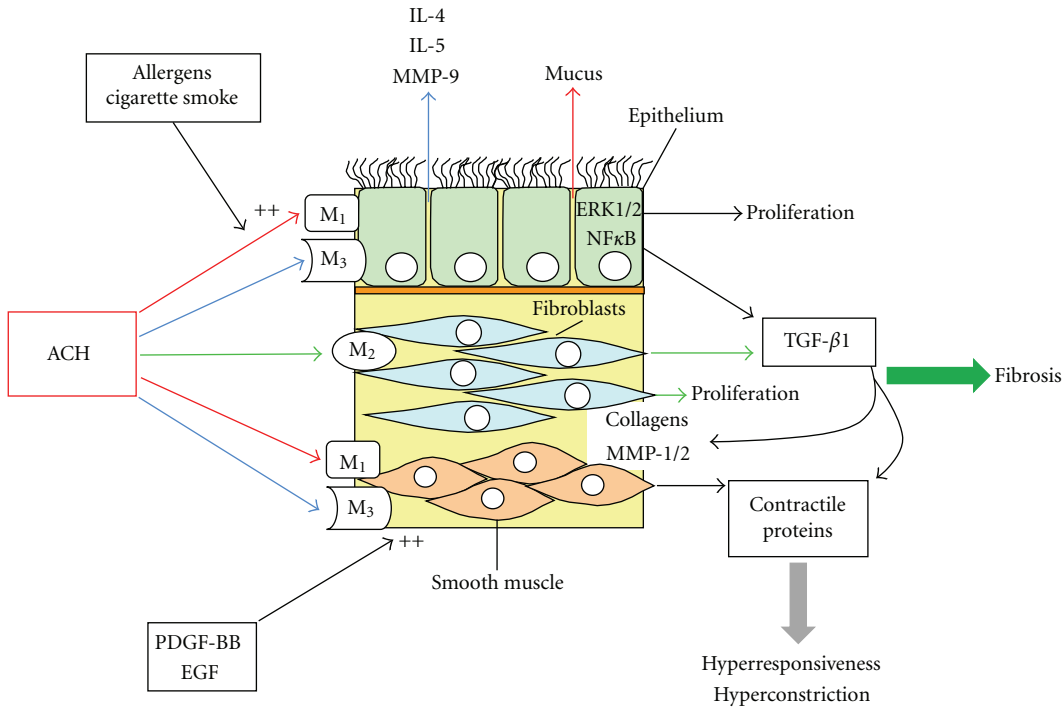

Figure 3: Cell type and muscarinic receptor specific effects on airway wall remodeling.

Furthermore, muscarinic receptor activation enhanced the mitogenic effect of platelet-derived growth factor (PDGF) and EGF on airway smooth muscle cells [66, 67]. However, the molecular interaction of the signalling cascades is not clear. Moreover, the expression of myosin light-chain kinase was augmented by carbachol in human airway smooth muscle cells exposed to cyclical mechanical strain [68] and stimulation of muscarinic receptors further enhanced the TGF-β1-induced expression of the contractile protein [69]. In animal models of asthma and COPD, tiotropium significantly inhibited airway smooth muscle remodeling and contractile protein expression in guineapigs [52, 60] and smooth muscle thickening and the expression of TGF-β1 in bronchoalveolar lavage fluid in an ovalbumine mouse model [70]. Similar effects have been described for the selective M_3 receptor antagonist bencycloquidium bromide, which inhibited ovalbumin-induced mRNA expression of IL-5, IL-4, and MMP-9, as well as lung tissue eosinophil infiltration, airway mucus production, and collagen deposition in lung tissues in a murine asthma model [71]. The cell-type-specific expressions of muscarinic receptors and their effect on airway remodeling and inflammation is summarized in Figure 3.

7. Muscarinic Receptor and Extracellular Matrix Molecules

Extracellular matrix molecules, such as collagenous proteins, matrix metalloproteases (MMP), glycosaminoglycans (GAG), and proteoglycans play a key role in airway remodeling, inflammation, and emphysema [72–76].

