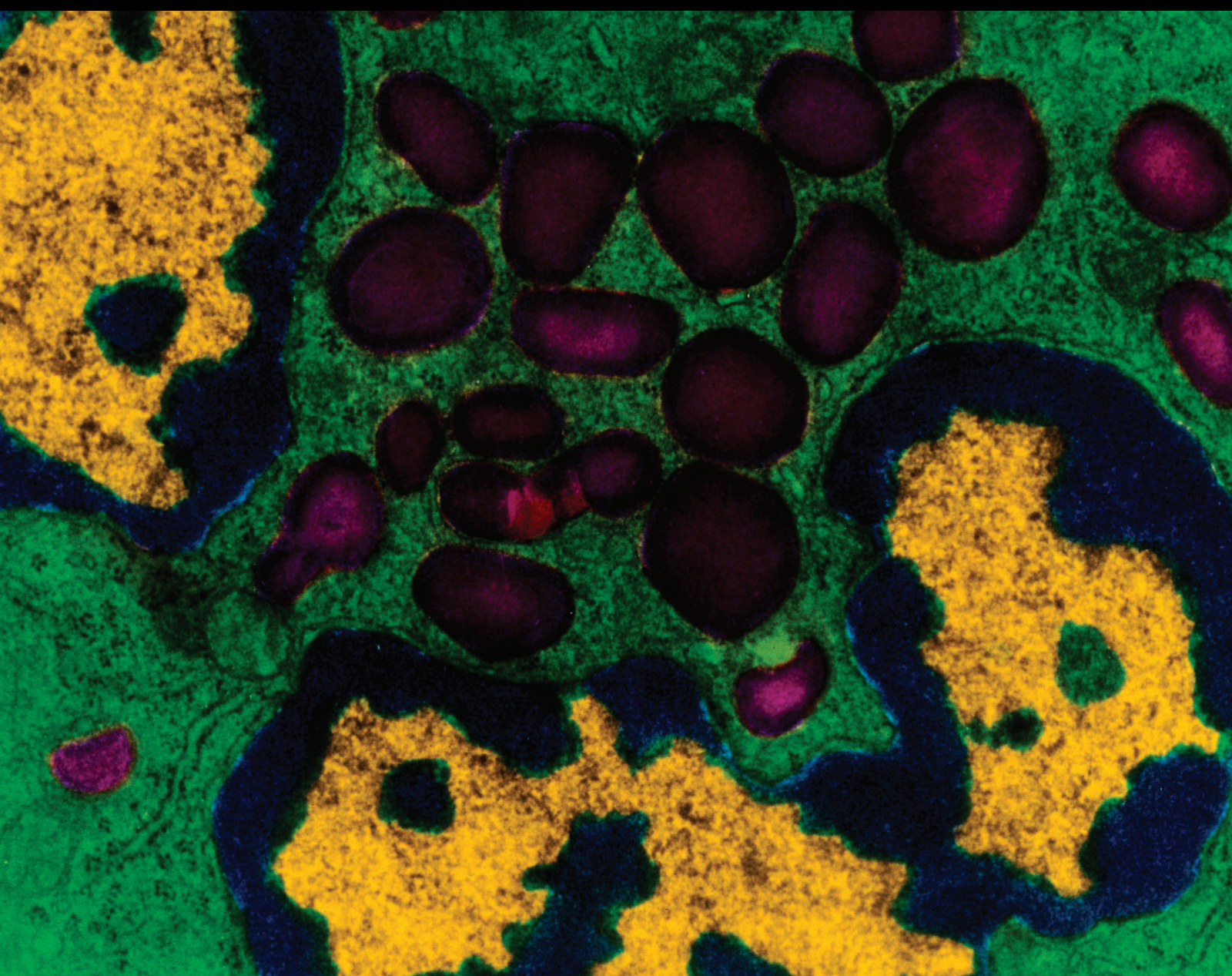
Mediators of Inflammation

Interplay between Hormones, the Immune System, and Metabolic Disorders

Special Issue Editor in Chief: Joilson O. Martins
Guest Editors: Naïma Moustaïd-Moussa and Francisco Rios



Interplay between Hormones, the Immune System, and Metabolic Disorders

Interplay between Hormones, the Immune System, and Metabolic Disorders

Special Issue Editor in Chief: Joilson O. Martins
Guest Editors: Naïma Moustaïd-Moussa and Francisco Rios

Editorial Board

Contents

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 8654212, 2 pages
https://doi.org/10.1155/2018/8654212

Editorial

Interplay between Hormones, the Immune System, and Metabolic Disorders

Francisco J. Rios,[1] Naïma Moustaïd-Moussa,[2] and Joilson O. Martins[3]

[1]*Institute of Cardiovascular and Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow, UK*
[2]*Department of Nutritional Sciences and Obesity Research Cluster at Texas Tech University, Lubbock, USA*
[3]*Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil*

Correspondence should be addressed to Joilson O. Martins; martinsj@usp.br

Received 5 August 2018; Accepted 5 August 2018; Published 30 September 2018

This special issue of Mediators of Inflammation focuses on diverse research areas related to the interplay between hormones, immune response, and metabolic disorders.

Hormones are metabolic components produced by different cell types, capable of regulating body homeostasis and the cross talk among the endocrine, cardiovascular, and immune systems. In patients with compromised immune response, inflammation may last longer or may be ineffective, leading to recurrent infections or other types of systemic dysfunctions associated with chronic inflammation. In the past few years, it became evident that hormones, neurotransmitters, and dietary factors are specific modulators of cells from the immune system by fine-tuning their activation and key functions. Of note, cells from the immune system present high expression of receptors for different hormones present in the blood circulation, such as aldosterone and glucocorticoids. This in turn might also affect the vascular function leading to cardiovascular diseases. Therefore, the main scope of this edition is to contribute to knowledge in this growing and innovative area, through reviews and original articles that will help to understand the diverse mechanisms by which hormones and/or diet can influence inflammatory response and immune activation.

This special issue covers the most current research aimed at elucidating how metabolites and dietary components such as vitamins, bioactive compounds, or lipid mediators influence inflammatory processes. Articles published in here explore the cell and molecular mechanisms underpinning the endocrine/paracrine networks of regulatory immune mediators, their targets on immune cell signaling, and how they contribute to metabolic dysregulations in obesity. The current issue also highlights the importance whereby hormones contribute to cellular homeostasis and immune system regulation, and that an imbalance in this well-regulated system can lead to cardiovascular and/or metabolic disorders.

In this edition of Mediators of Inflammation, N. V. Fedorova et al. studied the effects of insulin, glucagon, and 17β-estradiol (E2) on the activation of human neutrophils. They reported that hormones influence the activation of neutrophils and induce their adherence to blood vessels in diabetes and metabolic disorders. Extracellular matrix proteins play a crucial role in this process. Moreover, glucagon can contribute to the development of metabolic vascular disorders by initiating the secretion of cathepsin G, an important enzyme present in neutrophils with bactericide activity. In addition, cathepsin G may promote inflammatory response and stimulate further neutrophil adhesion via proteolysis of cell surface receptors. On the other hand, insulin and E2 can alter the adhesion of neutrophils initiating the secretion of metalloproteinases, which modify extracellular matrix proteins.

In newborns, the developing hypothalamic-pituitary-adrenal axis is activated after exposure to painful and stressful situations as a result of increased glucocorticoid secretion by the adrenal gland cortex. A high concentration of cortisol might also result in an increased risk factor for insulin resistance, hyperlipidaemia, immunologic deficiencies, and

destructive changes in the hippocampus. G. De Bernardo et al. presented a pilot but interesting study showing that a full-time rooming-in (for 24 hrs.) is better than a partial rooming-in (for 14 hrs.) in reducing neonatal stress response in hospitalized newborns. This was supported by their data showing that lower salivary cortisol levels (SCLs) may have long-term positive effects in reducing the risk of metabolic syndrome, high blood pressure, and cognitive and behavioural changes. In addition, in an experimental study, A. M. Balbino et al. evaluated long leptin receptor isoform (ObRb) expression in lung endothelial cells from low birth weight (LBW) rats and examined the production of lipid mediators and cytokines. They found that lung endothelial cells isolated from intrauterine undernourished rats with a LBW exhibit suppressed IL-1β and IL-6 production after applying inflammatory stimuli. They further demonstrate that these effects may be linked to a lack of ObRb receptor expression and mediated in part by the NF-κB and p38 MAPK signaling pathways.

Wound healing involves a series of tightly controlled biochemical and cellular events, divided in 3 concomitant and overlapping phases: inflammation, proliferation, and remodeling. Poor wound healing or chronic wounds are characterized by a full thickness in depth and a slow healing tendency. Examples of these include diabetic foot ulcers, venous leg ulcers, and pressure ulcers, and all represent a silent epidemic that affects a large fraction of the world population and represent a major public health problem. J. R. Silva et al. reported that omega-6 (ω-6) fatty acids can improve the wound healing process by modulating cellular responses, through increased endothelial and inflammatory cell migration and function as well as enhanced angiogenesis at the wound site, therefore accelerating the wound healing process.

Sex hormone-binding globulin (SHBG) is a serum protein released mainly by the liver, and a low serum level correlates with a higher risk for metabolic syndrome including diabetes, obesity, and cardiovascular events. H. Yamazaki et al. report that SHBG exhibits anti-inflammatory effects involving macrophages and adipocytes, as evidenced by suppressed mRNA levels for inflammatory cytokines such as IL-6, TNFα, and MCP-1, all known to be highly expressed in adipocytes, with major effects on the chronic low grade inflammation. Additionally, in a review article M. Mendes-Braz and J. O. Martins discuss recent publications addressing the effects of diabetes mellitus (DM) on oxidative stress response and inflammatory processes, which play an essential role in ischaemia-reperfusion injury and impaired hepatic regeneration after liver surgery. Authors highlight the need to expand the knowledge in this area, to benefit patients with DM who undergo surgical procedures that are increasing in clinical practice.

T. Zhu et al. showed that in lipopolysaccharide- (LPS-) induced pulmonary inflammation, there is a reduction in the surfactant protein-A (SP-A) expression, both *in vivo* and *in vitro*. Pulmonary surfactant (PS) is synthesized by type II alveolar epithelial (ATII) cells and plays a crucial role in the maintenance of pulmonary compliance and fluid balance in the lungs, in preventing the lung from collapsing at the end of expiration, and in regulating the size of alveoli and pulmonary immune defenses. They also reported that the SP-A-enhancing property of liraglutide was most likely mediated via the thyroid transcription factor-1 (TTF-1) signaling pathway.

Leishmania is an obligatory intracellular protozoan that is transmitted vectorially and causes a disease that affects two million people globally each year. This infection results in lesions on the skin, mucosa, or viscera, depending on the specie of the parasite as well as the host response. L. C. Reis et al. reported new insights into the immunology of leishmaniasis by showing that IGF-I is an effector element in cellular responses mediated by IL-4, leading to M2 macrophage polarization. Mechanisms underlying these effects are dependent on the PI3K/Akt pathway during *L. major* infection. In addition, they speculated that individuals in endemic areas might be more susceptible or resistant depending on the expression of basal IGF-I.

Histamine is a biogenic vasoactive amine, which may impact the immune system activation, by acting as a regulatory component to establish homeostasis after injury or preventing the inflammatory process. Histamine is the main mediator responsible for the clinical symptoms in type 1 hypersensitivity reactions and has pleiotropic effects that are dependent on the interaction with histamine receptors. A. C. C. C. Branco et al. discuss recent findings about histamine effects on inflammation through the activation of intracellular pathways to enhance the production of inflammatory mediators and cytokines in different immune cells. In addition, a review article written by L. M. Oliveira et al. discusses the effects of vitamin A on the innate and adaptive immunity with special emphasis on the inflammatory status, which is becoming a public health concern. Currently, more than 2 billion people are affected by micronutrient deficiency worldwide and vitamin A supplementation is highly effective in reducing mortality from different causes, such as intestinal diseases, neurodegenerative processes, skin aging, and cancer.

In summary, we hope that the original research and the review articles featured in this special issue will enhance the knowledge about the importance of different endocrine systems in inflammatory processes, and help shed light on potential avenues for the development of novel therapies for increasingly prevalent inflammatory and metabolic diseases.

Conflicts of Interest

The authors have no conflict of interest regarding the publication of this editorial.

Acknowledgments

We would like to thank all the authors and reviewers for their valuable contributions to this special issue.

Francisco J. Rios
Naïma Moustaïd-Moussa
Joilson O. Martins

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9524075, 10 pages
https://doi.org/10.1155/2018/9524075

Review Article

Role of Histamine in Modulating the Immune Response and Inflammation

Anna Cláudia Calvielli Castelo Branco, Fábio Seiti Yamada Yoshikawa, Anna Julia Pietrobon, and Maria Notomi Sato

Laboratory of Dermatology and Immunodeficiencies, LIM-56, Tropical Medical Institute, Department of Dermatology, School of Medicine, University of São Paulo, Sao Paulo, SP, Brazil

Correspondence should be addressed to Maria Notomi Sato; marisato@usp.br

Received 23 February 2018; Revised 15 June 2018; Accepted 4 July 2018; Published 27 August 2018

Academic Editor: Naïma Moustaïd-Moussa

Inflammatory mediators, including cytokines, histamine, bradykinin, prostaglandins, and leukotrienes, impact the immune system, usually as proinflammatory factors. Other mediators act as regulatory components to establish homeostasis after injury or prevent the inflammatory process. Histamine, a biogenic vasoactive amine, causes symptoms such as allergies and has a pleiotropic effect that is dependent on its interaction with its four histamine receptors. In this review, we discuss the dualistic effects of histamine: how histamine affects inflammation of the immune system through the activation of intracellular pathways that induce the production of inflammatory mediators and cytokines in different immune cells and how histamine exerts regulatory functions in innate and adaptive immune responses. We also evaluate the interactions between these effects.

1. Introduction

Histamine and its receptors represent a complex system of immunoregulation with distinct effects mediated by four GPCRs (G protein-coupled receptors HRs 1–4) and their differential expression, which changes according to the stage of cell differentiation and microenvironmental influences. Several host factors, in addition to genetic factors, may influence histamine/receptor effects, including the microbiota, gender, ageing, autoimmune diseases, inflammatory skin, cancer, gut, and pulmonary diseases.

Inflammatory conditions (e.g., allergy, asthma, and autoimmune diseases) have long been thought to be mainly mediated by the activation of histamine receptor 1 (H1R). However, in the treatment of diseases such as chronic pruritus, asthma, and allergic rhinitis, the use of selective H4R ligands and/or modulation of H1 and H4 receptor synergism may be more effective for such pathophysiological conditions. Recent evidence strongly suggests that H4R ligands might be exploited as potential therapeutics in allergy, inflammation, autoimmune disorders, and possibly cancer. Overall, exploiting the impact of histamine on innate and adaptive immune responses may be helpful for understanding receptor signaling and trends during inflammation or regulation.

Histamine shows a dichotomous nature, whereby it is able to promote inflammatory and regulatory responses that contribute to pathological processes, such as allergy induction, as well as homeostatic functions, such as intestinal regulation. In this review, we summarize recent findings about the regulation of the immune response by histamine. A general overview of the immune cascades triggered by histamine receptor activation is provided.

2. Histamine and Histamine Receptors

Histamine (2-[3H-imidazol-4-yl]ethanamine) is an important chemical mediator that causes vasodilation and increased vascular permeability and may even contribute to anaphylactic reactions [1]. It also acts on several physiological functions, such as cell differentiation, proliferation, haematopoiesis, and cell regeneration. Synthesis of histamine

occurs through decarboxylation of the amino acid histidine by the enzyme L-histidine decarboxylase (HDC), which is expressed in neurons, parietal cells, gastric mucosal cells, mast cells, and basophils; degradation of histamine is mediated by the enzyme diamine oxidase (DAO) and histamine N-methyltransferase (HNMT), which catalyses histamine deamination [2, 3]. HNMT is expressed in the central nervous system, where it may play a critical regulatory role because its deficiency is related to aggressive behaviour and abnormal sleep-wake cycles in mice [4].

The pleiotropic effects of histamine are mediated by 4 histamine receptors (HRs), H1R, H2R, H3R, and H4R, which are G protein-coupled receptors. The active and inactive conformations of these receptors coexist in equilibrium. Agonists of these receptors stabilize the active conformation, whereas antagonists stabilize the inactive conformation. Curiously, the ageing process impairs expression or activity of HRs, and the enzymes HDC and DAO may contribute to the progression of allergic reactions and various neurodegenerative disorders [5]. Chronic itch in the elderly is a common problem that is often multifactorial due to physiological changes in ageing skin, including impaired skin barrier function, and changes in immunological, neurological, and psychological systems associated with age.

H1R is expressed in various cell types, such as neurons, endothelial cells, adrenal medulla, muscle cells, hepatocytes, chondrocytes, monocytes, neutrophils, eosinophils, DCs, T cells, and B cells. H1R signaling results in the following: synthesis of prostacyclins; activation of platelet factor; synthesis of nitric oxide, arachidonic acid and its metabolites, and thromboxane; and contraction of smooth muscle cells. In addition, H1R activation leads to increased chemotaxis of eosinophils and neutrophils at the site of inflammation, higher functional capacity of antigen-presenting cells (APCs), activation of Th1 lymphocytes, and decreased humoral immunity but the promotion of IgE production [6]. As expected for such biological actions, H1R antagonists, including pyrilamine, fexofenadine, diphenhydramine, and promethazine, are commonly used for the treatment of allergic symptoms.

Signaling via H1R leads to the activation of intracellular transcription factors, such as IP3 (inositol triphosphate), PLC (phospholipase C), PKC (protein kinase C), DAG (diacylglycerol), and Ca^{2+}. Recently, H1R and H4R signaling was implicated in MAPK signaling and cAMP accumulation, leading to increased proinflammatory gene expression [7]. In addition, activation of H1R is important for the generation of Th1 responses, whereas H2R regulates Th2 responses. Mice genetically deficient for H1R (H1R−/−) have an exacerbated Th2 profile due to a decrease in Th1 responses [8]. In addition, H1R was demonstrated in an experimental allergy model to play a critical role together with histamine in orchestrating recruitment of Th2 cells to the site of allergic lung inflammation [9].

H2R is expressed by parietal cells of the gastric mucosa, muscle, epithelial, endothelial, neuronal, hepatocyte, and immune cells. H2R antagonizes some of the effects mediated by H1R and leads to the relaxation of smooth muscle cells, causing vasodilation. H2R activation regulates several of the functions mediated by histamine, including cardiac contraction, gastric acid secretion, cell proliferation, and differentiation [10]. It also acts as a suppressor molecule in DCs, increasing IL-10 production [11]. One recent study demonstrated that histamine acts on H2R and induces inhibition of leukotriene synthesis in human neutrophils through cAMP-dependent protein kinase (PKA) signaling [12]. In a murine lung inflammation model, H2R loss has an effect on invariant natural killer T (iNKT) cells, aggravating local inflammation [13].

In monocyte-derived DCs from healthy adult subjects, H2R activation counterbalances the Toll-like receptor (TLR) response, leading to inhibition of CXCL10, IL-12, and TNF-α stimulation of IL-10, which is likely associated with Th2 polarization [14]. Mechanistically, inhibition of TLR-associated NF-κB and AP-1 pathways occurs due to cAMP activation downstream of H2R activation [14].

While the activation H1R and H2R mainly accounts for mast cell- and basophil-mediated allergic disorders [15], H3R functions were identified in the central nervous system and peripheral and presynaptic receptors to control the release of histamine and other neurotransmitters. The asymmetry of histamine via H3R inhibits the acetylcholine released in the mouse cortex, which controls neurogenic inflammation by inhibiting cAMP formation and Ca^{2+} accumulation [16]. H3R knockout mice exhibit an obese phenotype, suggesting that H3R regulates insulin resistance and leptin release, as well as a decrease in homeostatic energy, the cellular process for coordinating homeostatic regulation of food intake (energy inflow) and energy expenditure (energy outflow), as associated with the UCP1 and UCP3 genes [17]. H3R expression may be associated with bronchoconstriction, pruritus (without involvement of mast cells), increased proinflammatory activity, and antigen-presentation capacity [18].

Neuromodulation and the waking state are related to histaminergic neurons. The waking state is maintained by continual activation of aminergics (such as histamine, dopamine, noradrenaline, and acetylcholine). Three subtypes of HRs are widely distributed in the brain, not only on neurons but also on astrocytes and blood vessels. Positive-allosteric modulators of $GABA_A$ receptors acting on histamine neurons in the posterior hypothalamus induce a natural NREM-like sleep [19]. Targeting the histamine and noradrenergic systems may aid in the design of more precise sedatives and, at the same time, may reveal more about the natural sleep-wake circuitry [19]. In fact, there is a potential utility of histamine H3R antagonist/inverse agonists for CNS disorders. An experimental study showed that when subjected to lipopolysaccharide (LPS) challenge, histamine inhibits the injurious effect of microglia-mediated inflammation by protecting dopaminergic neurons, highlighting the down-modulatory ability of histamine and/or HR agonists. This finding may be useful for the development of new therapeutic approaches to treat neurodegenerative disorders [20].

H4R is preferentially expressed in the intestine, spleen, thymus, bone marrow, peripheral haematopoietic cells, and cells of the innate and adaptive immune systems. Expression of H4R is regulated by stimulation with IFN, TNF-

α, IL-6, IL-10, and IL-13, leading to inhibition of cAMP accumulation and activation of MAPK (mitogen-activated protein kinases) by H4R. Activation of this receptor causes chemotaxis in mast cells and eosinophils, leading to an accumulation of inflammatory cells and control of cytokine secretion by DC and T cells. H4R is also involved in increased secretion of IL-31 by Th2 cells [21]. Treatment of mice with the H4R antagonist JNJ7777120 attenuates pruritus in response to histamine, IgE, and compound 48/80, and its inhibitory effect is greater than that observed with H1R antagonists [22]. The use of this synthetic H4R antagonist in a murine encephalomyelitis model resulted in an increase in the clinical and pathological signs of the disease, suggesting a modulatory role [23].

HRs are present on tumour cells, making them sensitive to variations in histamine. High levels of histamine are associated with bivalent behaviour in the regulation of several tumours (i.e., cervical, ovarian, vaginal, uterine, vulvar, colorectal, and melanoma cancers) by promoting or inhibiting their growth [24]. The presence of H3R and H4R in human mammary tissue suggests that H3R may be involved in regulating breast cancer growth and progression [25], emphasizing the possible use of antihistamines as adjuvants in cancer chemotherapy.