7.1. Matrix Metalloproteases. Increased levels of MMP-1, MMP-2, and MMP-9 have been reported in the sputum [77] and lung parenchyma [78] of asthma or COPD patients. Hypoxia, which is associated with extracellular matrix remodeling in inflammatory lung diseases, such as fibrosis, COPD, and asthma, upregulated the expression of MMP-1, MMP-2, and MMP-9 precursors without subsequent activation in human lung fibroblasts and pulmonary vascular smooth muscle cells. MMP-13 expression was increased only in fibroblasts and PDGF-BB inhibited the synthesis and secretion of all hypoxia-induced MMP via ERK1/2 MAP kinase activation [73]. Same evidence indicates that muscarinic receptors mediate the expression of MMP in obstructive pulmonary diseases. Tiotropium inhibited TGF-β-induced expression of MMP-1 and MMP-2 in human lung fibroblasts, but had no effect on TGF-β-induced TIMP-1 and TIMP-2 expression [79, 80]. In contrast, bencycloquidium bromide, a selective M_3 receptor antagonist, inhibited ovalbumin-induced expression of MMP-9 mRNA in a murine asthma model [71], indicating that M_1 and M_3 receptors mediate profibrotic and inflammatory response via specific MMPs. Evidence for the involvement of muscarinic receptors in the homeostasis of MMP comes also from other tissues. In human colon cancer, the activation of the M_3 receptors stimulated the expression of MMP-1, MMP-7, and MMP-10, with subsequent transactivation of the epidermal growth factor receptor and proliferation [81].

7.2. Collagenous Proteins. Hypoxia and PDGF-BB induced synthesis of soluble collagen type I via ERK1/2 and p38 MAP kinase in human lung fibroblasts and pulmonary vascular smooth muscle cells [73]. In human lung fibroblasts

stimulation of M_2 receptors induced cell proliferation and collagen synthesis [44, 64]. In a clinical trial, inhalation of methacholine induced airway remodeling in asthma patients, through the expression of TGF-β and collagen type-I as shown in bronchial biopsies [53]. Treatment with tiotropium inhibited the increased peribronchial collagen deposition in a guinea pig COPD model [59].

7.3. Glycosaminoglycans (GAG). GAG provide structural links between fibrous and cellular elements of the extracellular matrix. They contribute to viscoelastic properties, regulate permeability and retention of plasma components within the matrix, inhibit vascular cell growth, affect hemostasis, platelet aggregation, and interact with lipoproteins and various growth factors [82]. There are two main types of GAG: the nonsulphated hyaluronic acid and the sulphated GAG, heparan sulphate, heparin, chondroitin sulphate, dermatan sulphate, and keratan sulphate. With the exception of hyaluronic acid, GAG are usually covalently attached to a protein core, forming overall structures referred to as proteoglycans [82].

Evidence for the involvement of muscarinic receptors in the homeostasis of GAG comes from studies on various tissues, including the lung. In rat bladder, hyaluronic acid ameliorated H_2O_2-induced hyperactivity, possibly via the antioxidant activity and the inhibition of purinergic and muscarinic signaling pathway [83]. In rat vascular smooth muscle cells of the aorta, M_3 receptors were involved in heparin-dependent relaxation [32]. In rabbits, acetylcholine-induced reactive oxygen species generation in myocytes and the intact heart was mediated via transactivation of EGF receptors through MMP-dependent release of heparin-binding EGF via muscarinic receptors [84]. In mouse pancreatic beta cells, heparin inhibited a muscarine-dependent ionic current [85]. In humans, inhaled heparin inhibited the bronchoconstriction induced by methacholine [86], even though contrary results have also been reported [87].

8. Conclusion

Muscarinic receptors and their intracellular molecular pathways comprise a major drug target in obstructive lung diseases. There is a need for further pharmacological exploitation of this crucial family of receptors as targets for more effective treatment of asthma and COPD. This huge potential transcends the beneficiary effect of antimuscarinic agents on bronchoconstriction and expands to anti-inflammatory, antiproliferative, and antiremodeling effects. Extracellular matrix molecules, such as GAG and MMP may be valuable biomarkers to determine the effect of muscarinic receptor inhibitors in clinical studies investigating drugs with anti inflammatory and anti-remodeling effects in the human lung.