In HDC-deficient mice, a decrease in H4R expression on iNKT cells is associated with lower production of IL-4 and IFN-γ by those cells, which demonstrates regulation between these factors [26]. Several studies have shown that histamine is involved in regulating the function of DCs [27], such as by potentiating antigen endocytosis, inducing intracellular Ca^{2+} mobilization, promoting F-actin polymerization in immature DCs derived from monocytes [28], and promoting expression of MHC class II molecules. Strikingly, cross-presentation, the ability to drive MHC II-associated antigens towards the MHC I pathway, is blocked by H3R/H4R antagonists. Histamine also acts on T cell polarization by inhibiting IFN-γ or LPS-driven IL-12 production in a H1R/H2R dependent manner [29]. H4R has a modulatory role in APCs (DCs and monocytes) by exerting anti-inflammatory action and reducing IL-12 and CCL2 production [28, 30].

Asthma is prevalent in males during childhood but is more frequent in females during adolescence and adulthood. Furthermore, allergic diseases are common in women of childbearing age. Both asthma and atopic conditions may worsen, improve, or remain the same during pregnancy. Female hormones, such as estrogen, can modulate the inflammatory response, and histamine receptors can differ between males and females, which might explain the different incidence of allergy between the sexes [31]. For example, H2R and H3R are highly expressed in female compared to male rats and are downregulated in ovariectomized females, whereas H1R is equally expressed in both sexes [32].

The cascades and effects of different histamine receptors are summarized in Figure 1 and Table 1.

3. Histamine Stimulates Inflammation

Inflammatory mediators are molecules produced by activated cells that intensify and prolong the inflammatory response. Histamine is a potent inflammatory mediator, commonly associated with allergic reactions, promoting vascular and tissue changes and possessing high chemoattractant activity. The binding of histamine to eosinophil H4R induces increased expression of macrophage-1 antigen (Mac1) and ICAM-1 adhesion molecules, in addition to promoting actin filament rearrangement [33, 34]. These events favour the migration of eosinophils from the bloodstream to the site of inflammation. In mast cells, the binding of histamine to this same receptor promotes the intracellular release of calcium and recruitment of mast cells into tissues [35]. Mast cells from H4R-knockout mice lose the ability to migrate against a histamine gradient [35]. Recruitment of these cells to sites of inflammation amplifies the inflammatory reactions mediated by histamine and may favour the establishment of a chronic inflammatory response. In experimental models, histamine drives colitis via HR4 by promoting granulocyte infiltration into the colonic mucosa [36].

Histamine also modulates the inflammatory response by acting on other cellular populations, in human lung macrophages, binding of histamine to H1R induces production of the proinflammatory cytokine IL-6 and β-glucuronidase [37, 38], a marker of exocytosis, and the release of lysosomal enzymes is associated with epithelial damage and rupture of the basement membrane [39]. Together, these events suggest that histamine may contribute to the maintenance of inflammatory conditions in the airways. In contrast, activation of H2R in rat peritoneal macrophages inhibits production of TNF-α and IL-12 when stimulated with LPS [40].

In an animal model of allergic airway inflammation, H4R-knockout mice present lower inflammation, reduced pulmonary infiltrate of eosinophils and lymphocytes and an attenuated Th2 response [41]. The contribution of histamine to the induction of airway inflammation is also due to its effect on nonimmune cells. In nasal fibroblasts, there is a dose-dependent increase in IL-6 production in response to histamine stimulation [42]. This inflammatory mediator increases expression of phosphorylated p38, pERK, and pJNK and induces NF-κB activation. Treatment with H1R antagonists reduces expression of phosphorylated p38 and NF-κB and, consequently, IL-6 production.

Blocking H4R in a model of pulmonary fibrosis alleviates the inflammatory response, reducing COX2 expression and activity, leukocyte infiltration, production of TGF-β (profibrotic cytokine), and collagen deposition [43]. Histamine also activates pulmonary epithelial cells. Histamine binding to H1R enhances TLR3 expression in these cells, and treatment with the TLR agonist poly(I:C), along with histamine, potentiates NF-κB phosphorylation and IL-8 secretion, indicating an increased response of epithelial cells to microbial ligands [44].

In the nervous system, microglial activation is regulated by histamine in a dose-dependent manner, which leads to the production of proinflammatory cytokines, such as IL-6 and TNF-α [45]. This activation is mediated via H1R, and H4R and is dependent on MAPK and PI3K/AKT cascades. In addition to inducing iNOS expression and NO production, histamine promotes the loss of mitochondrial membrane potential and the production of ROS in microglia by

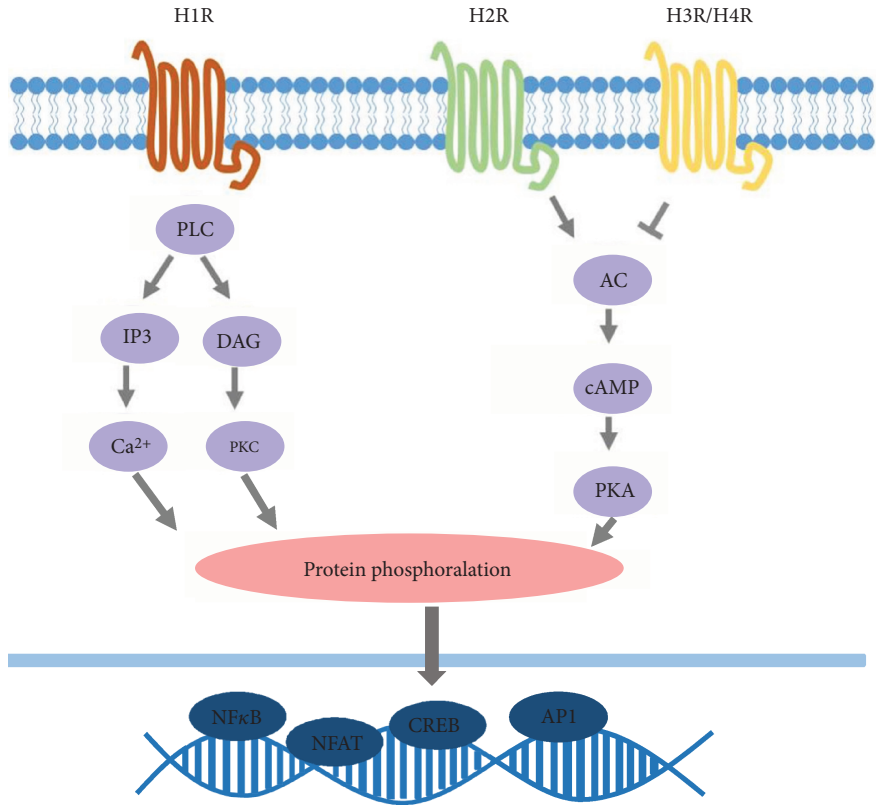


Figure 1: Intracellular activation cascades triggered by histamine receptors (HRs). The pleiotropic effects of histamine are mediated by four histamine receptors: H1R, H2R, H3R, and H4R, which are G protein-coupled receptors. Signaling via H1R leads to activation of intracellular transcription factors, such as PLC (phospholipase C), IP3 (inositol triphosphate), PKC (protein kinase C), DAG (diacylglycerol), and Ca^{2+}. H2R signaling acts through activation of adenylyl cyclase (AC), which increases cyclic adenosine monophosphate (cAMP) levels and activates protein kinase A (PKA), while H3R and H4R inhibit this cascade. Those intracellular signaling pathways culminate into protein phosphorylation and transcription of nuclear factor such as nuclear factor kappa B (NF-κB), nuclear factor of activated T-cells (NFAT), cAMP response element binding protein (CREB), and activator protein 1 (AP-1).

Table 1: Immunological functions mediated by histamine receptors signaling.

Receptor	Expression	Intracellular signaling	Immunological activity
H1R	Endothelial cells, nerve cells, epithelial cells, neutrophils, eosinophils, monocytes, macrophages, DCs, and T and B cells	PLC, PIP2, DAG, IP3, Ca^{2+}, and PKC	Allergic reactions and inflammation, histamine release, eosinophil and neutrophil chemotaxis, antigen presentation ability, Th1/IFN-γ activity, and recruitment of Th2 cells; decreases humoral immunity and IgE production
H2R	Endothelial cells, nerve, epithelial, neutrophils, eosinophils, monocytes, macrophages, DCs, and T cells and B	Adenyl cyclase, cAMP, PKA, CREB, and EPAC	Increases IL-10 production and humoral immunity; decreases cellular immunity; inhibits Th2 cells and cytokines, chemotaxis of eosinophils, and neutrophils; suppresses IL-12p70 of MoDCs
H3R	Histaminergic neurons, monocytes, eosinophils	Inhibitor of adenyl cyclase and cAMP; increases levels of Ca^{2+}	Control of neurogenic inflammation, increased proinflammatory activity, and antigen presentation capacity
H4R	Eosinophils, DCs, Langerhans cells, neutrophils, T cells, basophils, and mast cells	Inhibitor of adenyl cyclase and cAMP; increases levels of Ca^{2+}	Affects pDC and mDC functions, Th1/Th2 differentiation, eosinophil and mast cell chemotaxis, IL-6 production, leukotriene B4, and migration of Tγ/δ cells; increases IL-17 secretion by Th17 cells, and regulatory T recruitment; suppresses IL-12p70 of MoDCs

binding to these same receptors [46, 47]. Overall, the accumulation of these cytokines and proinflammatory molecules can be deleterious, leading to nerve damage.

Histamine also modulates the response of DCs. Stimulation of immature DCs induces expression of CD86, CD80, and MHC class II, increasing the efficiency of T cell activation [11]. In the presence of histamine, DCs exhibit a higher production of IL-6, IL-8, and CCL2 as well as induced expression of IL-1β, CCL5, and CCL4. However, the H2R pathway promotes IL-10 production and inhibits IL-12 synthesis by immature DCs, favouring the development of a Th2 response profile [29]. This modulation of cytokine production, suggests that histamine indirectly alters the Th1/Th2 balance through the stimulation of DCs. In a food allergy model, simultaneous blockage of H1R and H4R inhibited the development of intestinal inflammation and diarrhoea when the animals were exposed to the allergen by suppressing histamine-mediated DC antigen presentation and chemotaxis [48].

The Th1/Th2 balance is directly regulated by histamine, as described above. Th1 cells display higher H1R expression, and their binding to histamine promotes activation of Th1 responses, potentiating IFN-γ production [49]. In contrast, Th1 and Th2 responses are inhibited by histamine stimulation via H2R. Histamine also alters the response of other subpopulations of lymphocytes. Activation of CD8+ T cells via H4R induces secretion of IL-16, a chemoattractant molecule for CD4+ cells such as monocytes and DCs [50]. In addition, histamine stimuli induces IL-17 production in human Th17 cells, suggesting the contribution of this inflammatory mediator to the activation of lymphocytes present in skin lesions in atopic dermatitis and psoriasis [51].

Other evidence, indicates that histamine plays an important role in inflammatory skin diseases. When stimulated via H4R, the NK cells present in skin lesions, increase expression of the chemokines CCL3 and CCL4, favouring cell recruitment to injured tissue [52]. As H4R knockout mice display a lower influx of inflammatory cells and less cell proliferation at the lesion sites, H4R is associated with the inflammatory response in atopic dermatitis [53]. H4R is also involved in NK cell recruitment and induction of CCL17 production by DCs at lesion sites in murine models of atopic dermatitis, contributing to increased local inflammation [54]. In an experimental model of pruriginous dermatitis, H4R blockage decreases itching because the activation of this receptor is involved in increased IL-31 secretion by Th2 cells [21, 55]. Moreover, blocking H4R improves skin lesions and reduces the number of mast cells at lesion sites [55].

Within the context of vascular inflammatory diseases, histamine produced by the tunica intima stimulates the monocytes present in atherosclerotic plaques to express CCL2 and its receptor CCR2, via activation of H2R [56]. Furthermore, higher production of IL-6 and adhesion molecules, such as ICAM-1 and VCAM-1, occurs in endothelial cells stimulated by histamine, thereby favouring progression of the disease [56, 57]. In an experimental model, the absence of H1R reduced the development of atherosclerosis, whereas the absence of H2R exerted the opposite effect [58].

4. Regulatory and Immunomodulatory Functions of Histamine

As discussed above, the pleiotropic effects of histamine are a consequence of the existence of four different receptors that belong to the same family of G-coupled proteins and trigger different signaling cascades; these receptors are differentially distributed across tissues and cells [59]. Therefore, in addition to its classical roles in the inflammatory process, histamine is recognized as a key player in immune regulation.

Although histamine is commonly associated with skin inflammation processes, for example, allergic dermatitis [60], it may play a regulatory role in other clinical conditions such as psoriasis, which is a multifactorial Th1/Th17-driven inflammation of the skin [61]. In a murine psoriasis-like model induced by imiquimod administration, Kim et al. showed that the H4R agonist 4-methylhistamine ameliorated the clinical scores of psoriatic mice due to repression of Th1 cytokines and simultaneous induction of Treg cells [62]. These findings suggest that histamine targeting has pharmacological potential. In addition, histamine participates in the wound-healing process by increasing the viability of rat wound fibroblasts and promoting the production of TGF-β in an H1R-dependent manner [63].

The contribution of histamine to inflammatory neurological diseases, such as multiple sclerosis, is controversial: although H1R and H2R appear to favour the inflammatory response in brain lesions [64], H3R dampens neuroinflammation, mainly by modulating the production of chemokines and maintaining the integrity of the blood-brain barrier [65] in a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Pharmacological blockage of H4R exacerbates EAE, as observed by increased recruitment of inflammatory mediators, larger lesion areas, and enhanced activation of T cells, indicating that H4R also plays a regulatory role in a neurological context [23]. In a murine model of the motor neuron disorder amyotrophic lateral sclerosis (ALS), microglia from SOD1-G93A mice, which spontaneously develop an ALS phenotype, appear to be responsive to histamine through H1R and H4R, leading to a reduction in the inflammatory state. The findings suggest deregulation of the histaminergic pathway in ALS patients [66].

Regardless, the role of histamine in gut homeostasis maintenance is clearly essential, as exemplified in a murine model of HDC deficiency. Yang et al. showed that HDC$^{-/-}$ mice were prone to develop inflammation and had a higher tumour burden at mucosal sites (intestine and skin) in models of chemically induced carcinogenesis but that their phenotype was attenuated by histamine administration [67]. Most HDC expression in these tissues was found in immature myeloid cells (IMCs), rather than in resident mast cells; curiously, histamine administration, acting through H1R and H2R, was required to promote terminal differentiation of IMCs into monocytes and neutrophils [67].

Expression levels of HDC and H2R are positively associated with increased survival in colorectal cancer patients [68], and some epidemiological studies support the association of atopy with the reduced incidence of some cancers

[69, 70], reinforcing a possible role of histamine in blocking tumour development.

In addition to tumour biology, histamine has a regulatory function in gut inflammatory diseases, such as in a murine trinitrobenzene sulfonic acid- (TNBS-) induced model of colonic inflammation, which promotes a delayed hypersensitivity reaction that resembles the phenotype [71]. By employing H4R-deficient mice, Wunschel et al. demonstrated that histamine protects animals from an exacerbated inflammatory reaction in the colon, as illustrated by enhanced production of chemokines/cytokines and loss of intestinal architecture, which ultimately leads to death [72]. Strikingly, Schirmer et al. reported the opposite effect using a similar mouse background ($H4R^{-/-}$ mice), whereby histamine worsened dextran sodium sulfate- (DSS-) induced colitis and pharmacological inhibition of H4R ameliorated the health of the mice [73]. Differences between the models, such as the chemical stimulation used and the route of administration, may account for the opposite results. Overall, the response that prevails in humans requires further investigation.

Recently, the microbiota was revealed to be very important for gut pathophysiology. Indeed, the microbial community in the intestine shapes the health status of the host and its susceptibility to a broad range of local and systemic diseases [74]. The microbiota supplies the host with a number of metabolites, such as short-chain fatty acids, tryptophan metabolites and histamine [75, 76], and a recent work highlights the key role of microbe-derived histamine in the host response.

For example, histamine-secreting bacteria (*Escherichia coli*, *Lactobacillus vaginalis*, and *Morganella morganii*) are found at higher frequency in faecal samples from asthmatic patients, with a possible contribution to their atopic phenotype [76, 77], as microbial-derived histamine is indistinguishable from the human-produced form.

Lactobacillus reuteri is a member of the gut microbiota that belongs to the Firmicutes family; HDC gene expression by this commensal bacterium confers the ability to produce histamine from histidine [78]. Microbial histamine modulates the inflammatory response of the human monocyte cell line THP-1, mainly by acting through H2R, to promote activation of the cAMP/PKA cascade and block of ERK signaling [79]. *L. reuteri*-derived histamine relies on H2R to promote its regulatory function in the intestine [80]. Interestingly, histamine modulates the response of myeloid and plasmacytoid DCs to LPS, favouring IL-10 production over the secretion of inflammatory cytokines [14] and enhancing their ability to phagocytose soluble antigens and upregulate expression of the costimulatory molecules CD86 and inducible costimulator ligand (ICOS-L) [81]. Although the effects depend on H2R and cAMP, histamine requires the participation of exchange proteins activated by cAMP (Epac) in DCs, as opposed to PKA in monocytes, indicating that these signaling cascades are cell type specific [14].

In addition to H2R agonism, *L. reuteri* simultaneously blocks H1R signaling by secreting the enzyme diacylglycerol kinase (DGK). H1R leads to the production of inflammatory mediators by triggering PKC-dependent NF-κB activation [82]. PKC activation, in turn, requires the cofactor diacylglycerol (DAG); DGK degrades DAG into phosphatidic acid. Thus, *L. reuteri*-derived DGK interrupts H1R signaling and blocks the inflammatory effects of histamine but preserves H2R-associated immunomodulation [83].

L. reuteri administration protects HDC-deficient mice from colon tumours in a model of intestinal carcinogenesis with azoxymethane/dextran sodium sulfate (AOS/DSS) administration. *L. reuteri* produces or restores the histamine pool in the intestine, lowering recruitment of immature myeloid cells and production of inflammatory cytokines, which blocks tumour development [68]. Importantly, *L. reuteri* only produces histamine; therefore, it exerts regulatory activity regardless of whether the precursor (histidine) is exogenously provided, for example, by food intake [80]. This highlights the critical interplay between the host immune response, the microbiota, and environmental (dietary) influences on biological outcomes.

In addition to *L. reuteri*, other bacteria have probiotic potential. Another lactobacilli, *Lactobacillus rhamnosus*, is a source of histamine that promotes a regulatory Foxp3-T cell response profile in intestinal Peyer patches while dampening Th1 polarization in an H2R-dependent fashion [14].

The lung is a classical mucosal site under histamine control. Although histamine is associated with deleterious inflammation in asthmatic patients, triggering airway hyperresponsiveness and remodelling [84, 85], recent evidence suggests that the contribution of histamine to pulmonary homeostasis is not straightforward and depends on the receptor and cell type involved. H1R antagonism is well known for its beneficial effects in asthma management [86]; however, H2R deficiency or pharmacological blockade worsens lung inflammation in OVA-sensitized mice due to increased activation of iNKT cells, which promotes recruitment of macrophages and neutrophils and production of IL-4, IL-17, and IFN-γ by T cells [13].

Although the regulatory properties of histamine reveal a promising therapeutic potential, histamine may also exert deleterious effects in some clinical conditions. For example, histamine immunomodulation might contribute to higher susceptibility to sepsis in diabetic mice. Mast cell-derived histamine is increased in diabetic settings and reduces neutrophil recruitment due to repression of CXCR2 [87]. In addition, histamine may impair the oxidative burst of neutrophils [88]. Because neutrophils produce histamine once stimulated [89], a negative feedback loop is established, attenuating the microbicidal mechanism against invading bacteria.

5. Conclusion

Histamine research is an attractive perspective for the potential therapeutic exploitation of new drug targets. The main actions of histamine in controlling the immune response are summarized in Figure 2. The pleiotropic actions of histamine due to the different natures of its receptors allow this simple molecule to exert broad and oppose effects on the immune system, highlighting the importance of a fine-tune control that promotes a homeostatic environment in the

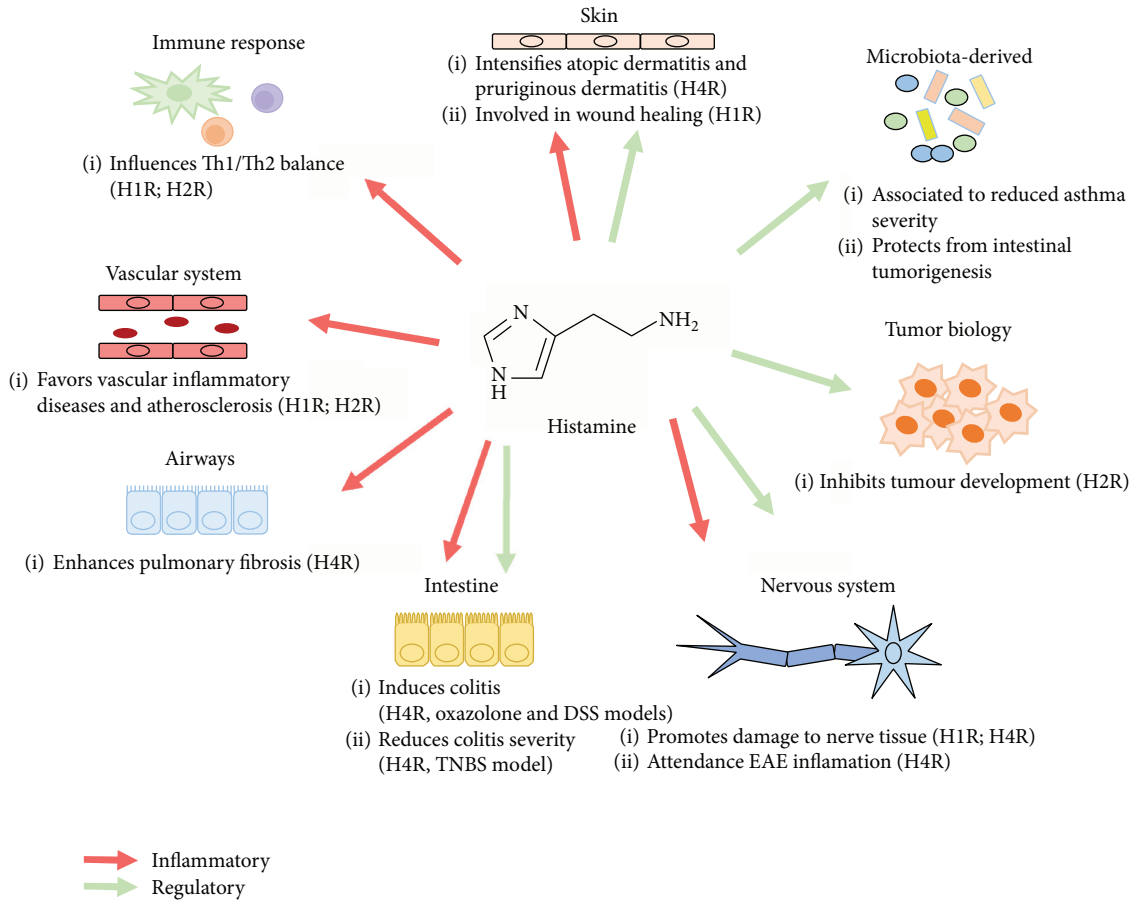


Figure 2: Inflammatory and regulatory functions of histamine on different body sites. Histamine plays dual functions according to the cell type and the receptor. As an inducer of inflammation, histamine can contribute to pulmonary fibrosis, cardiovascular diseases and atherosclerosis, atopic dermatitis, central nervous system damage, and colitis in some experimental models, besides favoring the polarization of the immune response to a Th1 profile. On the other hand, histamine can regulate inflammation in models of experimental autoimmune encephalomyelitis (EAE) and colitis, favor wound healing in skin lesions, and inhibit tumour development. Also, microbiota-derived histamine can regulate the inflammatory picture of asthma. Red arrows indicate proinflammatory action; green arrows indicate regulatory action of histamine.

body, balancing important inflammatory reactions to host protection as well as immunomodulation. The findings about the role of histamine in carcinogenesis, allergic regulation, and even behavioural regulation underscore histamine as a remarkable therapeutic target. However, as HRs are widely expressed in the body and change during cell differentiation and ageing and even differ between sexes, complex histamine/receptor agonism/antagonism should be exploited for therapeutic approaches with caution. New insight about the role of histamine in different body sites and cell types may result in more targeted therapies. Overall, recent findings about the microbial contribution to histamine homeostasis add a new layer of complexity to the picture. Ideal histaminergic therapy should be composed of a mixture of agonists and antagonists that would avoid deleterious side effects. Future studies may improve our understanding of the histamine network in organisms and its paradoxical nature.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo under Grant 2012/16524-6 and the Laboratório de Investigação Médica, Unidade 56.