List of Abbreviations

COPD: Chronic obstructive pulmonary disease
EGF: Epidermal growth factor
ERK1/2: Extracellular signal-regulated kinases 1 and 2
GAG: Glycosaminoglycans
G-proteins: GTP-binding proteins
IL: Interleukin
MMP: Matrix metalloproteinases
M_1, M_2, M_3, M_4, and M_5: Muscarinic receptors
NFκB: Nuclear factor-kappaB
PDGF: Platelet-derived growth factor
TGF: Transforming growth factor.

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Review Article

CC-Chemokine CCL15 Expression and Possible Implications for the Pathogenesis of IgE-Related Severe Asthma

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Airway inflammation is accompanied by infiltration of inflammatory cells and an abnormal response of airway smooth muscle. These cells secrete chemokines and express the cell surface chemokine receptors that play an important role in the migration and degranulation of inflammatory cells. Omalizumab is a monoclonal antibody directed against immunoglobulin E, and its blocking of IgE signaling not only reduces inflammatory cell infiltration mediated by the Th2 immune response but also inhibits other immune responses. The chemokine CCL15 is influenced by omalizumab, and the source of CCL15 has been reported to be airway smooth muscle cells and basophils. CCL15 binds to its receptor CCR1, which has been reported to be expressed by various inflammatory cells and also by airway smooth muscle cells. Therefore, CCL15/CCR1 signaling could be a target for the treatment of asthma. We review the role of CCL15 in the pathogenesis of asthma and also discuss the influence of IgE-mediated immunomodulation via CCL15 and its receptor CCR1.

1. Introduction

Chemokines play an important role in the accumulation of inflammatory cells. They belong to a superfamily of small (6–14 kDa) proteins that regulate trafficking in various cells [1]. The C-C motif chemokine ligand 15 (CCL15) is a member of the macrophage inflammatory protein-1 family of chemokines, and its gene is located on 17q11.2. The genetic sequence of CCL15 is similar to that of C-C motif chemokine ligand 5 (CCL5) which is known as regulated on activation normal T cell expressed and secreted (RANTES) and C-C motif chemokine ligand 3 (CCL3), which is named macrophage inflammatory protein-1α (MIP-1α). The CCL15 gene has four exons and three introns. CCL15 has also been variously termed macrophage inhibitory protein-5 (MIP-5), leukotactin-1 (Lkn-1), and human C-C chemokine 2 (HCC-2) [2–4]. CCL15 binds to two receptors known as CCR1 and CCR3, but it has a higher affinity for the former [2, 5, 6].

Although other chemokines, such as CCL3L1 and CCL5, have already been examined in detail to assess their role in asthma, little is known about the possible influence of CCL15 on asthma. Recently, the serum level of CCL15 was found to be elevated in patients with severe asthma and it was shown to be reduced by omalizumab, a humanized anti-IgE antibody [7]. In addition, airway smooth muscle cells (ASMCs) have been shown to produce CCL15 in vitro, and these cells express CCR1 in asthma patients [8, 9]. This paper reviews the role of CCL15 in the pathogenesis of asthma and also discusses the influence of IgE-mediated immunomodulation via CCL15 and its receptor CCR1.

1.1. CCL15 Expressions and Inflammatory Cells. CCL15 mRNA expression is abundant in the heart and skeletal muscle, and it is also detectable in the placenta, liver, pancreas, adrenal gland, bone marrow, colon, small intestine, lung, trachea, and ASMC [4, 8, 10]. Among the various inflammatory cells, CCL15 mRNA expression has been observed in human lung leukocytes, basophils, and alveolar macrophages, but no expression has been found in

lymphocytes, neutrophils, monocytes, lung dendritic cells, or endothelial cell, fibroblast, and leukemia cell lines [2, 8].