References

[1] L. O'Mahony, M. Akdis, and C. A. Akdis, "Regulation of the immune response and inflammation by histamine and histamine receptors," *Journal of Allergy and Clinical Immunology*, vol. 128, no. 6, pp. 1153–1162, 2011.

[2] T. Biegański, "Biochemical, physiological and pathophysiological aspects of intestinal diamine oxidase," *Acta Physiologica Polonica*, vol. 34, no. 1, pp. 139–154, 1983.

[3] T. Yoshikawa, F. Naganuma, T. Iida et al., "Molecular mechanism of histamine clearance by primary human astrocytes," *Glia*, vol. 61, no. 6, pp. 905–916, 2013.

[4] F. Naganuma, T. Nakamura, T. Yoshikawa et al., "Histamine *N*-methyltransferase regulates aggression and the sleep-wake cycle," *Scientific Reports*, vol. 7, no. 1, article 15899, 2017.

[5] L. Shan, D. F. Swaab, and A. M. Bao, "Neuronal histaminergic system in aging and age-related neurodegenerative disorders," *Experimental Gerontology*, vol. 48, no. 7, pp. 603–607, 2013.

[6] M. Jutel, K. Blaser, and C. A. Akdis, "The role of histamine in regulation of immune responses," *Chemical Immunology and Allergy*, vol. 91, pp. 174–187, 2006.

[7] S. Beermann, G. Bernhardt, R. Seifert, A. Buschauer, and D. Neumann, "Histamine H_1- and H_4-receptor signaling cooperatively regulate MAPK activation," *Biochemical Pharmacology*, vol. 98, no. 3, pp. 432–439, 2015.

[8] M. Jutel, T. Watanabe, S. Klunker et al., "Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors," *Nature*, vol. 413, no. 6854, pp. 420–425, 2001.

[9] P. J. Bryce, C. B. Mathias, K. L. Harrison, T. Watanabe, R. S. Geha, and H. C. Oettgen, "The H1 histamine receptor regulates allergic lung responses," *The Journal of Clinical Investigation*, vol. 116, no. 6, pp. 1624–1632, 2006.

[10] E. N. Schneider, B. R. Smoller, and L. W. Lamps, "Histiocytic subpopulations in the gastrointestinal tract: distribution and possible relationship to function," *Applied Immunohistochemistry & Molecular Morphology*, vol. 12, no. 4, pp. 356–359, 2004.

[11] G. Caron, Y. Delneste, E. Roelandts et al., "Histamine induces CD86 expression and chemokine production by human immature dendritic cells," *The Journal of Immunology*, vol. 166, no. 10, pp. 6000–6006, 2001.

[12] N. Flamand, H. Plante, S. Picard, M. Laviolette, and P. Borgeat, "Histamine-induced inhibition of leukotriene biosynthesis in human neutrophils: involvement of the H_2 receptor and cAMP," *British Journal of Pharmacology*, vol. 141, no. 4, pp. 552–561, 2004.

[13] R. Ferstl, R. Frei, W. Barcik et al., "Histamine receptor 2 modifies iNKT cell activity within the inflamed lung," *Allergy*, vol. 72, no. 12, pp. 1925–1935, 2017.

[14] R. Frei, R. Ferstl, P. Konieczna et al., "Histamine receptor 2 modifies dendritic cell responses to microbial ligands," *Journal of Allergy and Clinical Immunology*, vol. 132, no. 1, pp. 194–204.e12, 2013.

[15] N. Novak, N. Mete, C. Bussmann et al., "Early suppression of basophil activation during allergen-specific immunotherapy by histamine receptor 2," *Journal of Allergy and Clinical Immunology*, vol. 130, no. 5, pp. 1153–1158.e2, 2012.

[16] V. Dimitriadou, A. Rouleau, M. D. T. Tuong et al., "Functional relationship between mast cells and C-sensitive nerve fibres evidenced by histamine H_3-receptor modulation in rat lung and spleen," *Clinical Science*, vol. 87, no. 2, pp. 151–163, 1994.

[17] N. Takahashi, H. R. Patel, Y. Qi, J. Dushay, and R. S. Ahima, "Divergent effects of leptin in mice susceptible or resistant to obesity," *Hormone and Metabolic Research*, vol. 34, no. 11/12, pp. 691–697, 2002.

[18] C. A. Akdis and F. E. R. Simons, "Histamine receptors are hot in immunopharmacology," *European Journal of Pharmacology*, vol. 533, no. 1–3, pp. 69–76, 2006.

[19] X. Yu, N. P. Franks, and W. Wisden, "Sleep and sedative states induced by targeting the histamine and noradrenergic systems," *Frontiers in Neural Circuits*, vol. 12, p. 4, 2018.

[20] S. Barata-Antunes, A. C. Cristóvão, J. Pires, S. M. Rocha, and L. Bernardino, "Dual role of histamine on microglia-induced neurodegeneration," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1863, no. 3, pp. 764–769, 2017.

[21] R. Gutzmer, S. Mommert, M. Gschwandtner, K. Zwingmann, H. Stark, and T. Werfel, "The histamine H_4 receptor is functionally expressed on T_H2 cells," *Journal of Allergy and Clinical Immunology*, vol. 123, no. 3, pp. 619–625, 2009.

[22] P. J. Dunford, K. N. Williams, P. J. Desai, L. Karlsson, D. McQueen, and R. L. Thurmond, "Histamine H_4 receptor antagonists are superior to traditional antihistamines in the attenuation of experimental pruritus," *Journal of Allergy and Clinical Immunology*, vol. 119, no. 1, pp. 176–183, 2007.

[23] C. Ballerini, A. Aldinucci, I. Luccarini et al., "Antagonism of histamine H_4 receptors exacerbates clinical and pathological signs of experimental autoimmune encephalomyelitis," *British Journal of Pharmacology*, vol. 170, no. 1, pp. 67–77, 2013.

[24] A. I. Faustino-Rocha, R. Ferreira, A. Gama, P. A. Oliveira, and M. Ginja, "Antihistamines as promising drugs in cancer therapy," *Life Sciences*, vol. 172, pp. 27–41, 2017.

[25] V. Medina, M. Croci, E. Crescenti et al., "The role of histamine in human mammary carcinogenesis: H3 and H4 receptors as potential therapeutic targets for breast cancer treatment," *Cancer Biology & Therapy*, vol. 7, no. 1, pp. 28–35, 2008.

[26] M. C. Leite-de-Moraes, S. Diem, M. L. Michel et al., "Cutting edge: histamine receptor H4 activation positively regulates in vivo IL-4 and IFN-γ production by invariant NKT cells," *The Journal of Immunology*, vol. 182, no. 3, pp. 1233–1236, 2009.

[27] T. Simon, V. László, and A. Falus, "Impact of histamine on dendritic cell functions," *Cell Biology International*, vol. 35, no. 10, pp. 997–1000, 2011.

[28] R. Gutzmer, C. Diestel, S. Mommert et al., "Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells," *The Journal of Immunology*, vol. 174, no. 9, pp. 5224–5232, 2005.

[29] A. Mazzoni, H. A. Young, J. H. Spitzer, A. Visintin, and D. M. Segal, "Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization," *The Journal of Clinical Investigation*, vol. 108, no. 12, pp. 1865–1873, 2001.

[30] D. Dijkstra, R. Leurs, P. Chazot et al., "Histamine downregulates monocyte CCL2 production through the histamine H_4 receptor," *Journal of Allergy and Clinical Immunology*, vol. 120, no. 2, pp. 300–307, 2007.

[31] E. Ridolo, I. Martignago, G. Passalacqua, M. Mauro, and C. Incorvaia, "Evaluation of the safety of a protocol for switching venom immunotherapy products," *Annals of Allergy, Asthma & Immunology*, vol. 120, no. 4, pp. 429-430, 2018.

[32] J. N. Li, X. L. Li, J. He et al., "Sex- and afferent-specific differences in histamine receptor expression in vagal afferents of rats: a potential mechanism for sexual dimorphism in prevalence and severity of asthma," *Neuroscience*, vol. 303, pp. 166–177, 2015.

[33] P. Ling, K. Ngo, S. Nguyen et al., "Histamine H_4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation," *British Journal of Pharmacology*, vol. 142, no. 1, pp. 161–171, 2004.

[34] R. Barnard, A. Barnard, G. Salmon, W. Liu, and S. Sreckovic, "Histamine-induced actin polymerization in human eosinophils: an imaging approach for histamine H_4 receptor," *Cytometry Part A*, vol. 73A, no. 4, pp. 299–304, 2008.

[35] C. L. Hofstra, P. J. Desai, R. L. Thurmond, and W. P. Fung-Leung, "Histamine H_4 receptor mediates chemotaxis and calcium mobilization of mast cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 3, pp. 1212–1221, 2003.

[36] J. B. Wechsler, A. Szabo, C. L. Hsu et al., "Histamine drives severity of innate inflammation via histamine 4 receptor in murine experimental colitis," *Mucosal Immunology*, vol. 11, no. 3, pp. 861–870, 2018.

[37] G. Marone, M. Gentile, A. Petraroli, N. De Rosa, and M. Triggiani, "Histamine-induced activation of human lung macrophages," *International Archives of Allergy and Immunology*, vol. 124, no. 1–3, pp. 249–252, 2001.

[38] M. Triggiani, M. Gentile, A. Secondo et al., "Histamine induces exocytosis and IL-6 production from human lung macrophages through interaction with H_1 receptors," *The Journal of Immunology*, vol. 166, no. 6, pp. 4083–4091, 2001.

[39] M. Triggiani, F. Granata, A. Oriente et al., "Secretory phospholipases A_2 induce β-glucuronidase release and IL-6 production from human lung macrophages," *The Journal of Immunology*, vol. 164, no. 9, pp. 4908–4915, 2000.

[40] Y. Azuma, M. Shinohara, P. L. Wang, A. Hidaka, and K. Ohura, "Histamine inhibits chemotaxis, phagocytosis, superoxide anion production, and the production of TNFα and IL-12 by macrophages via H_2-receptors," *International Immunopharmacology*, vol. 1, no. 9-10, pp. 1867–1875, 2001.

[41] P. J. Dunford, N. O'Donnell, J. P. Riley, K. N. Williams, L. Karlsson, and R. L. Thurmond, "The histamine H_4 receptor mediates allergic airway inflammation by regulating the activation of $CD4^+$ T cells," *The Journal of Immunology*, vol. 176, no. 11, pp. 7062–7070, 2006.

[42] I. H. Park, J. Y. Um, J. S. Cho, S. H. Lee, S. H. Lee, and H. M. Lee, "Histamine promotes the release of Interleukin-6 via the H1R/p38 and NF-κB pathways in nasal fibroblasts," *Allergy, Asthma & Immunology Research*, vol. 6, no. 6, pp. 567–572, 2014.

[43] L. Lucarini, A. Pini, A. C. Rosa et al., "Role of histamine H_4 receptor ligands in bleomycin-induced pulmonary fibrosis," *Pharmacological Research*, vol. 111, pp. 740–748, 2016.

[44] Y. Hou, Y. Zhou, X. Zheng et al., "Modulation of expression and function of toll-like receptor 3 in A549 and H292 cells by histamine," *Molecular Immunology*, vol. 43, no. 12, pp. 1982–1992, 2006.

[45] H. Dong, W. Zhang, X. Zeng et al., "Histamine induces upregulated expression of histamine receptors and increases release of inflammatory mediators from microglia," *Molecular Neurobiology*, vol. 49, no. 3, pp. 1487–1500, 2014.

[46] S. M. Rocha, J. Pires, M. Esteves, B. Graça, and L. Bernardino, "Histamine: a new immunomodulatory player in the neuronglia crosstalk," *Frontiers in Cellular Neuroscience*, vol. 8, p. 120, 2014.

[47] J. Zhu, C. Qu, X. Lu, and S. Zhang, "Activation of microglia by histamine and substance P," *Cellular Physiology and Biochemistry*, vol. 34, no. 3, pp. 768–780, 2014.

[48] M. Wang, J. Han, J. Domenico, Y. S. Shin, Y. Jia, and E. W. Gelfand, "Combined blockade of the histamine H1 and H4 receptor suppresses peanut-induced intestinal anaphylaxis by regulating dendritic cell function," *Allergy*, vol. 71, no. 11, pp. 1561–1574, 2016.

[49] M. Jutel, S. Klunker, M. Akdis et al., "Histamine upregulates Th1 and downregulates Th2 responses due to different patterns of surface histamine 1 and 2 receptor expression," *International Archives of Allergy and Immunology*, vol. 124, no. 1–3, pp. 190–192, 2001.

[50] F. Gantner, K. Sakai, M. W. Tusche, W. W. Cruikshank, D. M. Center, and K. B. Bacon, "Histamine H_4 and H_2 receptors control histamine-induced interleukin-16 release from human $CD8^+$ T cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 303, no. 1, pp. 300–307, 2002.

[51] S. Mommert, M. Gschwandtner, B. Koether, R. Gutzmer, and T. Werfel, "Human memory Th17 cells express a functional histamine H4 receptor," *The American Journal of Pathology*, vol. 180, no. 1, pp. 177–185, 2012.

[52] S. Mommert, O. Dittrich-Breiholz, H. Stark, R. Gutzmer, and T. Werfel, "The histamine H4 receptor regulates chemokine production in human natural killer cells," *International Archives of Allergy and Immunology*, vol. 166, no. 3, pp. 225–230, 2015.

[53] K. Rossbach, K. Schaper, C. Kloth et al., "Histamine H4 receptor knockout mice display reduced inflammation in a chronic model of atopic dermatitis," *Allergy*, vol. 71, no. 2, pp. 189–197, 2016.

[54] S. Ehling, K. Roßbach, S. M. Dunston, H. Stark, and W. Bäumer, "Allergic inflammation is augmented via histamine H4 receptor activation: the role of natural killer cells in vitro and in vivo," *Journal of Dermatological Science*, vol. 83, no. 2, pp. 106–115, 2016.

[55] E. Suwa, K. Yamaura, M. Oda, T. Namiki, and K. Ueno, "Histamine H_4 receptor antagonist reduces dermal inflammation and pruritus in a hapten-induced experimental model," *European Journal of Pharmacology*, vol. 667, no. 1–3, pp. 383–388, 2011.

[56] S. Kimura, K. Y. Wang, A. Tanimoto, Y. Murata, Y. Nakashima, and Y. Sasaguri, "Acute inflammatory reactions caused by histamine via monocytes/macrophages chronically participate in the initiation and progression of atherosclerosis," *Pathology International*, vol. 54, no. 7, pp. 465–474, 2004.

[57] Y. Delneste, P. Lassalle, P. Jeannin, M. Joseph, A. B. Tonnel, and P. Gosset, "Histamine induces IL-6 production by human endothelial cells," *Clinical & Experimental Immunology*, vol. 98, no. 2, pp. 344–349, 1994.

[58] S. Yamada, K. Y. Wang, A. Tanimoto et al., "Histamine receptors expressed in circulating progenitor cells have reciprocal actions in ligation-induced arteriosclerosis," *Pathology International*, vol. 63, no. 9, pp. 435–447, 2013.

[59] S. Smolinska, M. Jutel, R. Crameri, and L. O'Mahony, "Histamine and gut mucosal immune regulation," *Allergy*, vol. 69, no. 3, pp. 273–281, 2014.

[60] H. Ohtsu and M. Seike, "Histamine and histamine receptors in allergic dermatitis," *Handbook of Experimental Pharmacology*, vol. 241, pp. 333–345, 2017.

[61] M. Mortezavi and C. Ritchlin, "Immunologic advances reveal new targets in psoriasis and psoriatic arthritis," *Discovery Medicine*, vol. 20, no. 110, pp. 169–175, 2015.

[62] C. H. Kim, J. M. Lee, J. K. Yoo et al., "Inhibitory effect of imiquimod-induced psoriasis-like skin inflammation in mice by histamine H4 receptor agonist 4-methylhistamine," *Scandinavian Journal of Immunology*, vol. 83, no. 6, pp. 409–417, 2016.

[63] M. Wolak, E. Bojanowska, T. Staszewska, J. Ciosek, M. Juszczak, and J. Drobnik, "The role of histamine in the regulation of the viability, proliferation and transforming growth

factor β1 secretion of rat wound fibroblasts," *Pharmacological Reports*, vol. 69, no. 2, pp. 314–321, 2017.

[64] R. Pedotti, J. J. DeVoss, S. Youssef et al., "Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 1867–1872, 2003.

[65] C. Teuscher, M. Subramanian, R. Noubade et al., "Central histamine H_3 receptor signaling negatively regulates susceptibility to autoimmune inflammatory disease of the CNS," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 24, pp. 10146–10151, 2007.

[66] S. Apolloni, P. Fabbrizio, S. Amadio et al., "Histamine regulates the inflammatory profile of SOD1-G93A microglia and the histaminergic system is dysregulated in amyotrophic lateral sclerosis," *Frontiers in Immunology*, vol. 8, p. 1689, 2017.

[67] X. D. Yang, W. Ai, S. Asfaha et al., "Histamine deficiency promotes inflammation-associated carcinogenesis through reduced myeloid maturation and accumulation of $CD11b^{+}$ $Ly6G^{+}$ immature myeloid cells," *Nature Medicine*, vol. 17, no. 1, pp. 87–95, 2011.

[68] C. Gao, B. P. Ganesh, Z. Shi et al., "Gut microbe–mediated suppression of inflammation-associated colon carcinogenesis by luminal histamine production," *The American Journal of Pathology*, vol. 187, no. 10, pp. 2323–2336, 2017.

[69] A. E. Prizment, A. R. Folsom, J. R. Cerhan, A. Flood, J. A. Ross, and K. E. Anderson, "History of allergy and reduced incidence of colorectal cancer, Iowa Women's Health Study," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 16, no. 11, pp. 2357–2362, 2007.

[70] Y. Cui and A. W. Hill, "Atopy and specific cancer sites: a review of epidemiological studies," *Clinical Reviews in Allergy & Immunology*, vol. 51, no. 3, pp. 338–352, 2016.

[71] S. Wirtz, C. Neufert, B. Weigmann, and M. F. Neurath, "Chemically induced mouse models of intestinal inflammation," *Nature Protocols*, vol. 2, no. 3, pp. 541–546, 2007.

[72] E. J. Wunschel, B. Schirmer, R. Seifert, and D. Neumann, "Lack of histamine H_4-receptor expression aggravates TNBS-induced acute colitis symptoms in mice," *Frontiers in Pharmacology*, vol. 8, p. 642, 2017.

[73] B. Schirmer, T. Rezniczek, R. Seifert, and D. Neumann, "Proinflammatory role of the histamine H_4 receptor in dextrane sodium sulfate-induced acute colitis," *Biochemical Pharmacology*, vol. 98, no. 1, pp. 102–109, 2015.

[74] K. J. Pflughoeft and J. Versalovic, "Human microbiome in health and disease," *Annual Review of Pathology*, vol. 7, no. 1, pp. 99–122, 2012.

[75] J. A. Gilbert, R. A. Quinn, J. Debelius et al., "Microbiome-wide association studies link dynamic microbial consortia to disease," *Nature*, vol. 535, no. 7610, pp. 94–103, 2016.

[76] W. Barcik, B. Pugin, P. Westermann et al., "Histamine-secreting microbes are increased in the gut of adult asthma patients," *Journal of Allergy and Clinical Immunology*, vol. 138, no. 5, pp. 1491–1494.e7, 2016.

[77] W. Barcik, M. Wawrzyniak, C. A. Akdis, and L. O'Mahony, "Immune regulation by histamine and histamine-secreting bacteria," *Current Opinion in Immunology*, vol. 48, pp. 108–113, 2017.

[78] J. K. Spinler, A. Sontakke, E. B. Hollister et al., "From prediction to function using evolutionary genomics: human-specific ecotypes of *Lactobacillus reuteri* have diverse probiotic functions," *Genome Biology and Evolution*, vol. 6, no. 7, pp. 1772–1789, 2014.

[79] C. M. Thomas, T. Hong, J. P. van Pijkeren et al., "Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling," *PLoS One*, vol. 7, no. 2, article e31951, 2012.

[80] C. Gao, A. Major, D. Rendon et al., "Histamine H2 receptor-mediated suppression of intestinal inflammation by probiotic *Lactobacillus reuteri*," *mBio*, vol. 6, no. 6, 2015.

[81] H. Schenk, D. Neumann, and C. Kloth, "Histamine regulates murine primary dendritic cell functions," *Immunopharmacology and Immunotoxicology*, vol. 38, no. 5, pp. 379–384, 2016.

[82] M. Matsubara, T. Tamura, K. Ohmori, and K. Hasegawa, "Histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine production through inhibition of Ca^{2+}-dependent protein kinase C, Raf/MEK/ERK and IKK/IκB/NF-κB signal cascades," *Biochemical Pharmacology*, vol. 69, no. 3, pp. 433–449, 2005.

[83] B. P. Ganesh, A. Hall, S. Ayyaswamy et al., "Diacylglycerol kinase synthesized by commensal *Lactobacillus reuteri* diminishes protein kinase C phosphorylation and histamine-mediated signaling in the mammalian intestinal epithelium," *Mucosal Immunology*, vol. 11, no. 2, pp. 380–393, 2017.

[84] A. B. Murray, A. C. Ferguson, and B. Morrison, "Airway responsiveness to histamine as a test for overall severity of asthma in children," *Journal of Allergy and Clinical Immunology*, vol. 68, no. 2, pp. 119–124, 1981.

[85] N. Tsurikisawa, C. Oshikata, T. Tsuburai et al., "Bronchial reactivity to histamine is correlated with airway remodeling in adults with moderate to severe asthma," *The Journal of Asthma*, vol. 47, no. 8, pp. 841–848, 2010.

[86] F. E. R. Simons and K. J. Simons, "H_1 antihistamines: current status and future directions," *World Allergy Organization Journal*, vol. 1, p. 145, 2008.

[87] D. Carlos, F. Spiller, F. O. Souto et al., "Histamine H_2 receptor signaling in the pathogenesis of sepsis: studies in a murine diabetes model," *The Journal of Immunology*, vol. 191, no. 3, pp. 1373–1382, 2013.

[88] M. Ciz and A. Lojek, "Modulation of neutrophil oxidative burst via histamine receptors," *British Journal of Pharmacology*, vol. 170, no. 1, pp. 17–22, 2013.

[89] C. Smuda, J. B. Wechsler, and P. J. Bryce, "TLR-induced activation of neutrophils promotes histamine production via a PI3 kinase dependent mechanism," *Immunology Letters*, vol. 141, no. 1, pp. 102–108, 2011.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3067126, 17 pages
https://doi.org/10.1155/2018/3067126

Review Article

Impact of Retinoic Acid on Immune Cells and Inflammatory Diseases

Luana de Mendonça Oliveira, Franciane Mouradian Emidio Teixeira, and Maria Notomi Sato

Laboratory of Dermatology and Immunodeficiencies, LIM-56, Department of Dermatology, School of Medicine, University of São Paulo, Institute of Tropical Medicine of São Paulo, São Paulo, SP, Brazil

Correspondence should be addressed to Maria Notomi Sato; marisato@usp.br

Received 29 March 2018; Revised 16 June 2018; Accepted 28 June 2018; Published 9 August 2018

Academic Editor: Naïma Moustaïd-Moussa

Vitamin A metabolite retinoic acid (RA) plays important roles in cell growth, differentiation, organogenesis, and reproduction and a key role in mucosal immune responses. RA promotes dendritic cells to express CD103 and to produce RA, enhances the differentiation of $Foxp3^+$ inducible regulatory T cells, and induces gut-homing specificity in T cells. Although vitamin A is crucial for maintaining homeostasis at the intestinal barrier and equilibrating immunity and tolerance, including gut dysbiosis, retinoids perform a wide variety of functions in many settings, such as the central nervous system, skin aging, allergic airway diseases, cancer prevention and therapy, and metabolic diseases. The mechanism of RA is interesting to explore as both a mucosal adjuvant and a combination therapy with other effective agents. Here, we review the effect of RA on innate and adaptive immunity with a special emphasis on inflammatory status.

1. Introduction

Vitamins are essential components of diet and are essential for the maintenance of various biological processes. For example, vitamin A, through its active metabolite, retinoic acid (RA), acts in several biological conditions, such as embryonic development, hormone function, the maintenance and modulation of the immune response, and the homeostasis of epithelial tissues and mucosa [1, 2].

Vitamin A is obtained through diet, and its deficiency, especially in childhood, increases the morbidity and mortality risk from infectious diseases, especially diseases of the gastrointestinal and pulmonary tracts, causes blindness and anemia, and impairs vaccine responses [1, 3]. In low-income countries, children receive insufficient amounts of vitamin A during breastfeeding and childhood, making vitamin A deficiency a public health problem. Studies have shown that vitamin A supplementation reduces the mortality rate by 24% among children aged 6 months to 5 years [4]. For this reason, the World Health Organization (WHO) recommends vitamin A supplementation for infants and children aged 6–59 months in underdeveloped countries [5].

Indeed, after the absorption and metabolization of vitamin A into RA in the gut, RA plays critical roles in the mucosal immune response as a regulatory signal in the intestinal mucosa by promoting Foxp3 regulatory T cell differentiation [6] and immunoglobulin (Ig) A production [7]. In addition, RA induces the homing of innate immune cells, such as innate lymphoid cells (ILCs) [8] besides regulatory and effector T and B cells, to the gut [9–11]. During infections, RA can induce the production of proinflammatory cytokines by dendritic cells (DCs), promoting the differentiation of effector T cells and the protection of the mucosa [12]. Thus, RA is crucial for maintaining homeostasis at the intestinal barrier and equilibrating immunity and tolerance. Due to the extensive role of RA in immune cells and the immune response, reducing mortality in children by vitamin A supplementation may be possible [4].

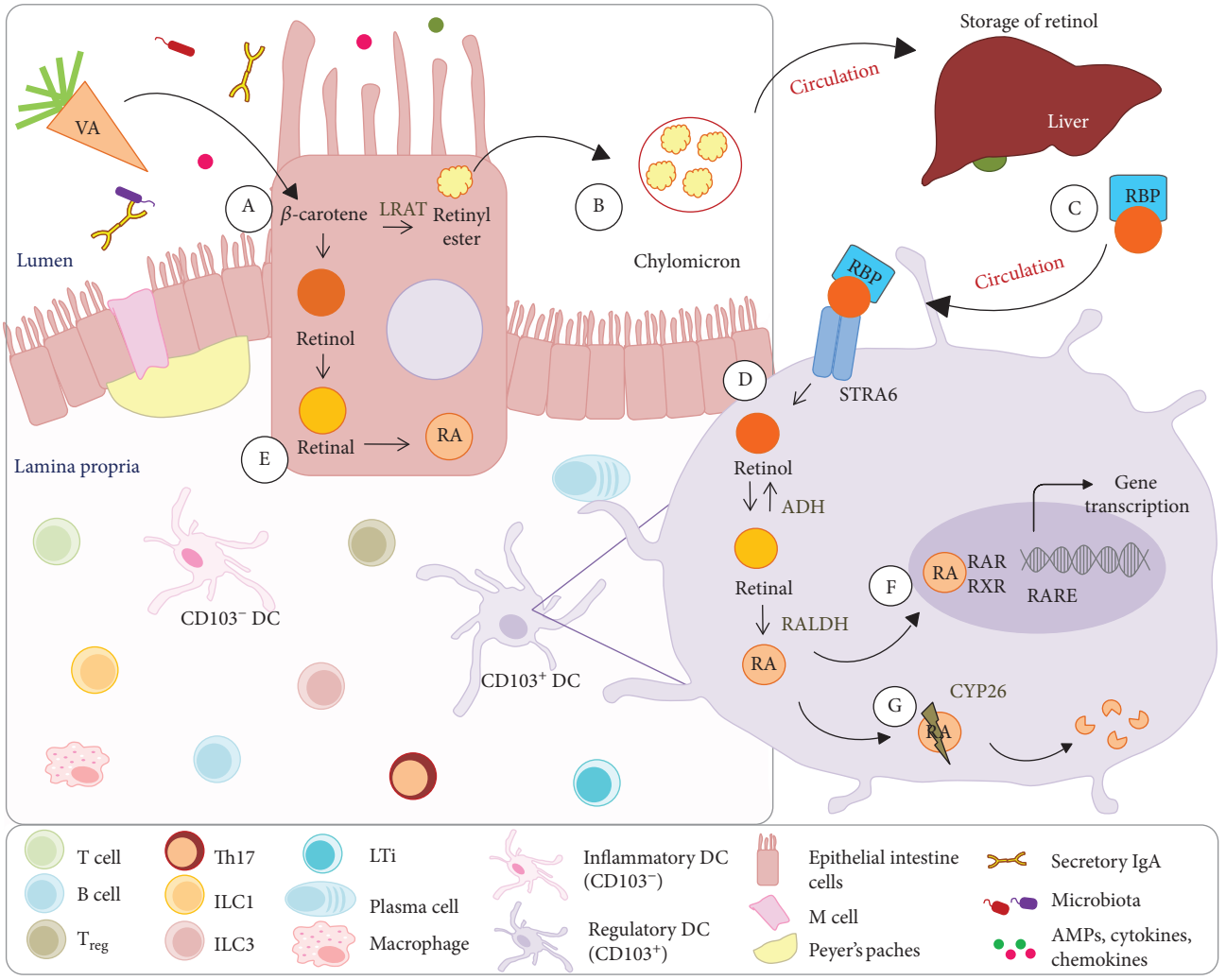


Figure 1: RA metabolism and signaling. (A) Vitamin A and its precursors (β-carotene) obtained from diet are absorbed by intestinal epithelium cells and esterified in retinyl esters by the enzyme lecithin retinol acyltransferase (LRAT). (B) Retinyl esters are packed with chylomicrons and enter general circulation where they are captured by hepatocytes and stored as retinol. (C) The retinol binds to retinol binding protein (RBP) in the liver and is carried through the bloodstream. This complex is recognized via the stimulated by retinoic acid 6 (STRA6) receptor, which mediates the absorption of extracellular retinol to cytosol. (D) After uptake, RA is generated from retinol by two sequential reactions. First, retinol is oxidized into retinal by enzyme alcohol dehydrogenase (ADH). Subsequently, in CD103$^+$ DCs, retinal is oxidized by the enzyme retinal dehydrogenase (RALDH) to generate RA. (E) Intestinal epithelium cells can also metabolize vitamin A after absorption into retinal and RA, which can be directly released into the intestinal mucosa. (F) RA interacts with nuclear receptors, such as the retinoic acid receptor (RAR) and retinoid receptor X (RXR), to regulate the transcription of several target genes by binding the retinoic acid-responsive elements (RAREs) in DNA. (G) Control of the RA concentration in tissues is performed by a group of enzymes that belong to the cytochrome P450 family 26 (CYP26), which catalyzes RA present in the cytosol to generate the oxidized forms.

In addition, due to its regulatory activity, RA has been shown to play an important role in the control of inflammatory diseases not only in the intestine [13, 14] but also in other tissues, such as the central nervous system [15–17] and pulmonary mucosa [18, 19].

Therefore, the roles of RA in the immune system, that is, both maintaining mucosal and epithelial homeostasis and contributing to anti-inflammatory function, are addressed in this review. The focus is on the role of RA in inflammatory responses, such as responses to inflammatory skin, intestinal, and airway diseases and its impact on immune cells. However, first, we discuss the metabolization of vitamin A into RA and its signaling pathways.

2. RA Metabolism and Signaling

Vitamin A is obtained from diet though the consumption of foods containing vitamin A precursors (mainly β-carotene) and vitamin A in the form of retinyl esters, which are derived from plant and animal food, respectively [20]. Vitamin A and its precursors are absorbed in the intestine by intestinal epithelium cells, and the vitamin A precursors are esterified

in retinyl esters by the enzyme lecithin retinol acyltransferase (LRAT). Retinyl esters are packed with chylomicrons and enter general circulation [21] (Figure 1). In the systemic circulation, the chylomicrons undergo the action of the lipoprotein lipase enzyme, resulting in their capture by hepatocytes and hydrolysis to retinol. Retinol is stored in the liver, mostly in hepatic stellate cells (HSCs) [1].

When RA is needed by the organism, the formed retinol binds retinol-binding protein (RBP) in the liver and is carried through the bloodstream [22]. This complex is recognized via the stimulated by retinoic acid 6 (STRA6) receptor, which mediates the absorption of extracellular retinol to cytosol [23]. However, the STRA6 receptor is only essential for maintaining RA homeostasis in the eye; therefore, other mechanisms are likely involved in the uptake of retinol into other tissues [24, 25].

After uptake, RA is generated from retinol by two sequential reactions. In the first reversible reaction, retinol is oxidized into retinal by the ubiquitously expressed enzyme alcohol dehydrogenase (ADH) [20]. Subsequently, in intestinal epithelium cells, DCs and macrophages associated with mesenteric lymph nodes (mLNs) and Peyer's patches (PPs), retinal is oxidized by the enzyme retinal dehydrogenase (RALDH) to generate RA [1]. There are three isoforms of RALDH (RALDH1, RALDH2, and RALDH3) [21], and their expression is tightly regulated and limited in the cells mentioned above. Thus, RALDH is considered the main enzyme that defines the populations of cells that are capable of producing RA [20]. Intestinal epithelium cells can also metabolize vitamin A after absorption into retinal and RA, which can be directly released into the intestinal mucosa [21].

RA can be generated in multiple forms as *all-trans*, *9-cis*, and *13-cis* RA [26, 27]; however, *all-trans* RA (atRA) is physiologically the most abundant [28]. RA interacts with nuclear receptors, such as the retinoic acid receptor (RAR) and retinoid receptor X (RXR), to regulate the transcription of several target genes [10, 29] by binding the retinoic acid-responsive elements (RAREs) in DNA [30]. These receptors form heterodimers; RAR comprises three major isoforms (α, β, and γ) that interact with all forms of RA, whereas RXR, which also has the α, β, and γ isoforms, mainly interacts with *9-cis* RA [31]. RA can also signal through peroxisome proliferator-activating receptor beta (PPAR-β) when it forms a heterodimer with RXR, which may be important for lipid metabolism and glucose homeostasis [1]. In addition, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) [32] and hepatocyte nuclear factor 4 (HNF-4) [33] receptors can forms a heterodimer with RXR and become low-affinity retinoic acid receptors. Similar to PPAR-β, their signals are important for lipid metabolism and glucose homeostasis [32].

Control of the RA concentration in tissues is performed by a group of enzymes that belong to the cytochrome P450 family 26 (CYP26), including subfamilies A1, B1, and C1 (CYP26A1, CYP26B1, and CYP26C1), which catalyze RA present in the cytosol to generate the oxidized forms (*5,8-epoxy* RA, *4-oxo* RA, *4-hydroxy* RA, and *18-hydroxy* RA) [34, 35]. The action of these enzymes prevents RA accumulation in the organism and maintains optimal physiological RA concentrations for the best performance.

3. Effects of RA on Immune Cells

RA can act on different cells of both the innate and adaptive immune systems (Figure 2), exerting local action at mucosal sites, mainly in the intestinal mucosa, and systemic action. In addition, RA plays a key role in the maintenance of immune homeostasis during inflammatory responses.

3.1. Tolerogenic Effect of RA on DCs and Macrophages. The balance between tolerance and effector responses is mainly regulated by antigen-presenting cells (APCs), especially DCs [36]. DCs in peripheral organs are characterized by the expression of CD103 and CD11b molecules [37]. RA can regulate the differentiation of bone marrow DC precursors (pre-DCs) into premucosal DC (pre-μDCs) by expression of gut-trafficking receptor $\alpha4\beta7$ and gives rise to intestinal $CD103^+CD11b^+$ DC, in mice [38].

Tolerogenic $CD103^+$ DCs, which are located mainly in the lamina propria of the small intestine and gut-associated lymphoid tissue (GALT), such as PPs and mLNs [39, 40], are responsible for the maintenance of homeostasis. This type of DC can promote the generation of $Foxp3^+$ regulatory T cells and the migration of regulatory and effector cells to the GALT [9–11]. The migration of T and B cells is mediated by $CD103^+$ DCs due to their ability to synthesize RA [10, 41] as these cells have a high expression of the RALDH1 and RALDH2 enzymes, which are responsible for the conversion of retinal to RA; thus, these cells are the main synthesizers of RA [42].

Other $RALDH^+$ DC populations that also produce RA are mainly located at mucosal interfaces, such as the skin, the lungs, and the corresponding draining lymph nodes [43, 44]. At the moment of antigen presentation in secondary lymphoid organs, $RALDH^+$ DCs (mainly $CD103^+$ DCs) release RA, which can freely diffuse across the cell membrane of the target cell. Then, RA signaling via the RARα receptor regulates the transcription of the promoter regions of the $\alpha4$ gene subunit of $\alpha4\beta7$ integrin and the CC chemokine receptor 9 (CCR9) gene on target cells [10], promoting the synthesis and expression of gut-trafficking receptors $\alpha4\beta7$ and CCR9 in the cellular membrane. $\alpha4\beta7$ and CCR9 can interact with mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and CC chemokine ligand 25 (CCL25), respectively [41, 45]. MAdCAM-1 is present in the venules of mLNs and PPs, while CCL25 is produced by intestinal epithelial cells; thus, RA imprints gut-homing specificity on immune cells [8, 40, 41, 45, 46].

However, not all DCs express the RALDH enzyme, such as inflammatory DCs, which can infiltrate or develop in the gut during inflammation due to chemokines and cytokines secreted by resident cells during the inflammatory process [47, 48]. In contrast to $CD103^+$ DCs, the proinflammatory $CD103^-$ DC population promotes the differentiation of interferon-gamma- (IFN-γ-) producing T cells and produces proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin- (IL-) 6, suggesting that these

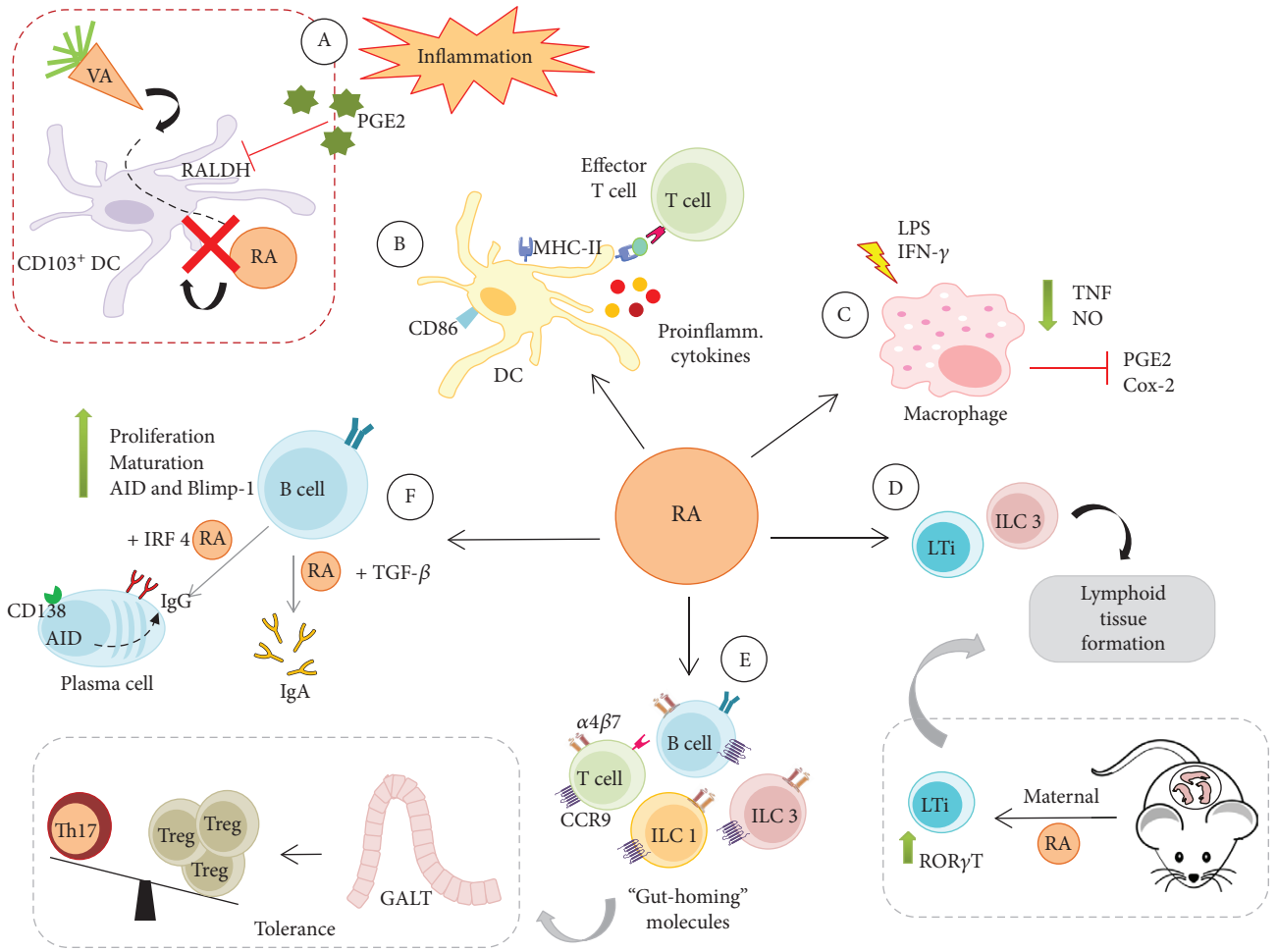


Figure 2: Role of RA in immune cells. RA can act on different cells of both the innate and adaptive immune systems exerting local action at mucosal sites and systemic action, which simultaneously, depending on where the RA-producing cells, mainly CD103$^+$ DCs, are located when it releases the RA. (A) However, in an inflammatory environment (red box), PGE2 released during the inflammatory response inhibits the RALDH enzyme that is required for RA synthesis. When RA is released, it acts as follows: (B) RA together with proinflammatory cytokines contributes to the activation of DCs and the generation of effector T cells; (C) RA promotes macrophage modulation, inhibiting inflammatory mediators and the release of TNF and NO; (D) RA also activates ILC3, especially LTi cells, which are required for the formation of lymphoid tissue, including during fetal development; (E) RA induces expression of the molecules $\alpha4\beta7$ and CCR9 in lymphocytes and ILCs and the homing of these cells into the intestine and promotes the balance of Th17/Treg cells in the GALT, assuring tolerance, but is also able to induce Th17 in the presence of infection and inflammation; and (F) RA promotes the activation of B cells and their differentiation into Ab-secreting plasma cells.

subsets of DCs can play a distinct role in promoting effector T cell response in the gut [36, 49].

Some inflammatory factors may influence RALDH expression, such as prostaglandin E2 (PGE2), which is produced by peripheral stromal cells and suppresses the differentiation of RA$^+$ DCs by directly antagonizing RALDH expression [50]. In addition, DCs that infiltrate the gut during inflammation do not acquire RALDH activity, which is required for RA synthesis. These inflammatory DCs express E-cadherin and the CD103 receptor, accumulate in the mesenteric lymph nodes and the inflamed colon, exhibit high expression of Toll-like receptors (TLRs) and produce cytokines IL-6 and IL-23, enhancing inflammation [47].

Mice with a vitamin A-deficient diet (VAD) exhibit reduced expression and activity of the RALDH enzyme in intestinal DCs, which is essential for the regulation of immune and inflammatory responses [51]. Others factors, including GM-CSF, IL-4, IL-13, and TLR ligands 2 and 5, may induce the *in vitro* expression of RALDH [52, 53], suggesting that the local microenvironment is able to modulate RA synthesis.