The major role of CCL15 as a chemoattractant (similar to CCL3L1) is mediated via its receptor. CCL15 elicits a transient increase of intracellular calcium $[Ca^{2+}]i$ in isolated human blood monocytes and eosinophils, but it has little effect on lymphocytes and neutrophils [4]. In agreement with these findings, CCL15 has a chemotactic effect on human blood monocytes and eosinophils, but little effect on lymphocytes [4]. Neutrophils isolated from the peripheral blood showed a transient increase of $[Ca^{2+}]i$ when exposed to CCL15, but they did not show a chemotactic response [4]. However, another study showed that CCR1, which is the receptor for CCL15, is expressed by neutrophils isolated from peripheral blood, and CCL15 has a high binding capacity for CCR1 and induces chemotaxis of human neutrophils. CCL15 elicits a stronger neutrophil response compared with CCL3, while neutrophils from CCR1 knockout mice fail to respond to this chemokine [11]. Thus, CCL15 plays a role in chemotaxis, but its effects on inflammatory cells are not fully understood.

1.2. CCL15 Receptors and Intracellular Signaling. CCL15 binds to the cell surface receptors CCR1 and CCR3. The genes for CCR1 and CCR3 form a cluster on chromosome 3p, and these receptors belong to the C-C chemokine receptor family, which are seven transmembrane proteins similar to G-coupled protein receptors [12–14]. CCR1 is expressed by monocytes, macrophages, dendritic cells, T cells, B cells, as well as at a low level on mast cells, eosinophils, neutrophils, and ASMC [2, 14–17]. CCL15 induces G protein (Gi/Go) signal transduction, increases phospholipase C (PLC) activity, increases protein kinase Cδ (PKCδ) activity, and activates the transcription factor nuclear factor-kappa B (NF-κB), resulting in chemotaxis or protein synthesis or degranulation [5, 18]. The kinase activity of mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase-1/2 (ERK1/2) and p38 contributes to CCL15-induced chemotaxis [19]. CCR3 is constitutively expressed at a high level on eosinophils, and is also expressed on basophils, mast cells, T cells (Th2), keratinocytes, and ASMC [14, 20]. Among the ligands of CCR3, the role of CCL3, CCL5, and CCL11 (eotaxin-1) in allergic inflammation has been extensively examined, but little is known about CCL15-induced CCR3 signal transduction. CCL11 induces the signal transduction pathway for G proteins (G$\beta\gamma$/Gαi), increases Ras activity, increases phosphorylation of ERK2 and p38 MAP kinases, and promotes chemotaxis, cell differentiation, and protein synthesis or release by degranulation [21–23].

CCL15 binds to both CCR1 and CCR3, but it has a lower affinity for the latter receptor [2, 5, 6]. CCR1 was equally expressed by Th1 and Th2 cells in human cord blood lymphocytes [24]. CCR1 knockout mice have smaller experimental lung granulomas, which is related to increased interferon-γ (IFN-γ) production (Th1) with decreased production of interleukin-4 (IL-4) (Th2) in pulmonary lymph node cells. These results suggested that CCR1 not only influences the inflamatory response through a direct effect on leukocyte chemotaxis, but also by modulating the Th1 or Th2 cytokine balance [25]. Thus, signaling via CCR1 plays a role in the modulation of inflammation.

1.3. Expression of CCL15 and Its Receptor (CCR1) by Airway Smooth Muscle Cells and Basophils. Elevation of CCL15 protein levels had been reported in patients with various pulmonary diseases. CCL15 was elevated in the bronchoalveolar lavage fluid (BALF) obtained from patients with stage III sarcoidosis [26] and in peripheral blood from patients with severe persistent asthma, while anti-IgE antibody therapy reduced the CCL15 level of severe asthma patients [7]. Treatment of nonsmall cell lung cancer reduced the plasma level of CCL15 protein, and this was thought to be related to the influence of CCL15 on angiogenesis [27]. A recent study showed that CCL15 protein was elevated in the supernatant of cultured human airway smooth muscle cells (ASMCs) after stimulation with tumor necrosis factor-α (TNF-α), while the level was synergistically enhanced by adding IFN-γ (the synergy of TNF-α with IFN-γ was NF-κB dependent). Expression of CCL15 mRNA was elevated in bronchial biopsy specimens from patients with moderate-to-severe asthma, and its level was higher in moderate asthma [8]. Elevation of CCL15 mRNA levels had been reported in human lung leukocytes and alveolar macrophages [2, 8], while basophils are also a source of CCL15. CCL15 protein production was observed when basophils isolated from the peripheral blood of asthma patients and nonasthmatic control subjects were stimulated with IL-3 [28]. Biopsy of the airways has demonstrated elevated expression of CCR1 mRNA in mild-to-severe asthma, and CCR1 expression in ASMC has been revealed by immunohistochemistry [9]. Basophils also express CCR1 in allergic responses [14, 29].