In infections, RA signaling may also induce the production of proinflammatory cytokines by DCs, promoting the differentiation of effector T cells [12] and enhancing the cellular activation state, in addition to the promotion of the formation of tertiary lymphoid structures [10]. These structures are formed in response to nonresolving inflammation generating lymphoid aggregates that drive adaptive immune reactions [54, 55]. RA also influences the maturation of monocyte-derived DCs (MoDCs) by increasing the expression of major histocompatibility complex (MHC) class II and CD86 and regulating the survival of DCs via the

RARα/RXR pathway [56]. In parallel to the activation of the innate immune response, RA promotes human DCs to induce IL-10-producing T cells to control inflammation and the maintenance of tissue homeostasis [57].

There are other sources of RA, such as lamina propria stromal cells, intestinal epithelial cells, and macrophages. Intestinal macrophages express RALDH1 and RALDH2, but that expression is dependent on external stimuli, such as cytokines and TLR ligands, whereas in $CD103^+$ DCs, the expression of these enzymes appears to be related to dietary vitamin A [51, 58]. In contrast, atRA treatment upregulates CD103 expression in human MoDCs, increasing the ability of the DCs to synthesize RA [59] and inducing tolerogenic DCs.

Mucosal macrophages, which constitute the most abundant population of phagocytic cells in the gut, show inflammatory anergy, avoiding inflammation in normal intestinal mucosa despite proximity to immunostimulatory microbiota [58]. RA also acts directly on macrophages at both mucosal sites and other immunological sites. atRA modulates peritoneal macrophage activation by endotoxin and IFN-γ by suppressing TNF production and nitric oxide (NO) synthesis [60]. In addition, atRA inhibits the expression of PGE2 and COX-2 and the release of TNF, which are induced by bacterial lipopolysaccharide (LPS) in murine peritoneal macrophages [61].

Retinoid treatment inhibits IL-12 production in LPS-activated macrophages by inhibiting nuclear factor kappa B (NFκB)-DNA interactions and the competitive recruitment of transcription integrators between NFκB and RXR [62]. atRA also inhibits the LPS-induced production of the proinflammatory cytokines TNF-α and IL-12 and potentiates IL-10 production in the THP-1 monocyte/macrophage cell line and human cord blood mononuclear cells (CBMCs) [63]. Plasma factors, such as transforming growth factor-(TGF-) β and PGE2, in combination with RA, act synergistically with IL-4 synthesized by basophils to increase the sensitivity of macrophages to IL-4, which contributes to M2 macrophage polarization and the regulation of the inflammatory process in mice [64].

Tissue-resident macrophages are highly heterogeneous in terms of their functions and phenotypes as a consequence of adaptation to different microenvironments [65]. Monocyte-derived inflammatory macrophages can be converted into the resident tissue phenotype in a vitamin A-dependent manner [66]. VAD mice fail to convert tissue-resident macrophages during infection, which may lead to a deregulated inflammatory process [66].

In general, the impact of RA on macrophages suggests that RA inhibits the production of inflammatory cytokines and favors the generation of tolerance.

3.2. Modulation of Innate Lymphoid Cells (ILCs) by RA. ILCs constitute a group of tissue-resident innate immune cells that can regulate inflammation and repair tissues in multiple anatomical compartments, particularly on the barrier surfaces of the skin, airways, and intestine [67]. ILCs are derived from the same DNA-binding 2- (Id2-) dependent precursor and are characterized by the expression of the IL-7 receptor [68].

These cells are subdivided into the following three main groups: group 1 ILCs (ILC1), which include natural killer (NK) cells, are induced by transcription factor T-box expressed in T cells (T-bet) and produce IFN-γ; ILC2 require the GATA-binding protein 3 (GATA3) transcription factor and produce IL-5 and IL-13; and ILC3 depend on the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma (RORγt) and secrete IL-17 and IL-22 [69].

ILC1 are accumulated during chronic inflammation in the gut (inflammatory bowel disease) and lung (chronic obstructive pulmonary disease), where they contribute to IFN-γ-mediated inflammation; ILC2 are mainly found in the lung but can also be present in the skin and gut, are related to helminth defense, and are mostly involved in tissue repair, allergy, and asthma, and ILC3 are implicated in gut barrier defense and skin inflammation [70].

In addition, ILC3 include lymphoid tissue-inducing cells (LTi) that contribute to the formation of secondary lymphoid organs [71]. RA during gestation is necessary for the development of fetal LTi cells during the embryonic stage, since maternal RA upregulates the RORγt transcription factor and favors the formation of lymphoid tissue, promoting greater efficiency in the immune responses of adult offspring [72]. Similarly, RA is required during the postnatal phase for the generation of intestinal ILC3 and LTi cells in adult mice, since its deficiency or the blockade of RA-RAR signaling reduces the development of enteric lymphoid tissue [73].

RA also induces the expression of $\alpha 4\beta 7$ and CCR9 in ILCs 1 and 3, which is crucial during antiviral and antibacterial responses in the intestinal mucosa; this effect is not observed in ILC2 since the homing receptors expression of these cells is determined during development in the bone marrow [8]. RA associated with IL-2 *in vitro* contributes to the synthesis of IL-5 and IL-13 by ILC2 and IFN-γ by ILCs 1 and 3, which are important for the functional maintenance of ILCs in allergic and inflammatory diseases [74].

Intestinal tolerance induced by RA could be obtained by modulating ILC3 function in the GALT by increasing IL-22 production during colon inflammation induced by dextran sulfate sodium (DSS) or pathogenic bacteria, in mice [75]. In addition, the RAR receptor, which acts as a transcription factor, is able to bind the IL-22 promoter and directly promote its transcription. Moreover, human intestinal ILC1 can differentiate into ILC3 *in vitro* in response to IL-2, IL-23, IL-1β, and RA [59], which is important for tolerance.

In NK cells, RA acts tolerogenically and suppresses the human NK cell cytotoxicity activated by IFN-α [76]. NK cells are cytotoxic cells that act against tumor cells and virus-infected cells by a complex process of signaling mediated by activating and inhibitory receptors [77]. Additionally, antibody-dependent cellular cytotoxicity (ADCC) directs the cytotoxicity of NK cells toward antibody-coated target cells [78]. RA can influence the activity of NK cells by inhibiting ADCC and its natural cytotoxicity *in vitro* [79]. In addition, high concentrations of atRA inhibit NFκB signaling in NK cells, negatively regulating the secretion of IFN-γ, which is important for granzyme B release [80]. *In vitro* treatment with *13-cis* RA also regulates NK cell activity by increasing CD158b, which is a killer inhibitory receptor [81].

On the other hand, RA increases the expression of MHC class I chain-related proteins A and B (MICA and MICB) in tumor cells that bind the natural killer group 2D (NKG2D) receptor in NK cells, promoting their activation [82]. In addition, the number of circulating NK cells in humans is positively regulated by the level of retinol stocks [83]. Thus, RA exerts a bidirectional effect on NK cells, which may contribute to its inhibition or activation.

3.3. Effect of RA on B Cell Differentiation. RA plays an important role in the humoral response and is essential for B cell production of IgA antibodies playing a multifactorial role in mucosal immunity [10, 84]. Oral administration of RA in VAD mice proved to be efficient in reestablishing IgA production after influenza vaccination [7]. In addition, vitamin A and zinc deficiency leads to a decrease of serum IgA and a drastic reduction of humoral mucosal immunity [85]. During vaccination, the association with RA potentiates the immune response in both adult and neonatal mice, suggesting an important role of RA as a vaccine adjuvant, especially during the early stages of life [86, 87].

Retinoids are described as important cofactors for the stimulation and proliferation of B cells, accelerating B cell lymphopoiesis [88, 89]. RA increases the number of peripheral B cells in the spleen while decreasing lymphoid progenitors in the marrow; these effects are mediated by an increase of the early B cell factor 1 (EBF1) and paired box protein-5 (Pax-5) transcription factors, which are crucial for B cell development [89]. Moreover, RA accelerates the maturation of human B cells and their differentiation into plasma cells [90].

The development of an effective long-lasting humoral response requires the formation of germinal centers (GCs) in the lymphoid follicles, where interactions between B cells and follicular helper T cells guarantee the development of memory B cells and long-lived plasma cells [91]. Thus, B cells undergo somatic hypermutation and immunoglobulin class-switching recombination as a part of the GC reactions mediated by the activation-induced cytidine deaminase (AID) enzyme [92]. RA may increase more differentiated B cell phenotypes by upregulating the expression of AID and B lymphocyte-induced maturation protein-1 (Blimp-1) and increasing the expression of CD138 and IgG in splenic B cells [93]. RA also induces the expression of interferon regulatory factor 4 (IRF4), which is involved in the generation of plasma cells and RA-mediated IgG production, favoring AID expression [94]. In addition, RA may increase IgM and IgG syntheses in human B cells from CBMCs and adult peripheral blood mononuclear cells (PBMCs), respectively [95].

The microenvironment may directly affect the modulation of the humoral response. The combined effects of bacterial products and RA on the intestinal mucosa trigger signaling cascades via TLRs and RAR, respectively, activating follicular dendritic cells (FDCs) [96]. This process enhances the synthesis of CXC chemokine ligand 13 (CXCL13), which is a chemoattractant of B cell follicles in lymphoid tissues, and increases the expression of B cell-activating factor (BAFF), which is an important factor for B cell survival and TGF-β [96]. Collectively, RA favors the migration and survival of B cells and leads to the preferential generation of IgA in the intestine [84]. Other components present in the mucosa contribute to the generation of IgA, such as lactoferrin [97], which, together with RA, leads to the production of IgA by peritoneal B-1 cells [98].

On barrier surfaces, the humoral response is the main effector response to frequent microbial challenges from both the host microbiota and the external environment. RA plays a key role in the modulation of mucosal inflammatory responses by contributing to the synthesis of antibodies, especially IgA, ensuring immunity and tolerance.

3.4. Effects of RA on the T Cell Population. The effects of RA on the balance of Th1/Th2 responses are controversial. Some studies indicate that high levels of RA can promote the differentiation of naïve T cells into Th2 cells by inducing IL-4 gene expression [1]. In addition, RA modulates IL-12 production by APCs, inhibiting Th1 cell differentiation [99], and induces the expression of GATA3 and signal transducer and activator of transcription 6 (STAT6), which is important for the maintenance of the Th2 response [1, 36]. RXR agonists and *9-cis* RA also favor the development of Th2 cells [100]. However, an experimental ovalbumin-induced asthma murine model suggests that vitamin A deficiency is related to increased pulmonary inflammation by inducing type 2 cytokines [101]. In addition, some studies have shown that oral vitamin A treatment (vitamin A supplementation diet) indirectly reduces pulmonary inflammation as a result of the anti-inflammatory effects of RA on other immune cells and Treg cell generation in the lung without directly affecting the Th2 population [18, 19].

Although RA inhibits Th1 responses, it is essential for the stability and maintenance of Th1 cells, repressing transcription factor RORγt, which is important for the induction of Th17 cells [102]. Furthermore, RA plays an important role in the maintenance of Th1 responses since VAD mice exhibit a negative Th1 response after infection with *Toxoplasma gondii* [12].

The impact of RA on the Th17/Treg balance has a known mechanism. Small intestinal lamina propria DCs synthesize RA and have the ability to generate Tregs in the presence of TGF-β [6]. Thus, elevated levels of TGF-β promote the generation of Tregs from naïve CD4 T cells by an RA-dependent mechanism [9, 39, 103, 104] in which atRA promotes the acetylation of histones on the promoter of the Foxp3 gene. In addition, atRA, which activates STAT6 through IL-4 signaling, also promotes the acetylation of histones on the Foxp3 gene promoter, increasing its expression in the cell [105, 106].

At the steady state, RA inhibits the differentiation of naïve T cells into Th17 cells by blocking IL-23 and IL-6 signaling [107]. RA also indirectly induces Treg conversion by inhibiting the $CD44^{hi}CD4^{+}$ T cell population of memory cells, which blocks the differentiation of naïve T cells into Tregs via the secretion of IL-4, IL-21, and IFN-γ [108]. In addition, RA controls the generation of T cells with an inflammatory profile in the GALT, suppressing the differentiation of naïve T cells into Th17 cells in the mucosa to maintain tolerance [45]. In contrast, IL-6 inhibits the generation of Tregs, favoring the expansion of Th17 cells in colitis [105, 109].

Th17 cells are generated in the presence of IL-6 and IL-21 and low levels of TGF-β in the intestinal mucosa, mainly during chronic inflammation [109, 110]. These cells can secrete cytokines, such as IL-17A, IL-17F, IL-21, and IL-22, which can control bacterial and fungal infections at mucosal sites [10]. Although RA inhibits Th17 generation, during infection of the intestinal mucosa, low concentrations of RA produced by $TLR5^+$ lamina propria DCs induce the generation of Th17 cells, potentiating the protective response in the mucosa [111]. In addition, RA is essential for the in situ generation of Th17 cells in the intestinal mucosa during infection caused by *Toxoplasma gondii* [12].

Oral supplementation with RA in mice with chronic inflammation in the ileum may attenuate inflammation by restoring the balance between the Th17 and Treg populations, increasing the number of $CD103^+$ DCs and RALDH2 expression by a positive feedback mechanism [45, 109]. Microbial stimuli, such as the TLR-2 receptor ligand in mice, also increase RALDH2 expression and RA production, promoting regulatory T cells and inhibiting the generation of Th17 cells [42, 52]. Thus, RA balances the generation of subsets of T cells depending on the conditions and factors of the microenvironment to maintain homeostasis.

4. Immunomodulatory Effect of RA during Inflammatory Processes

Inflammation during immune responses is an important way to remove tissue injuries and promote restoration. Inflammation can occur as a physiological process in which dead cells are removed from tissues, keeping the tissues healthy, but it can also be caused by several other stimuli, such as pathogen infections, damaged cells, toxic compounds, or irradiation [112].

Typically, organisms undergo acute inflammatory responses in which molecular and cellular interactions resolve injury and infections without tissue damage, contributing to the restoration of tissue homeostasis. However, uncontrolled acute inflammation or nonresolution of infection may generate chronic inflammation, contributing to a variety of chronic inflammatory diseases [113, 114].

Simultaneously, some components of the diet act as anti-inflammatory mediators by attenuating acute and chronic inflammatory processes, promoting homeostasis and, thus, ameliorating the harmful effects of inflammatory responses. Here, we focus on how RA modulates the inflammatory response at different mucosal sites and different tissues (Figure 3).

4.1. Intestinal Mucosa. Inflammatory bowel disease (IBD), which is mainly referred to as Crohn's disease and ulcerative colitis, is a result of chronic inflammation characterized by excessive innate immune cells activation, tissue damage, and the induction of adaptive immune responses against the intestinal microbiota. Gut dysbiosis, mainly among commensal bacteria, initiates an exacerbated inflammatory response. This disorder can be caused by multiple factors, including abnormal immune responses, genetic susceptibility, infection, dietary habits, and the administration of antibiotics [115, 116].

RA has been shown to regulate immune responses and restore the Th17/Treg balance, mainly in the intestinal mucosa, showing that RA plays an important role in intestinal mucosal homeostasis [45, 109]. Vitamin A impairs the reprogramming of inducible Tregs (iTregs) into Th17 cells during intestinal inflammation induced in a T cell-dependent colitis model [13, 14]. In addition, DCs were more efficient for Treg differentiation after the restoration of intestinal RA by diet in intestinal tumor models [14].

In an experimental model of DSS- or pathogenic bacteria-induced colitis, RA was shown to attenuate inflammation by increasing IL-22 production by ILC3 and T$\gamma\delta$ cells and, consequently, increasing the synthesis of antimicrobial peptides [75] or by decreasing TNF levels and NFκB activation [115]. Moreover, in inflamed intestinal tissues from Crohn's disease patients, the number of $CD127^+$ ILC1 increased at the cost of ILC3 [59]; however, ILC1 can be differentiated into ILC3 *in vitro* and *in vivo* upon IL-2, IL-23, and IL-1β stimulation and this process was enhanced in the presence of RA, reducing inflammation [117]. In addition, *in vitro* atRA treatment of inflamed colonic mucosa from patients with ulcerative colitis and colitis-associated cancer modulates the LPS/TLR4/NFκB signaling pathway and decreases nitric oxide synthase 2 (NOS2) and TNF-α expression [118]. This finding suggests that RA may be a target for future colorectal cancer treatments.

The absence of RA in VAD mice makes them more susceptible to the development of DSS-induced colitis and colon cancer due to worsening of chronic inflammation in the intestine [119]. In this context, CYP26B1, which is responsible for the catabolism of RA [35], has been shown to regulate the differentiation and function of CD4 T cells during experimental colitis, driving the cells towards an inflammatory profile. The passive transfer of naïve cytochrome p450 family 26 subfamily b1-deficient ($Cyp26b1^{-/-}$) mice CD4 T cells into recombination-activating gene 1-deficient ($Rag1^{-/-}$) mice resulted in a significantly reduced disease state in a model of T cell-dependent colitis [120].

In humans and murine models of ulcerative colitis associated with colorectal cancer, alterations of atRA metabolism mediated by microbiota-induced intestinal inflammation, with increasing levels of CYP26A1, another atRA-catabolizing enzyme, reduced colonic atRA and promoted tumorigenesis [121]. Supplementation with atRA reduced the tumor burden, and this effect was mediated by cytotoxic CD8 T cells activated by MHC I upregulation on tumor cells [121]. In a case-control study with 898 colon cancer cases, 501 rectal cancer cases, and 1399 matched controls, an association between higher plasma retinol concentrations and a lower risk of colon cancer was observed, mainly in proximal colon cancer [122]. This evidence suggests a role of RA in the prevention of colon cancer.

Serum retinol levels in adults and children with Crohn's disease are lower than those in healthy people probably due to a deficiency in nutrient absorption [123, 124]; similar results were reported for ulcerative colitis [125]. Considering the tolerogenic role of RA in the intestinal mucosa and the

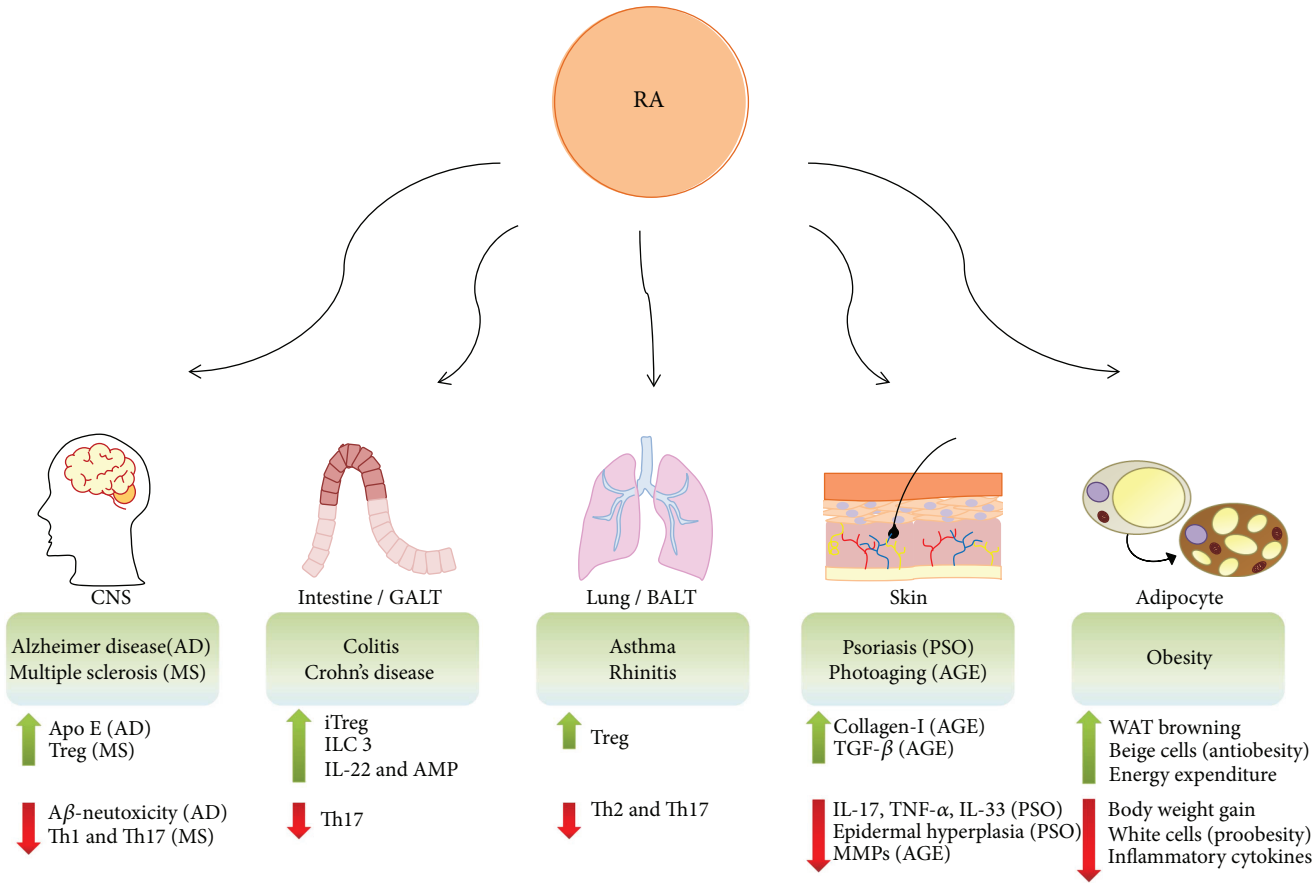


Figure 3: Potential anti-inflammatory effects of RA. RA can decrease inflammatory processes, promoting homeostasis and attenuating harmful inflammatory responses in mucosa and tissues. RA shows immunosuppressive effect on Th1/Th17 cells in multiple sclerosis (MS) and induces Apo E in microglia, protecting from the neurotoxic effects mediated by amyloid β (Aβ) in Alzheimer disease (AD), contributing to neuronal homeostasis. RA is crucial for intestinal tolerance, by inducing Treg, cytokines IL-10 and IL-22, and antimicrobial peptide (AMP) synthesis, which may lead to Th17 inhibition. RA modulates inflammatory airway diseases (asthma and rhinitis) by inhibiting Th2/Th17 response and enhancing Treg cells. Retinoids increases type I collagen and TGF-β, reducing matrix metalloproteinase (MMPs) in photoaging (AGE), and reduces IL-1 family cytokines (IL-17 and TNF-α), IL-33, and epidermal hyperplasia in psoriatic (PSO) lesions. RA also has effects in adipocytes, promoting white adipose tissue (WAT) browning by differentiation into beige cells (antiobesity) instead of white cells (proobesity). The formation of brown adipocytes within WAT enhances energy expenditure and reduces obesity. In addition, RA can repress the expression of inflammatory chemokines and cytokines, inhibiting inflammatory responses triggered by obesity.

fact that IBD patients have low levels of serum retinoids, RA administration should be an adjuvant treatment for inflammatory diseases.