Thus, ASMC and basophils express the CCR1, and CCL15 has a high binding affinity for this receptor. These findings suggest that ASMC and basophils are important sources of CCL15, which might have a role in asthma and contribute to the severity and persistence of this condition through targeting its receptors (especially CCR1) in an autocrine manner.

1.4. Interaction of CCL15/CCR1 with Immunoglobulin E in Asthma. ASMCs not only express CCR1 and CCR3, but also express high-affinity (FcεRI) and low-affinity (FcεRII) IgE-Fc receptors. It has been reported that IgE induces abnormal smooth muscle contraction, while sensitization of ASMC to IgE elicits the sequential autocrine release of IL-4, IL-5, and IL-13, but not IFN-γ [30–32]. IgE cross-linking also induces the production of IL-6, IL-8, and TNF-α via ERK1/2 and p38 MAP kinases in ASMC isolated from the bronchial tissue of asthma patients, while cytokine production is inhibited by the anti-IgE antibody omalizumab [33]. Moreover, CCL15 protein is produced by ASMC after stimulation with TNF-α, and its production was synergistically enhanced by IFN-γ [8].

The serum level of IFN-γ is related to the decline of FEV1 in asthma, and IFN-γ expression in the airway wall is higher in severe asthma than moderate asthma. IFN-γ mediates