Moreover, we must consider that RA also plays an important role during intestinal inflammation caused by pathogen infection, as observed during *Salmonella typhimurium*-mediated gastroenteritis in mice [126] and during *Vibrio cholera* infection after pretreatment with RA prior to immunization [127].

Overall, RA displays an important anti-inflammatory activity in the control of inflammation in the intestinal mucosa, but more studies are necessary to better understand the role of RA in inflammatory processes.

4.2. Airways and the Lung. Inflammatory airway diseases, such as asthma and allergic rhinitis, have a high prevalence around the world. Airway inflammation is mediated by Th2 cells that characteristically produce IL-4, IL-5, and IL-13 [128]. Moreover, in chronic airway inflammatory diseases, the massive infiltration of eosinophils is mediated by allergen-specific Th2 cells and neutrophils also participate in chronic obstructive pulmonary disease mediated by Th17 cells [129].

The role of RA in the pulmonary mucosa is controversial. Supplementation with vitamin A has been shown to increase the severity of asthma in experimental models with high levels of IgE and IgG1 antibodies and pulmonary inflammation [29]. In addition, RA has the ability to induce Th2 responses and inhibit Th1 responses [99, 100]. In contrast, oral administration of Net-4IB, an RXR partial agonist, suppressed aryl hydrocarbon receptor (AHR) and inflammatory cell accumulation in the airways and attenuated TNF-α levels in the lung and IL-5, IL-13, and NO levels in bronchoalveolar lavage fluid from mice [130]. The RXR partial agonist Net-4IB may be a promising candidate for the treatment of allergic airway inflammation.

Moreover, vitamin A deficiency has been related to an increased asthma incidence in children due to damage to the pulmonary mucosa and to the maintenance of the airway epithelium [131]. During vitamin A deficiency, a mouse asthma model revealed the induction of Th2 cytokines, such as IL-5 and IL-13, and an increase in pulmonary inflammation [101]. The administration of RA may attenuate inflammation by increasing the population of regulatory T cells in the lung and decreasing the tissue damage caused by inflammation [18, 19]. The association between RA and ovalbumin in oral tolerance in a murine model of bronchial asthma efficiently inhibited the inflammatory response and decreased eosinophilic infiltration besides Treg cells induction in the lung [132]. Treatment with atRA was able to attenuate airway inflammation by inhibiting Th2 and Th17 cytokines and downregulating the expression of the GATA3 and RORγt transcription factors in the lung [128]. Similar results were observed in a murine model of allergic rhinitis [133]. Interestingly, lung-resident tissue macrophages that coexpress TGF-β and retinal dehydrogenases (RALDH1 and RALDH2) are able to induce Treg cells at a steady state, favoring airway tolerance [134].

In human studies, the retinoid concentrations in the serum were significantly lower in patients with asthma than those in healthy control subjects and were even lower in patients with severe asthma than those in patients with mild asthma [135, 136], highlighting the importance of equilibrating physiological RA concentrations in airway diseases.

4.3. Central Nervous System. Although the brain is an immunologically privileged site, in pathologic conditions of the central nervous system (CNS), an organized immunologic response can develop within the CNS to eliminate inflammation without tissue damage [137]. However, in some cases, a persistent inflammatory response develops during neurodegenerative processes, such as multiple sclerosis (MS) and Alzheimer's disease (AD).

MS is an autoimmune disease characterized by recurrent episodes of demyelination and axonal lesions mediated by Th1 and Th17 cells, macrophages, and immune inflammatory mediators [138]. Taking into account the immunosuppressive role of RA for Th1/Th17 cells and macrophages, it is not unreasonable to think that RA may exert beneficial effects in MS.

Indeed, treatment with atRA alone or in combination with calcitriol (an active vitamin D metabolite) in murine autoimmune encephalomyelitis (EAE), which is an experimental model of MS, increased the expression of the Foxp3 and TGF-β genes in splenocytes while reducing RORγt gene expression [139]. PBMCs from patients with MS who were supplemented with vitamin A for 6 months also showed an upregulation of TGF-β and Foxp3 gene expression [15] and a reduction in IFN-γ and T-bet gene expression [16]. RA treatment also suppresses T$\gamma\delta$ cell pathogenic activity by decreasing IL-17 production, which is important for the maintenance of EAE [17]. Moreover, the combination of atRA and atorvastatin, which is a lipid-lowering agent with anti-inflammatory, immunomodulatory, and neuroprotective properties, causes the regression of the clinical and neuropathological features of EAE with reduced secretion of IL-17 and increased production of IL-10 and Foxp3$^+$ Treg cells in splenocytes [140].

In MS, activated astrocytes participate in promoting lesion progression by secreting proinflammatory mediators and chemokines. In cocultures with inflamed endothelial cells, primary astrocyte-derived RA attenuated oxidative stress [141]. In addition, murine astrocytes that were stimulated with LPS and treated with atRA expressed no or very low levels of CCL and CXCL chemokines [142].

All these data suggest that retinoids are candidates for the treatment of neuroinflammation. Indeed, the use of RA-loaded polymeric nanoparticles (RA-NPs) modulates the murine microglial response towards an anti-inflammatory and neuroprotective phenotype (M2-like) in organotypic hippocampal slice cultures [143]. Recently, the intravenous administration of RA-NPs was shown to prevent ischemic injury in the immature brains of 2-day-old mice, demonstrating the role of RA in the control of neuroinflammation [144].

RA also has neuroprotective activity and is capable of increasing barrier tightness in human-induced pluripotent stem cell-derived brain endothelial cells by RARα, RARγ, and RXRα activation [145]. In addition, treatment with RA in experimental models of AD and *in vitro* was beneficial.

AD is a progressive neurodegenerative disease characterized by neuroinflammation with reactive microglia, astrogliosis, proinflammatory cytokines, amyloid-β (Aβ) peptide deposition, and progressive memory loss [146]. Treatment with RA in an experimental model of AD was beneficial by inhibiting microglial activation in the hippocampus and improving the proliferation of stem cells [147], as well as increasing the synthesis of apolipoprotein E (Apo E) in human macrophages [148]. Apo E acts on microglia, protecting them from the neurotoxic effects of amyloid β, and contributes to neuronal homeostasis [149].

Oral coadministration of Am80 (an RAR-α/β agonist) and HX630 (an RXR agonist) reduced the level of insoluble Aβ peptide in the brain by promoting the differentiation of IL-4-responsive M2-like microglia and increasing their activity for the clearance of oligomeric Aβ peptides in an experimental model of AD. This finding showed that combination treatment with RAR and RXR agonists could be an effective approach for AD therapy [146].

atRA administration prevents LPS-induced neuroinflammation, NO production, amyloidogenesis, and memory impairment in aged rats [150]. PBMCs from patients with AD in cultures with atRA showed downregulated spontaneous NO production and iNOS expression, which was associated with a reduction in IL-17A production and increased IL-10 release [151].

All these data suggest that RA may be a potential target in both MS and AD treatments.

4.4. The Skin. The skin is the primary barrier that provides protection against microbial pathogens and physical and chemical insults to organisms [152]. The skin is composed of the following layers: epidermis, basement membrane, dermis, and subcutaneous fatty region. Each layer has several

structures, such as hair follicles, sweat glands (in humans but not mice), sebaceous glands, nerves, blood vessels, and lymphatics. The epidermis and dermis have a variety of cell types, including immune cells. Together, these cells form an orchestrated defense against invading pathogens [153]. In the epidermis, in addition to melanocytes that produce melanin and keratinocytes, there are Langerhans cells, which are the main skin-resident immune cells, and are more involved in tolerogenic than inflammatory responses [154]. The other types of immune cells, such as DC subpopulations, macrophages, ILC2, NK, and B and T cells, reside in the dermis [155].

In the epidermis, keratinocytes also play an important role in defense against pathogens. Epidermal keratinocytes are proinflammatory effector cells with a large production of antimicrobial peptides (AMPs), proinflammatory cytokines, and chemokines [155]. Keratinocytes also express TLRs [156], which are crucial for promoting skin immune responses and Th1 responses [152]. However, an imbalance in the immune response and microbiota or persistent infections can generate skin inflammations, causing several diseases.

The use of retinoids has long been established for the treatment of immune-mediated skin diseases. In dermatological treatment, retinoids are typically classified into three generations according to how they were developed [157]. The first-generation retinoids are the naturally occurring nonaromatic retinoids, including retinol, retinal, isotretinoin (*13-cis* RA), tretinoin (atRA), and alitretinoin (*9-cis* RA). The second-generation retinoids are the monoaromatics (etretinate, acitretin, and motretinate). The third-generation retinoids are the polyaromatics (bexarotene, adapalene, and tazarotene) [157, 158].

Photoaging is a process mainly triggered by ultraviolet radiation from chronic sun exposure that leads to DNA damage and the production of reactive oxygen species, which both promote inflammation [159] and result in increased matrix metalloproteinases (MMPs) and collagen degradation [160]. Retinoids have demonstrated efficacy in the treatment of the photoaged skin. The effects of RA include the inhibition of the expression of MMPs [161]; inhibition of tyrosinase activity, which increases epidermal cell turnover and leads to increased shedding of melanin-laden keratinocytes; reduction of inflammatory cytokine production; and enhancement of type 1 collagen and TGF-β [162]. All these effects contribute to the improvement of symptoms in photoaging.

In addition to aging/photoaging, the application of retinoids in skin diseases is very diverse and retinoids have been used in treatments for acne, rosacea, psoriasis, lichen planus, basal cell carcinoma, and so on [158]. Acne vulgaris is a common chronic inflammatory cutaneous disease that involves the pilosebaceous unit with abnormal keratinization leading to follicular plugging [163]. Retinoids act by increasing the turnover of follicular epithelial cells and accelerating the shedding of corneocytes, which helps normalize keratinization. Retinoids also exert a sebum-suppressive effect following oral isotretinoin administration [164]. The use of isotretinoin also induces the remission of acne by normalizing the innate immune response to the commensal bacterium *P. acnes* [165]. This remission occurs due to decreased monocyte TLR-2 expression and the subsequent inflammatory cytokines response to *P. acnes*. Combining retinoids with other components and antibacterial agents can decrease irritation and increase the efficacy of retinoid treatment [163, 166]. Importantly, retinoids regulate the transcription factor AP-1, resulting in the inhibition of MMPs, which are responsible for scar formation in acne [165].

Psoriasis is a prototype inflammatory skin disease characterized by marked keratinocyte hyperproliferation and altered differentiation associated with dermal and epidermal infiltration of leukocytes [166]. Tazarotene is the most commonly used retinoid for topical treatment [167–169] and is usually used in combination with phototherapy, corticosteroids, vitamin D, and other treatments [170–172]. Tazarotene acts by reducing plaque elevation and inflammation probably due to its anti-inflammatory role in immune cells [158].

In general, the use of retinoids in immune-mediated skin diseases has been highly beneficial for the patients.

4.5. Obesity. Obesity is a global health issue, and overnutrition and excess bodyweight are associated with an increased risk of developing metabolic disorders, such as diabetes and cardiovascular diseases. Several inflammatory markers have been consistently associated with obesity, suggesting that persistent low-grade inflammation is present in obesity [173, 174].

RA also plays an important role in the modulation of inflammatory processes at other sites and in other tissues. In human adipocytes, atRA represses chemokine and inflammatory cytokines expression by inhibiting NFκB signaling. Since inflammatory responses triggered by obesity play a major role in the onset of insulin resistance, atRA supplementation may represent a preventive nutritional strategy for controlling obesity and its complications [175].

Besides the liver, adipose tissue contains a substantial amount of retinol and its metabolites [176]. The RARs are highly expressed in adipose tissues; therefore, the RARs are directly influenced by atRA [177]. atRA and *9-cis* RA, *in vitro*, inhibit proliferation and induce apoptosis in a human preadipocyte cell lineage [178]. In addition, RA enhances lipid oxidation and inhibits lipid's biosynthesis capacity [178], as well as, decreases body weight gain in an obese rat model independent of Stearoyl-CoA desaturase 1 (SCD1) gene regulation, which is an enzyme involved in the biosynthesis of monounsaturated fatty acids [179].

RA also promotes the remodeling of white adipose tissue (WAT) [180], which is associated with metabolic disorders. In a VAD model, a marked increase in adiposity and hypertrophy of WAT was observed [181]. In addition, RA induces white adipose tissue browning by increasing adipose vascularity, which promotes the differentiation into beige cells (antiobesity) instead of white cells (proobesity) [182]. The formation of brown adipocytes within WAT enhances energy expenditure, reduces obesity, and could help improve metabolic health [183].

RA treatment of obese mice induces RAR target genes involved in the regulation of lipid homeostasis, leading to the suppression of obesity and insulin resistance [184].

Moreover, in an experimental obesity model, RA produced by DCs and macrophages upon IL-13 stimulation from IL-33-activated islet-resident ILC2 cells induced insulin secretion by β cells [185]. The IL-33-ILC2 axis was activated after acute β cell stress but was defective during chronic obesity. However, the fact that RA increases insulin secretion shows its potential modulatory role in metabolic diseases.

5. Conclusion and Future Perspectives

Globally, more than 2 billion people are affected by micronutrient deficiency and at least half of children aged 6 months to 5 years worldwide suffer from one or more micronutrient deficiencies. Vitamin A deficiency is a public health concern, and vitamin A supplementation in children is highly effective in reducing mortality from all causes. In adults, the anti-inflammatory effects of RA, which favor immune homeostasis, are a treatment strategy alone or in association with other drugs for inflammatory intestinal diseases, neurodegenerative processes, skin aging, and cancer. Furthermore, alterations of serum RA levels are not only indicators of homeostasis disequilibrium but also biomarkers for the intestinal inflammatory process. An uncontrolled vitamin supply and micronutrient deficiencies reinforce the need to better understand the effects of RA on the immune system and inflammatory diseases.

Conflicts of Interest

No conflicts of interest are declared.

Acknowledgments

This work was supported by the Laboratório de Investigação Médica, Unidade 56, Department of Dermatology, School of Medicine, University of São Paulo, Brazil and Fundação de Amparo à Pesquisa do Estado de São Paulo.

References

[1] J. R. Mora, M. Iwata, and U. H. von Andrian, "Vitamin effects on the immune system: vitamins A and D take centre stage," *Nature Reviews Immunology*, vol. 8, no. 9, pp. 685–698, 2008.

[2] B. Cassani, E. J. Villablanca, J. De Calisto, S. Wang, and J. R. Mora, "Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance," *Molecular Aspects of Medicine*, vol. 33, no. 1, pp. 63–76, 2012.

[3] E. Villamor and W. W. Fawzi, "Effects of vitamin a supplementation on immune responses and correlation with clinical outcomes," *Clinical Microbiology Reviews*, vol. 18, no. 3, pp. 446–464, 2005.

[4] A. Imdad, E. Mayo-Wilson, K. Herzer, and Z. A. Bhutta, "Vitamin A supplementation for preventing morbidity and mortality in children from six months to five years of age," *Cochrane Database of Systematic Reviews*, no. 3, article CD008524, 2017.

[5] WHO, *Guideline: Vitamin A Supplementation in Infants and Children 6–59 Months of Age*, World Health Organization, Geneva, 2011.

[6] C. M. Sun, J. A. Hall, R. B. Blank et al., "Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid," *The Journal of Experimental Medicine*, vol. 204, no. 8, pp. 1775–1785, 2007.

[7] S. L. Surman, B. G. Jones, R. E. Sealy, R. Rudraraju, and J. L. Hurwitz, "Oral retinyl palmitate or retinoic acid corrects mucosal IgA responses toward an intranasal influenza virus vaccine in vitamin a deficient mice," *Vaccine*, vol. 32, no. 22, pp. 2521–2524, 2014.

[8] M. H. Kim, E. J. Taparowsky, and C. H. Kim, "Retinoic acid differentially regulates the migration of innate lymphoid cell subsets to the gut," *Immunity*, vol. 43, no. 1, pp. 107–119, 2015.

[9] H. von Boehmer, "Oral tolerance: is it all retinoic acid?," *The Journal of Experimental Medicine*, vol. 204, no. 8, pp. 1737–1739, 2007.

[10] J. A. Hall, J. R. Grainger, S. P. Spencer, and Y. Belkaid, "The role of retinoic acid in tolerance and immunity," *Immunity*, vol. 35, no. 1, pp. 13–22, 2011.

[11] V. Cerovic, C. C. Bain, A. M. Mowat, and S. W. F. Milling, "Intestinal macrophages and dendritic cells: what's the difference?," *Trends in Immunology*, vol. 35, no. 6, pp. 270–277, 2014.

[12] J. A. Hall, J. L. Cannons, J. R. Grainger et al., "Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha," *Immunity*, vol. 34, no. 3, pp. 435–447, 2011.

[13] G. Tejón, V. Manríquez, J. De Calisto et al., "Vitamin A impairs the reprogramming of Tregs into IL-17-producing cells during intestinal inflammation," *BioMed Research International*, vol. 2015, Article ID 137893, 8 pages, 2015.

[14] H. L. Penny, T. R. Prestwood, N. Bhattacharya et al., "Restoring retinoic acid attenuates intestinal inflammation and tumorigenesis in APCMin/+ mice," *Cancer Immunology Research*, vol. 4, no. 11, pp. 917–926, 2016.

[15] A. A. Saboor-Yaraghi, M. H. Harirchian, N. Mohammadzadeh Honarvar et al., "The effect of vitamin A supplementation on FoxP3 and TGF-β gene expression in Avonex-treated multiple sclerosis patients," *Journal of Molecular Neuroscience*, vol. 56, no. 3, pp. 608–612, 2015.

[16] N. Mohammadzadeh Honarvar, M. H. Harirchian, M. Abdolahi et al., "Retinyl palmitate supplementation modulates T-bet and interferon gamma gene expression in multiple sclerosis patients," *Journal of Molecular Neuroscience*, vol. 59, no. 3, pp. 360–365, 2016.

[17] M. Raverdeau, C. J. Breen, A. Misiak, and K. H. Mills, "Retinoic acid suppresses IL-17 production and pathogenic activity of $\gamma\delta$ T cells in CNS autoimmunity," *Immunology and Cell Biology*, vol. 94, no. 8, pp. 763–773, 2016.

[18] S. Goswami, P. Angkasekwinai, M. Shan et al., "Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma," *Nature Immunology*, vol. 10, no. 5, pp. 496–503, 2009.

[19] C. Niu, N. Liu, J. Liu et al., "Vitamin A maintains the airway epithelium in a murine model of asthma by suppressing glucocorticoid-induced leucine zipper," *Clinical & Experimental Allergy*, vol. 46, no. 6, pp. 848–860, 2016.

[20] M. Bono, G. Tejon, F. Flores-Santibañez, D. Fernandez, M. Rosemblatt, and D. Sauma, "Retinoic acid as a modulator of T cell immunity," *Nutrients*, vol. 8, no. 6, 2016.

[21] M. N. Erkelens and R. E. Mebius, "Retinoic acid and immune homeostasis: a balancing act," *Trends in Immunology*, vol. 38, no. 3, pp. 168–180, 2017.

[22] V. Azais-Braesco and G. Pascal, "Vitamin A in pregnancy: requirements and safety limits," *The American Journal of Clinical Nutrition*, vol. 71, no. 5, pp. 1325S–1333S, 2000.

[23] D. C. Berry, C. M. Croniger, N. B. Ghyselinck, and N. Noy, "Transthyretin blocks retinol uptake and cell signaling by the holo-retinol-binding protein receptor STRA6," *Molecular and Cellular Biology*, vol. 32, no. 19, pp. 3851–3859, 2012.

[24] R. Kawaguchi, M. Zhong, M. Kassai, M. Ter-Stepanian, and H. Sun, "Vitamin A transport mechanism of the multitransmembrane cell-surface receptor STRA6," *Membranes*, vol. 5, no. 3, pp. 425–453, 2015.

[25] D. C. Berry, H. Jacobs, G. Marwarha et al., "The STRA6 receptor is essential for retinol-binding protein-induced insulin resistance but not for maintaining vitamin A homeostasis in tissues other than the eye," *Journal of Biological Chemistry*, vol. 288, no. 34, pp. 24528–24539, 2013.

[26] M. Berggren Soderlund, G. A. Fex, and P. Nilsson-Ehle, "Concentrations of retinoids in early pregnancy and in newborns and their mothers," *The American Journal of Clinical Nutrition*, vol. 81, no. 3, pp. 633–636, 2005.

[27] F. Campo-Paysaa, F. Marletaz, V. Laudet, and M. Schubert, "Retinoic acid signaling in development: tissue-specific functions and evolutionary origins," *Genesis*, vol. 46, no. 11, pp. 640–656, 2008.

[28] S. L. M. Arnold, J. K. Amory, T. J. Walsh, and N. Isoherranen, "A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS," *Journal of Lipid Research*, vol. 53, no. 3, pp. 587–598, 2012.

[29] G. U. Schuster, N. J. Kenyon, and C. B. Stephensen, "Vitamin A deficiency decreases and high dietary vitamin A increases disease severity in the mouse model of asthma," *The Journal of Immunology*, vol. 180, no. 3, pp. 1834–1842, 2008.

[30] J. E. Balmer and R. Blomhoff, "Gene expression regulation by retinoic acid," *Journal of Lipid Research*, vol. 43, no. 11, pp. 1773–1808, 2002.

[31] M. Hjertson, L. Dimberg, K. Nilsson, G. Nilsson, P. K. Kivinen, and I. T. Harvima, "Retinoic acid inhibits in vitro development of mast cells but has no marked effect on mature human skin tryptase- and chymase-positive mast cells," *Journal of Investigative Dermatology*, vol. 120, no. 2, pp. 239–245, 2003.

[32] S. W. Kruse, K. Suino-Powell, X. E. Zhou et al., "Identification of COUP-TFII orphan nuclear receptor as a retinoic acid-activated receptor," *PLoS Biology*, vol. 6, no. 9, article e227, 2008.

[33] R. Zolfaghari and A. C. Ross, "Hepatocyte nuclear factor 4α (HNF4α) in coordination with retinoic acid receptors increases all-trans-retinoic acid-dependent CYP26A1 gene expression in HepG2 human hepatocytes," *Journal of Cellular Biochemistry*, vol. 115, no. 10, pp. 1740–1751, 2014.

[34] Y. Sakai, C. Meno, H. Fujii et al., "The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo," *Genes & Development*, vol. 15, no. 2, pp. 213–225, 2001.

[35] H. Takeuchi, A. Yokota, Y. Ohoka, and M. Iwata, "Cyp26b1 regulates retinoic acid-dependent signals in T cells and its expression is inhibited by transforming growth factor-β," *PLoS One*, vol. 6, no. 1, article e16089, 2011.