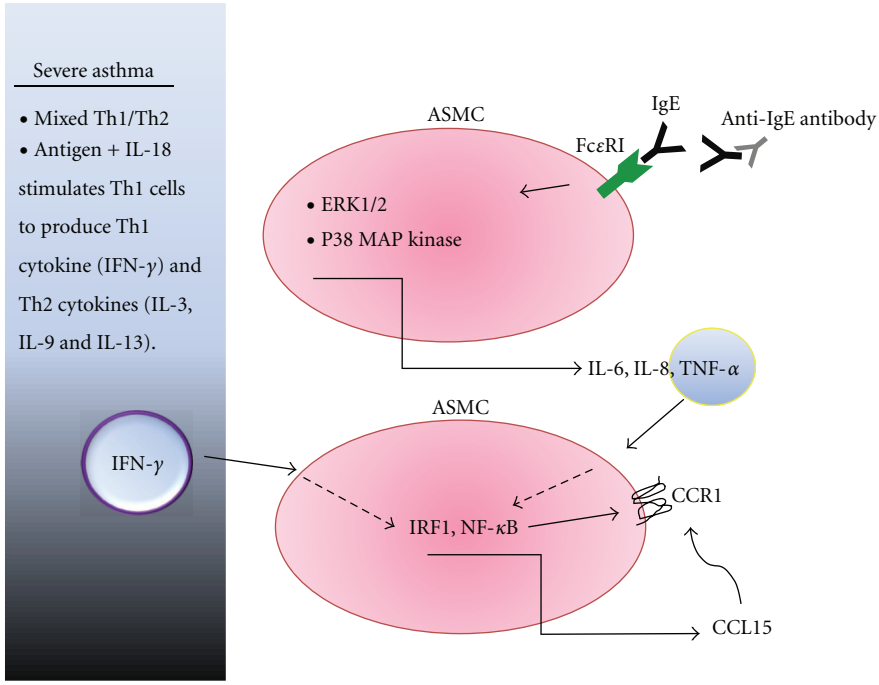

Figure 1: CCL15/CCR1-mediated inflammatory response associated with IgE in ASMCs from an asthma patient. IgE-stimulated ASMC produce IL-6, IL-8, and TNF-α via the activation of extracellularly regulated MAP kinase-1/2 (ERK1/2) and p38 MAP kinase. TNF-α also plays a role in CCL15 production by synergistically enhancing the effect of IFN-γ via interferon regulatory factor-1 (IRF-1) and NF-κB. IFN-γ production is abundant in patients with mixed Th1/Th2 or Th1 asthma. TNF-α and IFN-γ upregulate CCR1 expression by ASMC, while binding of CCL15 to CCR1 might influence the severity and persistence of asthma.

Th1 immune responses, while airway inflammation is a Th2 immune response that involves IL-4 and IL-13. However, the Th2 immune response is not essential in severe asthma and IFN-γ could be involved [34, 35]. Asthma is thus becoming recognized as a heterogenous disorder that presents a mixed Th1/Th2 phenotype with a contribution from Th17 (IL-17) cells [36, 37]. Th1 cells stimulated by antigens and IL-18 produce IFN-γ (a Th1 cytokine) as well as IL-9 and IL-13 (Th2 cytokines) [38, 39]. Based on these reports, the mechanism of IgE-mediated CCL15 production could be as follows. IgE stimulates ASMC to produce IL-6, IL-8, and TNF-α, after which TNF-α promotes CCL15 production by ASMC with a synergistic enhancement of this effect in the presence of IFN-γ (Figure 1).

TNF-α and IFN-γ upregulate CCR1 expression by ASMC, so binding of CCL15 to CCR1 might contribute to the severity and pesistence of asthma. Thus, IgE may indirectly modulate the CCL15/CCR1 axis in ASMC, while inhibition of these mechanisms by omalizumab might contribute to a reduction of CCL15 in patients with severe persistent asthma [7].

As described above, basophils are a source of CCL15, and these cells express CCR1, CCR3, and FcεRI. Although omalizumab therapy reduces FcεRI-mediated production of IL-4, IL-13, and IL-8 by basophils, CCL15 mRNA expression is not altered by FcεRI cross-linking [28]. The mechanism that regulates CCL15 production by basophils in response to IgE signaling remains unknown.

It has been reported that Th1 cells not only produce Th1 cytokines, but also Th2 cytokines (IFN-γ, IL-3, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor, GM-CSF) [38, 39], and novel CD4+ subsets that include Th17 cells, Th9 cells, and regulator T cells (Tregs) have emerged as being involved in the pathogenesis of asthma (Figure 2). Th17 cells differentiate from Th0 cells in response to interleukin-6 (IL-6), and these cells produce interleukin-17 (IL-17), interleukin-21 (IL-21), and interleukin-22 (IL-22) [40]. IL-17 attracts neutrophils [41], while IL-21 influences IgE production by B cells [42].

Some Th2 cells switch to Th9 cells after stimulation by transforming growth factor-β (TGF-β) and IL-4 [43]. Th9 cells secrete IL-4 and IL-9, which enhance IgE production by B cells, and IL-9 also promotes the production of IL-8, CCL11, and prostaglandin E_2 (PGE_2) by ASMC [44]. Tregs are induced by TGF-β, and these cells secrete interleukin-10 (IL-10) and TGF-β. Tregs have an inhibitory effect on Th2 cells and also inhibit IgE production by B cells [45]. Although Th17 cells, Th9 cells, and Tregs may be involved in IgE-mediated asthma and seem to have an influence on ASMC, the associations of these Th subsets with the CCL15/CCR1 axis has not been explored in relation to the pathogenesis of asthma.