[36] K. R. R. Siddiqui and F. Powrie, "CD103+ GALT DCs promote Foxp3+ regulatory T cells," *Mucosal Immunology*, vol. 1, no. 1, pp. S34–S38, 2008.

[37] M. R. Beijer, G. Kraal, and J. M. M. den Haan, "Vitamin A and dendritic cell differentiation," *Immunology*, vol. 142, no. 1, pp. 39–45, 2014.

[38] P. Czarnewski, S. Das, S. Parigi, and E. Villablanca, "Retinoic acid and its role in modulating intestinal innate immunity," *Nutrients*, vol. 9, no. 1, 2017.

[39] J. L. Coombes and K. J. Maloy, "Control of intestinal homeostasis by regulatory T cells and dendritic cells," *Seminars in Immunology*, vol. 19, no. 2, pp. 116–126, 2007.

[40] M. Iwata, A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song, "Retinoic acid imprints gut-homing specificity on T cells," *Immunity*, vol. 21, no. 4, pp. 527–538, 2004.

[41] B. Eksteen, J. Rodrigo Mora, E. L. Haughton et al., "Gut homing receptors on CD8 T cells are retinoic acid dependent and not maintained by liver dendritic or stellate cells," *Gastroenterology*, vol. 137, no. 1, pp. 320–329, 2009.

[42] W. W. Agace and E. K. Persson, "How vitamin A metabolizing dendritic cells are generated in the gut mucosa," *Trends in Immunology*, vol. 33, no. 1, pp. 42–48, 2012.

[43] M. Guilliams, K. Crozat, S. Henri et al., "Skin-draining lymph nodes contain dermis-derived CD103(−) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells," *Blood*, vol. 115, no. 10, pp. 1958–1968, 2010.

[44] D. Ruane, L. Brane, B. S. Reis et al., "Lung dendritic cells induce migration of protective T cells to the gastrointestinal tract," *The Journal of Experimental Medicine*, vol. 210, no. 9, pp. 1871–1888, 2013.

[45] C. Wang, S. G. Kang, H. HogenEsch, P. E. Love, and C. H. Kim, "Retinoic acid determines the precise tissue tropism of inflammatory Th17 cells in the intestine," *The Journal of Immunology*, vol. 184, no. 10, pp. 5519–5526, 2010.

[46] S. I. Hammerschmidt, M. Friedrichsen, J. Boelter et al., "Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice," *Journal of Clinical Investigation*, vol. 121, no. 8, pp. 3051–3061, 2011.

[47] K. R. R. Siddiqui, S. Laffont, and F. Powrie, "E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis," *Immunity*, vol. 32, no. 4, pp. 557–567, 2010.

[48] A. Rivollier, J. He, A. Kole, V. Valatas, and B. L. Kelsall, "Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon," *The Journal of Experimental Medicine*, vol. 209, no. 1, pp. 139–155, 2012.

[49] O. Annacker, J. L. Coombes, V. Malmstrom et al., "Essential role for CD103 in the T cell-mediated regulation of experimental colitis," *The Journal of Experimental Medicine*, vol. 202, no. 8, pp. 1051–1061, 2005.

[50] A. Stock, S. Booth, and V. Cerundolo, "Prostaglandin E2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans," *The Journal of Experimental Medicine*, vol. 208, no. 4, pp. 761–773, 2011.

[51] R. Molenaar, M. Knippenberg, G. Goverse et al., "Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin a," *The Journal of Immunology*, vol. 186, no. 4, pp. 1934–1942, 2011.

[52] S. Manicassamy, R. Ravindran, J. Deng et al., "Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity," *Nature Medicine*, vol. 15, no. 4, pp. 401–409, 2009.

[53] A. Yokota, H. Takeuchi, N. Maeda et al., "GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity," *International Immunology*, vol. 21, no. 4, pp. 361–377, 2009.

[54] C. Pitzalis, G. W. Jones, M. Bombardieri, and S. A. Jones, "Ectopic lymphoid-like structures in infection, cancer and autoimmunity," *Nature Reviews Immunology*, vol. 14, no. 7, pp. 447–462, 2014.

[55] G. W. Jones, D. G. Hill, and S. A. Jones, "Understanding immune cells in tertiary lymphoid organ development: it is all starting to come together," *Frontiers in Immunology*, vol. 7, 2016.

[56] F. Geissmann, P. Revy, N. Brousse et al., "Retinoids regulate survival and antigen presentation by immature dendritic cells," *The Journal of Experimental Medicine*, vol. 198, no. 4, pp. 623–634, 2003.

[57] G. Bakdash, L. T. Vogelpoel, T. M. van Capel, M. L. Kapsenberg, and E. C. de Jong, "Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells," *Mucosal Immunology*, vol. 8, no. 2, pp. 265–278, 2015.

[58] L. E. Smythies, M. Sellers, R. H. Clements et al., "Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity," *Journal of Clinical Investigation*, vol. 115, no. 1, pp. 66–75, 2005.

[59] M. M. Roe, S. Swain, T. A. Sebrell et al., "Differential regulation of CD103 (αE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands," *Journal of Leukocyte Biology*, vol. 101, no. 5, pp. 1169–1180, 2017.

[60] K. Mehta, T. McQueen, S. Tucker, R. Pandita, and B. B. Aggarwal, "Inhibition by all-trans-retinoic acid of tumor necrosis factor and nitric oxide production by peritoneal macrophages," *Journal of Leukocyte Biology*, vol. 55, no. 3, pp. 336–342, 1994.

[61] S. Y. Na, B. Y. Kang, S. W. Chung et al., "Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFκB," *Journal of Biological Chemistry*, vol. 274, no. 12, pp. 7674–7680, 1999.

[62] B. Y. Kang, S. W. Chung, S. H. Kim, S. N. Kang, Y. K. Choe, and T. S. Kim, "Retinoid-mediated inhibition of interleukin-12 production in mouse macrophages suppresses Th1 cytokine profile in CD4(+) T cells," *British Journal of Pharmacology*, vol. 130, no. 3, pp. 581–586, 2000.

[63] X. Wang, C. Allen, and M. Ballow, "Retinoic acid enhances the production of IL-10 while reducing the synthesis of IL-12 and TNF-α from LPS-stimulated monocytes/macrophages," *Journal of Clinical Immunology*, vol. 27, no. 2, pp. 193–200, 2007.

[64] V. W. Ho, E. Hofs, I. Elisia et al., "All trans retinoic acid, transforming growth factor β and prostaglandin E2 in mouse plasma synergize with basophil-secreted interleukin-4 to M2 polarize murine macrophages," *PLoS One*, vol. 11, no. 12, article e0168072, 2016.

[65] L. C. Davies, S. J. Jenkins, J. E. Allen, and P. R. Taylor, "Tissue-resident macrophages," *Nature Immunology*, vol. 14, no. 10, pp. 986–995, 2013.

[66] U. M. Gundra, N. M. Girgis, M. A. Gonzalez et al., "Vitamin A mediates conversion of monocyte-derived macrophages into tissue-resident macrophages during alternative activation," *Nature Immunology*, vol. 18, no. 6, pp. 642–653, 2017.

[67] E. D. Tait Wojno and D. Artis, "Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine," *Cell Host & Microbe*, vol. 12, no. 4, pp. 445–457, 2012.

[68] L. Rankin, J. Groom, L. A. Mielke, C. Seillet, and G. T. Belz, "Diversity, function, and transcriptional regulation of gut innate lymphocytes," *Frontiers in Oncology*, vol. 4, 2013.

[69] H. Spits and J. P. Di Santo, "The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling," *Nature Immunology*, vol. 12, no. 1, pp. 21–27, 2011.

[70] J. Mjösberg and H. Spits, "Human innate lymphoid cells," *Journal of Allergy and Clinical Immunology*, vol. 138, no. 5, pp. 1265–1276, 2016.

[71] H. Takatori, Y. Kanno, W. T. Watford et al., "Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22," *The Journal of Experimental Medicine*, vol. 206, no. 1, pp. 35–41, 2009.

[72] S. A. van de Pavert, M. Ferreira, R. G. Domingues et al., "Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity," *Nature*, vol. 508, no. 7494, pp. 123–127, 2014.

[73] G. Goverse, C. Labao-Almeida, M. Ferreira et al., "Vitamin A controls the presence of RORγ+ innate lymphoid cells and lymphoid tissue in the small intestine," *The Journal of Immunology*, vol. 196, no. 12, pp. 5148–5155, 2016.

[74] B. Ruiter, S. U. Patil, and W. G. Shreffler, "Vitamins A and D have antagonistic effects on expression of effector cytokines and gut-homing integrin in human innate lymphoid cells," *Clinical & Experimental Allergy*, vol. 45, no. 7, pp. 1214–1225, 2015.

[75] L. A. Mielke, S. A. Jones, M. Raverdeau et al., "Retinoic acid expression associates with enhanced IL-22 production by $\gamma\delta$ T cells and innate lymphoid cells and attenuation of intestinal inflammation," *The Journal of Experimental Medicine*, vol. 210, no. 6, pp. 1117–1124, 2013.

[76] J. Abb, H. Abb, and F. Deinhardt, "Effect of retinoic acid on the spontaneous and interferon-induced activity of human natural killer cells," *International Journal of Cancer*, vol. 30, no. 3, pp. 307–310, 1982.

[77] L. L. Lanier, "Up on the tightrope: natural killer cell activation and inhibition," *Nature Immunology*, vol. 9, no. 5, pp. 495–502, 2008.

[78] W. Wang, A. K. Erbe, J. A. Hank, Z. S. Morris, and P. M. Sondel, "NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy," *Frontiers in Immunology*, vol. 6, 2015.

[79] O. Eremin, J. Ashby, and J. Rhodes, "Inhibition of antibody-dependent cellular cytotoxicity and natural cytotoxicity by retinoic acid," *International Archives of Allergy and Immunology*, vol. 75, no. 1, pp. 2–7, 1984.

[80] A. Li, M. He, H. Wang et al., "All-trans retinoic acid negatively regulates cytotoxic activities of nature killer cell line 92," *Biochemical and Biophysical Research Communications*, vol. 352, no. 1, pp. 42–47, 2007.

[81] G. Konjevic, K. Mirjacic-Martinovic, A. Vuletic, and N. Babovic, "In vitro increased natural killer cell activity of metastatic melanoma patients with interferon-α alone as opposed to its combination with 13-cis retinoic acid is associated with modulation of NKG2D and CD161 activating receptor expression," *Journal of BUON*, vol. 17, no. 4, pp. 761–769, 2012.

[82] M. Jinushi, T. Takehara, T. Tatsumi et al., "Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid," *International Journal of Cancer*, vol. 104, no. 3, pp. 354–361, 2003.

[83] S. M. Ahmad, M. J. Haskell, R. Raqib, and C. B. Stephensen, "Markers of innate immune function are associated with vitamin a stores in men," *The Journal of Nutrition*, vol. 139, no. 2, pp. 377–385, 2009.

[84] J. R. Mora, M. Iwata, B. Eksteen et al., "Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells," *Science*, vol. 314, no. 5802, pp. 1157–1160, 2006.

[85] S. Kheirouri and M. Alizadeh, "Decreased serum and mucosa immunoglobulin A levels in vitamin Aand zinc-deficient mice," *Central European Journal of Immunology*, vol. 39, no. 2, pp. 165–169, 2014.

[86] Y. Ma, Q. Chen, and A. C. Ross, "Retinoic acid and polyriboinosinic:polyribocytidylic acid stimulate robust anti-tetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations," *The Journal of Immunology*, vol. 174, no. 12, pp. 7961–7969, 2005.

[87] Y. Ma and A. C. Ross, "The anti-tetanus immune response of neonatal mice is augmented by retinoic acid combined with polyriboinosinic:polyribocytidylic acid," *Proceedings of the National Academy of Sciences*, vol. 102, no. 38, pp. 13556–13561, 2005.

[88] J. Buck, G. Ritter, L. Dannecker et al., "Retinol is essential for growth of activated human B cells," *Journal of Experimental Medicine*, vol. 171, no. 5, pp. 1613–1624, 1990.

[89] X. Chen, B. L. Esplin, K. P. Garrett, R. S. Welner, C. F. Webb, and P. W. Kincade, "Retinoids accelerate B lineage lymphoid differentiation," *The Journal of Immunology*, vol. 180, no. 1, pp. 138–145, 2008.

[90] K. Morikawa and M. Nonaka, "All-trans-retinoic acid accelerates the differentiation of human B lymphocytes maturing into plasma cells," *International Immunopharmacology*, vol. 5, no. 13-14, pp. 1830–1838, 2005.

[91] C. C. Goodnow, C. G. Vinuesa, K. L. Randall, F. Mackay, and R. Brink, "Control systems and decision making for antibody production," *Nature Immunology*, vol. 11, no. 8, pp. 681–688, 2010.

[92] M. Muramatsu, K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo, "Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme," *Cell*, vol. 102, no. 5, pp. 553–563, 2000.

[93] Q. Chen and A. C. Ross, "Retinoic acid promotes mouse splenic B cell surface IgG expression and maturation stimulated by CD40 and IL-4," *Cellular Immunology*, vol. 249, no. 1, pp. 37–45, 2007.

[94] R. L. Indrevær, J. Ø. Moskaug, I. Paur et al., "IRF4 is a critical gene in retinoic acid-mediated plasma cell formation and is deregulated in common variable immunodeficiency-derived B cells," *The Journal of Immunology*, vol. 195, no. 6, pp. 2601–2611, 2015.

[95] W. Wang and M. Ballow, "The effects of retinoic acid on in vitro immunoglobulin synthesis by cord blood and adult peripheral blood mononuclear cells," *Cellular Immunology*, vol. 148, no. 2, pp. 291–300, 1993.

[96] K. Suzuki, M. Maruya, S. Kawamoto et al., "The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut," *Immunity*, vol. 33, no. 1, pp. 71–83, 2010.

[97] B. Lönnerdal, "Nutritional roles of lactoferrin," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 12, no. 3, pp. 293–297, 2009.

[98] S. H. Kang, B. R. Jin, H. J. Kim et al., "Lactoferrin combined with retinoic acid stimulates B1 cells to express IgA isotype and gut-homing molecules," *Immune Network*, vol. 15, no. 1, pp. 37–43, 2015.

[99] M. Iwata, Y. Eshima, and H. Kagechika, "Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors," *International Immunology*, vol. 15, no. 8, pp. 1017–1025, 2003.

[100] C. B. Stephensen, A. D. Borowsky, and K. C. K. Lloyd, "Disruption of Rxra gene in thymocytes and T lymphocytes modestly alters lymphocyte frequencies, proliferation, survival and T helper type 1/type 2 balance," *Immunology*, vol. 121, no. 4, pp. 484–498, 2007.

[101] W. Cui, P. Zhang, J. Gu et al., "Vitamin A deficiency promotes inflammation by induction of type 2 cytokines in experimental ovalbumin-induced asthma murine model," *Inflammation*, vol. 39, no. 5, pp. 1798–1804, 2016.

[102] C. C. Brown, D. Esterhazy, A. Sarde et al., "Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program," *Immunity*, vol. 42, no. 3, pp. 499–511, 2015.

[103] J. L. Coombes, K. R. R. Siddiqui, C. V. Arancibia-Cárcamo et al., "A functionally specialized population of mucosal CD103+DCs induces Foxp3+regulatory T cells via a TGF-β– and retinoic acid–dependent mechanism," *The Journal of Experimental Medicine*, vol. 204, no. 8, pp. 1757–1764, 2007.

[104] K. Pino-Lagos, Y. Guo, C. Brown et al., "A retinoic acid-dependent checkpoint in the development of CD4+ T cell-mediated immunity," *The Journal of Experimental Medicine*, vol. 208, no. 9, pp. 1767–1775, 2011.

[105] D. M. Duriancik, D. E. Lackey, and K. A. Hoag, "Vitamin A as a regulator of antigen presenting cells," *The Journal of Nutrition*, vol. 140, no. 8, pp. 1395–1399, 2010.

[106] Y. F. Zhu, J. Z. Hu, P. N. Zhao, L. X. Liu, and Y. Li, "All-transretinoic acid regulates Th1/Th2 balance in CD4+ T cells when GATA-3 is deficient," *Biomedical and Environmental Sciences*, vol. 26, no. 9, pp. 774–777, 2013.

[107] S. Xiao, H. Jin, T. Korn et al., "Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression," *The Journal of Immunology*, vol. 181, no. 4, pp. 2277–2284, 2008.

[108] J. A. Hill, J. A. Hall, C. M. Sun et al., "Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition

from CD4+CD44hi cells," *Immunity*, vol. 29, no. 5, pp. 758–770, 2008.

[109] C. B. Collins, C. M. Aherne, D. Kominsky et al., "Retinoic acid attenuates ileitis by restoring the balance between T-helper 17 and T regulatory cells," *Gastroenterology*, vol. 141, no. 5, pp. 1821–1831, 2011.

[110] C. Moore, C. Fuentes, D. Sauma et al., "Retinoic acid generates regulatory T cells in experimental transplantation," *Transplantation Proceedings*, vol. 43, no. 6, pp. 2334–2337, 2011.

[111] S. Uematsu, K. Fujimoto, M. H. Jang et al., "Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5," *Nature Immunology*, vol. 9, no. 7, pp. 769–776, 2008.

[112] L. Chen, H. Deng, H. Cui et al., "Inflammatory responses and inflammation-associated diseases in organs," *Oncotarget*, vol. 9, no. 6, pp. 7204–7218, 2018.

[113] X. Chen, C. Tu, T. Qin, L. Zhu, Y. Yin, and Q. Yang, "Retinoic acid facilitates inactivated transmissible gastroenteritis virus induction of CD8(+) T-cell migration to the porcine gut," *Scientific Reports*, vol. 6, no. 1, article 24152, 2016.

[114] Y. Zhou, Y. Hong, and H. Huang, "Triptolide attenuates inflammatory response in membranous glomerulo-nephritis rat via downregulation of NF-κB signaling pathway," *Kidney and Blood Pressure Research*, vol. 41, no. 6, pp. 901–910, 2016.

[115] K. Hong, Y. Zhang, Y. Guo et al., "All-trans retinoic acid attenuates experimental colitis through inhibition of NF-κB signaling," *Immunology Letters*, vol. 162, no. 1, pp. 34–40, 2014.

[116] T. Eom, Y. S. Kim, C. H. Choi, M. J. Sadowsky, and T. Unno, "Current understanding of microbiota- and dietary-therapies for treating inflammatory bowel disease," *Journal of Microbiology*, vol. 56, no. 3, pp. 189–198, 2018.

[117] J. H. Bernink, L. Krabbendam, K. Germar et al., "Interleukin-12 and -23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria," *Immunity*, vol. 43, no. 1, pp. 146–160, 2015.

[118] H. Rafa, S. Benkhelifa, S. AitYounes et al., "All-trans retinoic acid modulates TLR4/NF-all-trans retinoic acid modulates TLR4/NF-κB signaling pathway targeting TNF-α and nitric oxide synthase 2 expression in colonic mucosa during ulcerative colitis and colitis associated cancer," *Mediators of Inflammation*, vol. 2017, Article ID 7353252, 16 pages, 2017.

[119] I. Okayasu, K. Hana, N. Nemoto et al., "Vitamin A inhibits development of dextran sulfate sodium-induced colitis and colon cancer in a mouse model," *BioMed Research International*, vol. 2016, Article ID 4874809, 11 pages, 2016.

[120] A. Chenery, K. Burrows, F. Antignano, T. M. Underhill, M. Petkovich, and C. Zaph, "The retinoic acid-metabolizing enzyme Cyp26b1 regulates CD4 T cell differentiation and function," *PLoS One*, vol. 8, no. 8, article e72308, 2013.

[121] N. Bhattacharya, R. Yuan, T. R. Prestwood et al., "Normalizing microbiota-induced retinoic acid deficiency stimulates protective CD8(+) T cell-mediated immunity in colorectal cancer," *Immunity*, vol. 45, no. 3, pp. 641–655, 2016.

[122] M. Leenders, A. M. Leufkens, P. D. Siersema et al., "Plasma and dietary carotenoids and vitamins a, C and E and risk of colon and rectal cancer in the European prospective investigation into cancer and nutrition," *International Journal of Cancer*, vol. 135, no. 12, pp. 2930–2939, 2014.

[123] M. Soares-Mota, T. A. Silva, L. M. Gomes et al., "High prevalence of vitamin A deficiency in Crohn's disease patients according to serum retinol levels and the relative dose-response test," *World Journal of Gastroenterology*, vol. 21, no. 5, pp. 1614–1620, 2015.

[124] G. Rumi Jr, I. Szabó, Á. Vincze, Z. Matus, G. Tóth, and G. Mózsik, "Decrease of serum carotenoids in Crohn's disease," *Journal of Physiology-Paris*, vol. 94, no. 2, pp. 159–161, 2000.

[125] A. Bousvaros, D. Zurakowski, C. Duggan et al., "Vitamins A and E serum levels in children and young adults with inflammatory bowel disease: effect of disease activity," *Journal of Pediatric Gastroenterology & Nutrition*, vol. 26, no. 2, pp. 129–135, 1998.

[126] R. Sinha, D. R. Howlader, P. Mukherjee, S. Rai, D. Nag, and H. Koley, "Retinoic acid decreases the severity of Salmonella enterica serovar typhimurium mediated gastroenteritis in a mouse model," *Immunobiology*, vol. 221, no. 7, pp. 839–844, 2016.

[127] R. Sinha, D. R. Howlader, A. Ta, S. Mitra, S. Das, and H. Koley, "Retinoic acid pre-treatment down regulates V. cholerae outer membrane vesicles induced acute inflammation and enhances mucosal immunity," *Vaccine*, vol. 35, no. 28, pp. 3534–3547, 2017.

[128] J. Wu, Y. Zhang, Q. Liu, W. Zhong, and Z. Xia, "All-trans retinoic acid attenuates airway inflammation by inhibiting Th2 and Th17 response in experimental allergic asthma," *BMC Immunology*, vol. 14, no. 1, p. 28, 2013.

[129] H. Na, H. Lim, G. Choi et al., "Concomitant suppression of T H 2 and T H 17 cell responses in allergic asthma by targeting retinoic acid receptor–related orphan receptor γt," *Journal of Allergy and Clinical Immunology*, vol. 141, no. 6, pp. 2061–2073.e5, 2018.

[130] U. Fujii, N. Miyahara, A. Taniguchi et al., "Effect of a retinoid X receptor partial agonist on airway inflammation and hyperresponsiveness in a murine model of asthma," *Respiratory Research*, vol. 18, no. 1, p. 23, 2017.

[131] H. A. Marquez and W. V. Cardoso, "Vitamin A-retinoid signaling in pulmonary development and disease," *Molecular and Cellular Pediatrics*, vol. 3, no. 1, p. 28, 2016.