Blocking of IgE signaling also has effects on leukotrienes and prostaglandins. Omalizumab decreases the circulating levels of several leukotrienes (C4, D4, and E4) in children with allergic rhinitis [46], while prostaglandin D_2 levels

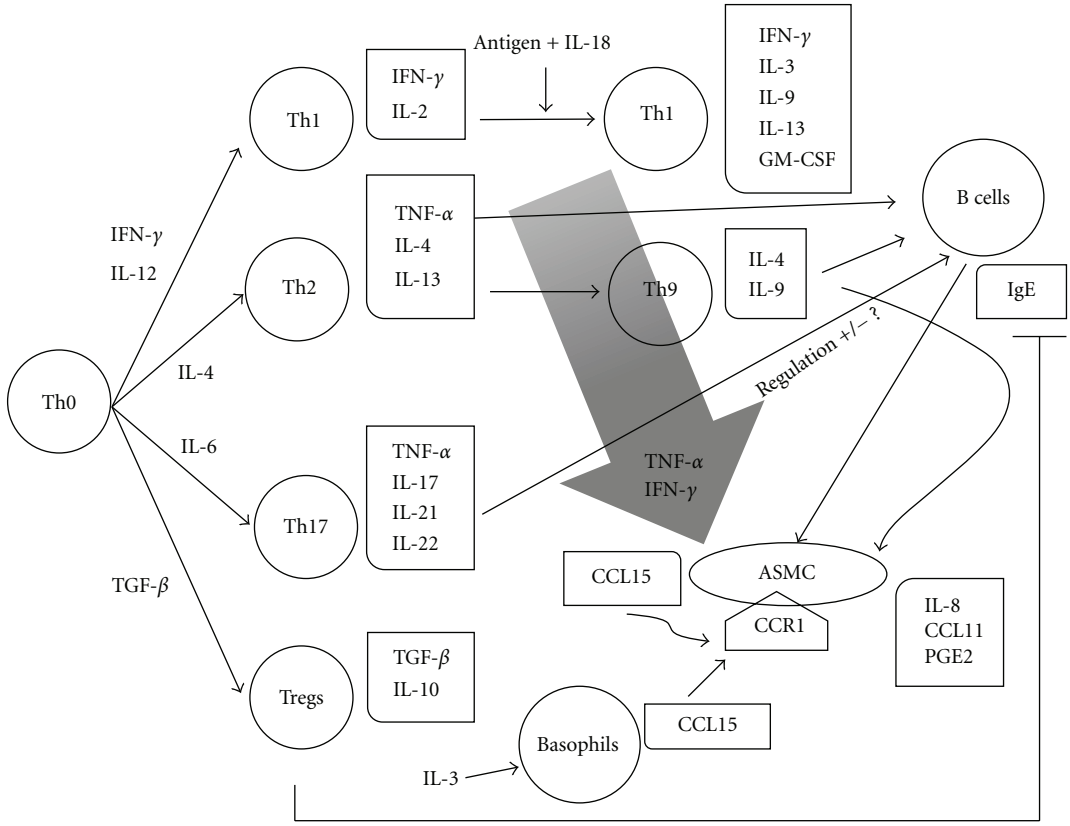

Figure 2: CD4+ Th subsets in asthma and the potential role of CCL15 production by ASMC. Mixed Th1 cells produce both Th1 and Th2 cytokines (IFN-γ, IL-3, IL-9, IL-13, GM-CSF) when stimulated by exposure to an antigen plus IL-18. Th2 and Th17 cells secrete TNF-α, while Th9 cells are differentiated from Th2 cells secrete IL-9, which promotes IgE production by B cells and the production of IL-8, CCL11, and prostaglandin E_2 (PGE_2) by ASMC. IgE promotes the inflammatory phenotype of ASMC, and these cells produce CCL15 when stimulated with both TNF-α and IFN-γ. IL-21 from Th17 cells has been suggested to regulate IgE production by B cells, and Tregs have an inhibitory effect on it.

in nasal lavage fluid from allergic rhinitis patients are reduced by omalizumab therapy [47]. These mediators are also important in asthma, but their relation with the CCL15/CCR1 axis remains unknown.

Omalizumab has been reported to be effective for food allergies, allergic rhinitis, atopic dermatitis, and urticaria caused by various triggers [48]. An asthma patient on high-dose beclomethasone (>800 μg/day) with a low forced expiratory volume (<65% of the predicted value) was reported to respond to omalizumab [49], but whether there is a different response depending on the trigger for asthma or a difference between moderate and severe asthma has not yet been determined.

2. Other Effects of CCL15

Airway remodeling is involved in the pathogenesis of severe persistent asthma. Omalizumab therapy reduced airway wall thickening in patients with severe asthma, along with a reduction of the sputum eosinophil count [50]. CCL15 has also been reported to contribute to plaque instability during the progression of atherosclerosis by promoting the release of matrix metalloproteinase-9 from THP-1 cells [51]. Furthermore, CCL15 promotes angiogenesis in lung cancer [27]. Moreover, CCL15 is elevated in patients with advanced (stage III) sarcoidosis [26], as well as in patients with moderate-to-severe asthma [7, 8]. Thus, CCL15 may contribute to both chronic inflammation and airway remodeling (Figure 3).

2.1. Clinical Trials of Omalizumab Therapy for Asthma. There have been many studies investigating the efficacy of omalizumab for moderate-to-severe asthma. The Investigation of Omalizumab in severe Asthma TrEatment study (INNOVATE study) showed a decrease of exacerbations in patients with severe asthma and improvement of the asthma quality of life questionnaire (AQLQ) [52]. In addition, regular use of asthma medications was reduced in patients with severe asthma [53], and the use of rescue medications was also decreased in patients with moderate-to-severe asthma [54].

A large-scale prospective study of omalizumab is ongoing (The Epidemiologic study of Xolair (omalizumab): evaluating Clinical Effectiveness and Long-term Safety in patients with moderate-to-severe asthma (EXCELS study)) [55]. According to a recent report on 2-year data from this study, patients initiating omalizumab therapy experienced

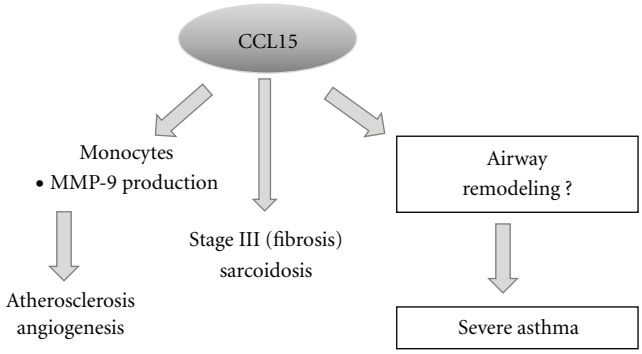

Figure 3: Other effects of CCL15. It has been shown that CCL15 has a role in atherosclerosis via macrophage activation, and that it promotes angiogenesis in lung cancer. CCL15 has also been suggested to show an association with the fibrotic stage of sarcoidosis, but its role in airway remodeling remains unknown.

clinically relevant improvement, whereas established users of omalizumab maintained control of their asthma along with slight improvement or a similar outcome to that seen in nonusers of this agent [56]. A study of the effect of omalizumab on clinical improvement of asthma and inflamatory mediators showed a reduction in the release of cytokines (IL-4, Il-8, and IL-13) by basophils from omalizumab-treated patients, along with the reduction of IL-5 and IL-13 release in cocultures of plasmacytoid dendritic cells and T cells. These studies also suggested that IgE probably facilitates the presentation of allergens by dendritic cells in vivo and has an important role in regulating DC-dependent T cell cytokines during the effector phases of allergic disease [57].

3. Conclusion

CCL15 was reduced in asthma patients by omalizumab and ASMCs were considered to be the source of CCL15. Anti-IgE therapy with omalizumab improves asthma, and several possible mechanisms of immunomodulation by omalizumab have been reported. As omalizumab is used more widely, further effects of this agent may be discovered. Although the chief role of the CCL15/CCR1 axis has been considered to involve promoting the accumulation of inflammatory cells in the airways of asthma patients; CCL15 may also make a contribution to the severity of asthma and to airflow limitation via effects on ASMC. Thus, CCL15 could be a potential target for asthma therapy, although little is known about its contribution to the pathophysiology of this disease.

Authors' Contribution

Y. Shimizu wrote the paper and K. Dobashi gave useful suggestions for this work.

Disclosure

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