[132] H. Sakamoto, T. Koya, K. Tsukioka et al., "The effects of all-trans retinoic acid on the induction of oral tolerance in a murine model of bronchial asthma," *International Archives of Allergy and Immunology*, vol. 167, no. 3, pp. 167–176, 2015.

[133] H. L. Son, H. R. Park, Y. J. Park, and S. W. Kim, "Effect of retinoic acid in a mouse model of allergic rhinitis," *Allergy, Asthma & Immunology Research*, vol. 7, no. 6, pp. 590–598, 2015.

[134] P. Soroosh, T. A. Doherty, W. Duan et al., "Lung-resident tissue macrophages generate Foxp3+ regulatory T cells and promote airway tolerance," *The Journal of Experimental Medicine*, vol. 210, no. 4, pp. 775–788, 2013.

[135] S. Allen, J. R. Britton, and J. A. Leonardi-Bee, "Association between antioxidant vitamins and asthma outcome measures: systematic review and meta-analysis," *Thorax*, vol. 64, no. 7, pp. 610–619, 2009.

[136] A. M. Al Senaidy, "Serum vitamin a and β-carotene levels in children with asthma," *Journal of Asthma*, vol. 46, no. 7, pp. 699–702, 2009.

[137] L. L. Muldoon, J. I. Alvarez, D. J. Begley et al., "Immunologic privilege in the central nervous system and the blood-brain barrier," *Journal of Cerebral Blood Flow & Metabolism*, vol. 33, no. 1, pp. 13–21, 2013.

[138] F. Jadidi-Niaragh and A. Mirshafiey, "Th17 cell, the new player of neuroinflammatory process in multiple sclerosis," *Scandinavian Journal of Immunology*, vol. 74, no. 1, pp. 1–13, 2011.

[139] K. Parastouei, A. Mirshafiey, M. R. Eshraghian et al., "The effect of 1, 25(OH)2 D3 (calcitriol) alone and in combination with all-trans retinoic acid on ROR-γt, IL-17, TGF-β, and FOXP3 gene expression in experimental autoimmune encephalomyelitis," *Nutritional Neuroscience*, vol. 21, no. 3, pp. 210–218, 2018.

[140] S. M. Abtahi Froushani, N. Delirezh, R. Hobbenaghi, and G. Mosayebi, "Synergistic effects of atorvastatin and all-trans retinoic acid in ameliorating animal model of multiple sclerosis," *Immunological Investigations*, vol. 43, no. 1, pp. 54–68, 2014.

[141] M. R. Mizee, P. G. Nijland, S. M. A. van der Pol et al., "Astrocyte-derived retinoic acid: a novel regulator of blood-brain barrier function in multiple sclerosis," *Acta Neuropathologica*, vol. 128, no. 5, pp. 691–703, 2014.

[142] S. van Neerven, A. Nemes, P. Imholz et al., "Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid," *Journal of Neuroimmunology*, vol. 229, no. 1-2, pp. 169–179, 2010.

[143] M. Machado-Pereira, T. Santos, L. Ferreira, L. Bernardino, and R. Ferreira, "Anti-inflammatory strategy for M2 microglial polarization using retinoic acid-loaded nanoparticles," *Mediators of Inflammation*, vol. 2017, Article ID 6742427, 11 pages, 2017.

[144] M. Machado-Pereira, T. Santos, L. Ferreira, L. Bernardino, and R. Ferreira, "Intravenous administration of retinoic acid-loaded polymeric nanoparticles prevents ischemic injury in the immature brain," *Neuroscience Letters*, vol. 673, pp. 116–121, 2018.

[145] M. J. Stebbins, E. S. Lippmann, M. G. Faubion, R. Daneman, S. P. Palecek, and E. V. Shusta, "Activation of RARα, RARγ, or RXRα increases barrier tightness in human induced pluripotent stem cell-derived brain endothelial cells," *Biotechnology Journal*, vol. 13, no. 2, 2018.

[146] K. Kawahara, M. Suenobu, H. Ohtsuka et al., "Cooperative therapeutic action of retinoic acid receptor and retinoid x receptor agonists in a mouse model of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 42, no. 2, pp. 587–605, 2014.

[147] R. Takamura, N. Watamura, M. Nikkuni, and T. Ohshima, "All-trans retinoic acid improved impaired proliferation of neural stem cells and suppressed microglial activation in the hippocampus in an Alzheimer's mouse model," *Journal of Neuroscience Research*, vol. 95, no. 3, pp. 897–906, 2017.

[148] V. Clemens, F. Regen, N. Le Bret, I. Heuser, and J. Hellmann-Regen, "Retinoic acid enhances apolipoprotein E synthesis in human macrophages," *Journal of Alzheimer's Disease*, vol. 61, no. 4, pp. 1295–1300, 2018.

[149] S. Qin, C. Colin, I. Hinners, A. Gervais, C. Cheret, and M. Mallat, "System Xc- and apolipoprotein E expressed by microglia have opposite effects on the neurotoxicity of amyloid-beta peptide 1-40," *Journal of Neuroscience*, vol. 26, no. 12, pp. 3345–3356, 2006.

[150] N. Behairi, M. Belkhelfa, H. Rafa et al., "All-trans retinoic acid (ATRA) prevents lipopolysaccharide-induced neuroinflammation, amyloidogenesis and memory impairment in aged rats," *Journal of Neuroimmunology*, vol. 300, pp. 21–29, 2016.

[151] N. Behairi, M. Belkhelfa, H. Mesbah-Amroun et al., "All-trans-retinoic acid modulates nitric oxide and interleukin-17A production by peripheral blood mononuclear cells from patients with Alzheimer's disease," *Neuroimmunomodulation*, vol. 22, no. 6, pp. 385–393, 2015.

[152] F. O. Nestle, P. Di Meglio, J. Z. Qin, and B. J. Nickoloff, "Skin immune sentinels in health and disease," *Nature Reviews Immunology*, vol. 9, no. 10, pp. 679–691, 2009.

[153] W. R. Heath and F. R. Carbone, "The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells," *Nature Immunology*, vol. 14, no. 10, pp. 978–985, 2013.

[154] E. Shklovskaya, B. J. O'Sullivan, L. G. Ng et al., "Langerhans cells are precommitted to immune tolerance induction," *Proceedings of the National Academy of Sciences*, vol. 108, no. 44, pp. 18049–18054, 2011.

[155] A. Matejuk, "Skin immunity," *Archivum Immunologiae et Therapiae Experimentalis*, vol. 66, no. 1, pp. 45–54, 2018.

[156] M. C. Lebre, A. M. G. van der Aar, L. van Baarsen et al., "Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9," *Journal of Investigative Dermatology*, vol. 127, no. 2, pp. 331–341, 2007.

[157] D. Rigopoulos, D. Ioannides, D. Kalogeromitros, and A. D. Katsambas, "Comparison of topical retinoids in the treatment of acne," *Clinics in Dermatology*, vol. 22, no. 5, pp. 408–411, 2004.

[158] S. Khalil, T. Bardawil, C. Stephan et al., "Retinoids: a journey from the molecular structures and mechanisms of action to clinical uses in dermatology and adverse effects," *Journal of Dermatological Treatment*, vol. 28, no. 8, pp. 684–696, 2017.

[159] F. Awad, E. Assrawi, C. Louvrier et al., "Photoaging and skin cancer: is the inflammasome the missing link?," *Mechanisms of Ageing and Development*, vol. 172, pp. 131–137, 2018.

[160] W. Gao, Y. S. Wang, Z. Y. Qu et al., "Orobanche cernuaLoefling attenuates ultraviolet B-mediated photoaging in human dermal fibroblasts," *Photochemistry and Photobiology*, vol. 94, no. 4, pp. 733–743, 2018.

[161] J. Varani, R. L. Warner, M. Gharaee-Kermani et al., "Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin," *Journal of Investigative Dermatology*, vol. 114, no. 3, pp. 480–486, 2000.

[162] R. R. Riahi, A. E. Bush, and P. R. Cohen, "Topical retinoids: therapeutic mechanisms in the treatment of photodamaged skin," *American Journal of Clinical Dermatology*, vol. 17, no. 3, pp. 265–276, 2016.

[163] L. W. Fu and R. B. Vender, "Newer approaches in topical combination therapy for acne," *Skin Therapy Letter*, vol. 16, no. 9, pp. 3–6, 2011.

[164] T. Karlsson, A. Vahlquist, N. Kedishvili, and H. Törmä, "13-cis-retinoic acid competitively inhibits 3α-hydroxysteroid oxidation by retinol dehydrogenase RoDH-4: a mechanism for its anti-androgenic effects in sebaceous glands?," *Biochemical and Biophysical Research Communications*, vol. 303, no. 1, pp. 273–278, 2003.

[165] M. C. Dispenza, E. B. Wolpert, K. L. Gilliland et al., "Systemic isotretinoin therapy normalizes exaggerated TLR-2-mediated innate immune responses in acne patients," *Journal of Investigative Dermatology*, vol. 132, no. 9, pp. 2198–2205, 2012.

[166] S. A. Sacchidanand, K. Lahiri, K. Godse et al., "Synchronizing pharmacotherapy in acne with review of clinical care," *Indian Journal of Dermatology*, vol. 62, no. 4, pp. 341–357, 2017.

[167] G. D. Weinstein, J. Y. M. Koo, G. G. Krueger et al., "Tazarotene cream in the treatment of psoriasis: two multicenter, double-blind, randomized, vehicle-controlled studies of the safety and efficacy of tazarotene creams 0.05% and 0.1% applied once daily for 12 weeks," *Journal of the American Academy of Dermatology*, vol. 48, no. 5, pp. 760–767, 2003.

[168] M. Lebwohl, E. Ast, J. P. Callen et al., "Once-daily tazarotene gel versus twice-daily fluocinonide cream in the treatment of plaque psoriasis," *Journal of the American Academy of Dermatology*, vol. 38, no. 5, pp. 705–711, 1998.

[169] L. Green and W. Sadoff, "A clinical evaluation of tazarotene 0.1% gel, with and without a high- or mid-high-potency corticosteroid, in patients with stable plaque psoriasis," *Journal of Cutaneous Medicine and Surgery*, vol. 6, no. 2, pp. 95–102, 2002.

[170] S. Behrens, M. Grundmann-Kollmann, R. Schiener, R. U. Peter, and M. Kerscher, "Combination phototherapy of psoriasis with narrow-band UVB irradiation and topical tazarotene gel," *Journal of the American Academy of Dermatology*, vol. 42, no. 3, pp. 493–495, 2000.

[171] L. F. Stein Gold, "Topical therapies for psoriasis: improving management strategies and patient adherence," *Seminars in Cutaneous Medicine and Surgery*, vol. 35, no. 2S, pp. S36–S44, 2016.

[172] R. Torsekar and M. M. Gautam, "Topical therapies in psoriasis," *Indian Dermatology Online Journal*, vol. 8, no. 4, pp. 235–245, 2017.

[173] A. J. Cox, N. P. West, and A. W. Cripps, "Obesity, inflammation, and the gut microbiota," *The Lancet Diabetes & Endocrinology*, vol. 3, no. 3, pp. 207–215, 2015.

[174] P. D. Cani and B. F. Jordan, "Gut microbiota-mediated inflammation in obesity: a link with gastrointestinal cancer," *Nature Reviews Gastroenterology & Hepatology*, 2018.

[175] E. Karkeni, L. Bonnet, J. Astier et al., "All-trans-retinoic acid represses chemokine expression in adipocytes and adipose tissue by inhibiting NF-κB signaling," *The Journal of Nutritional Biochemistry*, vol. 42, pp. 101–107, 2017.

[176] C. Tsutsumi, M. Okuno, L. Tannous et al., "Retinoids and retinoid-binding protein expression in rat adipocytes," *Journal of Biological Chemistry*, vol. 267, no. 3, pp. 1805–1810, 1992.

[177] M. L. Bonet, J. Ribot, F. Felipe, and A. Palou, "Vitamin A and the regulation of fat reserves," *Cellular and Molecular Life Sciences (CMLS)*, vol. 60, no. 7, pp. 1311–1321, 2003.

[178] J. Amengual, J. Ribot, M. L. Bonet, and A. Palou, "Retinoic acid treatment enhances lipid oxidation and inhibits lipid biosynthesis capacities in the liver of mice," *Cellular Physiology and Biochemistry*, vol. 25, no. 6, pp. 657–666, 2010.

[179] S. M. Jeyakumar, A. Vajreswari, and N. V. Giridharan, "Vitamin a regulates obesity in WNIN/Ob obese rat; independent of stearoyl-CoA desaturase-1," *Biochemical and Biophysical Research Communications*, vol. 370, no. 2, pp. 243–247, 2008.

[180] J. Mercader, J. Ribot, I. Murano et al., "Remodeling of white adipose tissue after retinoic acid administration in mice," *Endocrinology*, vol. 147, no. 11, pp. 5325–5332, 2006.

[181] J. Ribot, F. Felipe, M. L. Bonet, and A. Palou, "Changes of adiposity in response to vitamin A status correlate with changes of PPAR gamma 2 expression," *Obesity Research*, vol. 9, no. 8, pp. 500–509, 2001.

[182] B. Wang, X. Fu, X. Liang et al., "Retinoic acid induces white adipose tissue browning by increasing adipose vascularity and inducing beige adipogenesis of PDGFRα+ adipose progenitors," *Cell Discovery*, vol. 3, article 17036, 2017.

[183] A. Bartelt and J. Heeren, "Adipose tissue browning and metabolic health," *Nature Reviews Endocrinology*, vol. 10, no. 1, pp. 24–36, 2014.

[184] D. C. Berry and N. Noy, "All-trans-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor beta/delta and retinoic acid receptor," *Molecular and Cellular Biology*, vol. 29, no. 12, pp. 3286–3296, 2009.

[185] E. Dalmas, F. M. Lehmann, E. Dror et al., "Interleukin-33-activated islet-resident innate lymphoid cells promote insulin secretion through myeloid cell retinoic acid production," *Immunity*, vol. 47, no. 5, pp. 928–942.e7, 2017.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9787128, 17 pages
https://doi.org/10.1155/2018/9787128

Research Article

Insulin-Like Growth Factor-I as an Effector Element of the Cytokine IL-4 in the Development of a *Leishmania major* Infection

Luiza C. Reis,[1] Eduardo Milton Ramos-Sanchez,[1] Fabricio Petitto-Assis,[1] Audun H. Nerland,[2] Maria Hernandez-Valladares,[3] Frode Selheim,[3] Lucile Maria Floeter-Winter,[4] and Hiro Goto [1,5]

[1]*Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, IMTSP-USP, São Paulo, SP, Brazil*
[2]*Department of Clinical Science, University of Bergen, Bergen, Norway*
[3]*Departament of Biomedicine, University of Bergen, Bergen, Norway*
[4]*Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil*
[5]*Departamento de Medicina Preventiva, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil*

Correspondence should be addressed to Hiro Goto; hgoto@usp.br

Luiza C. Reis and Eduardo Milton Ramos-Sanchez contributed equally to this work and are co-first authors.

Received 16 February 2018; Revised 11 May 2018; Accepted 8 July 2018; Published 29 July 2018

Academic Editor: Naïma Moustaïd-Moussa

Certain cytokines modulate the expression of insulin-like growth factor- (IGF-) I. Since IL-4 and IGF-I promote growth of the protozoan *Leishmania major*, we here addressed their interaction in downregulating the expression of *Igf-I* mRNA using small interfering RNA (siRNA) in *Leishmania major*-infected macrophages. Parasitism was decreased in the siRNA-treated cells compared with the nontreated cells, reversed by the addition of recombinant IGF-I (rIGF-I). In IL-4-stimulated macrophages, parasitism and the *Igf-I* mRNA amount were increased, and the effects were nullified upon siRNA transfection. IGF-I downregulation inhibited both parasite and macrophage arginase activation even in IL-4-stimulated cells. Searching for intracellular signaling components shared by IL-4 and IGF-I, upon siRNA transfection, phosphorylated p44, p38, and Akt proteins were decreased, affecting the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. In *L. major*-infected C57BL6-resistant mice, the preincubation of the parasite with rIGF-I changed the infection profile to be similar to that of susceptible mice. We conclude that IGF-I constitutes an effector element of IL-4 involving the PI3K/Akt pathway during *L. major* infection.

1. Introduction

Certain physiological processes are reciprocally controlled by the immune and endocrine systems, where the actions of cytokines and hormones efficiently regulate these processes [1–3]. In this context, the observation that certain cytokines modulate the expression of the hormone insulin-like growth factor- (IGF-) I [4–6] led us to explore this cross talk in the interaction of the *Leishmania* parasite with host cells since the essential roles of different cytokines and the effect of IGF-I on the development of *Leishmania* infection are known [7].

Leishmania is an obligatory intracellular protozoan that is transmitted vectorially and causes diseases called leishmaniases that affect two million people globally each year. These infections result in lesions on the skin, mucosa, or viscera, depending on the parasite species and the host response and features [8].

Leishmania infection leads to specific activation of the host cellular immune response, in which macrophages that harbor the parasite play a fundamental role in infection progression. Cytokines and growth factors, including IGF-I, act on macrophage leishmanicidal or parasite growth-promoting

mechanisms. The metabolic products of L-arginine exert primary roles in these processes. Nitric oxide (NO), a main leishmanicidal element, is generated through the oxidation of L-arginine catalyzed by the inducible nitric oxide synthase (NOS2). In contrast, polyamines are essential nutrients for the growth of *Leishmania*, which are generated through the hydrolysis of L-arginine by arginase [9–11].

We began studying the role of IGF-I in *Leishmania* infection based on the observation that IGF-I is produced by different cell types, including macrophages, and is present in the skin, which is the tissue where the parasite initiates infection [12]. The addition of extrinsic recombinant IGF-I to cultures at a physiological concentration induces the proliferation of various species of *Leishmania* promastigotes and axenic amastigotes [13–15], promotes an increase in macrophage arginase-1 (ARG1) expression as well as in *Leishmania* arginase (*Larg*), *in vitro* [16, 17], and induces a significant increase in the lesion size and the number of viable parasites at the lesion site in experimental models [18]. Furthermore, macrophages contain endogenous IGF-I in the cytoplasm, which interacts with intracellular *Leishmania* parasites [19].

The initial data on the adaptive immune response in different mouse strains infected with *L. major* have shown that the susceptibility or resistance to this infection is related to the production of certain cytokines of the Th1 or Th2 profile, respectively, IFN-γ, or IL-4 and IL-13 [20–24]. Nevertheless, studies have indicated changes in the simplicity of this model with time. The production of IL-4 in resistant mice does not alter the evolution towards progressive disease, as expected. C3H mice that were treated with IL-4 or anti-IL-12 early in the infection developed a strong but transient increase in IL-4 level, with no change in their resistant phenotype [25–27]. Furthermore, the transfer of BALB/c cells with the high expression of IL-4 to genetically resistant chimeric mice on a C57BL/6 background did not result in susceptibility [28]. The findings from these studies thus suggest that the profiles of resistance and susceptibility are not exclusively due to Th1 and Th2 cytokines. Considering our previous results revealing an important role for IGF-I in the development of *Leishmania* infections and considering the differences in IGF-I expression in different strains of mice [29], IGF-I may emerge as a factor that could explain these differences. Furthermore, IFN-γ stimulation decreases IGF-I levels, whereas IL-4 and IL-13 stimulation increase IGF-I expression [4–6].

In the present work, we evaluated the role of intrinsic IGF-I in *L. major*-infected macrophages *in vitro* and its participation in modulating the effects of cytokines by silencing the expression of the *Igf-I* mRNA using a small interfering RNA. We studied the effects on parasitism and the L-arginine pathway upon cytokine stimulation. We next evaluated the effect on lesion development upon the injection of *L. major* promastigotes preincubated with recombinant IGF-I (rIGF-I) into the footpads of BALB/c and C57BL/6 mice, which differ in basal *Igf*-I mRNA amount.

Focusing on immune-endocrine cross talk, we are the first to observe a process in which a growth factor (i.e., IGF-I) acts as an effector element for the cytokine IL-4 in the induction of susceptibility to *L. major* infection since the expected parasite growth upon IL-4 stimulation was halted by silencing the IGF-I mRNA. The mechanism was directly related to the expression and activation of arginase and by the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway in macrophages. Furthermore, the injection of *L. major* promastigotes preincubated with rIGF-I into resistant C57BL/6 mice rendered the animals susceptible to the infection. The data thus suggest an essential role for IGF-I in the effect of IL-4 on the alternative macrophage activation pathway during *L. major* infection.

2. Materials and Methods

2.1. Mice. Six- to 8-week-old inbred BALB/c and C57BL/6 mice were obtained from the Animal Facility of the Faculdade de Medicina, Universidade de São Paulo (USP), and maintained in the animal facility of the Instituto de Medicina Tropical de São Paulo, USP.

2.2. Parasites. L. major LV39 (MRHO/Su/59/P) was maintained through regular passaging in BALB/c mice, and promastigotes were derived from amastigotes purified from the footpad lesions of BALB/c mice and expanded and maintained in 199 medium (Cultilab, Brazil) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Cultilab, Brazil) at 26°C. The promastigotes used in the experiments were in the stationary phase of growth and had undergone no more than four passages in culture. All procedures were approved by the institutional guidelines for animal care and use and by the ethics committee of the institution.

2.3. Evaluation of the Effect of IGF-I on Promastigotes in Culture. L. major promastigote cultures (5×10^5/mL) were established in 24-well plates (Corning Costar, USA) in 199 medium (Cultilab, Brazil) supplemented with 2% heat-inactivated fetal calf serum (FCS) (Cultilab, Brazil) at 26°C, and parasites were stimulated with or without 50 ng/mL IGF-I (R&D Systems, USA). Parasites were counted daily under a light microscope (Carl Zeiss, Gottingen, Germany), and the result was expressed as the number of parasites $\times$ 10^7/mL followed for 10 days of culture.

2.4. Infection of Macrophages with L. major. The RAW 264.7 macrophage cell line (ATCC) and BALB/c and C57BL/6 peritoneal macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% bovine serum albumin (BSA, insulin-free, Sigma, USA). Cells (5×10^5 or 2×10^6) were dispensed onto round 13 mm^2 glass cover slips, which were placed in the wells of 24-well plates (Corning Costar, USA) and incubated for 30 minutes at 37°C in a humid atmosphere with 5% CO_2 to allow adhesion. Thereafter, the wells were washed twice with culture medium to remove nonadherent cells. Then, the amastigote or promastigote suspensions (eight parasites per cell) were dispensed into the wells, and infection was allowed to occur for 4 hours at 33°C in a humid atmosphere with 5% CO_2. After incubation, the noninternalized parasites were washed away. In one set of experiments, macrophages were treated with the Th1 cytokine IFN-γ (200 U/mL) or the Th2

cytokines IL-4 (2 ng/mL) and IL-13 (5 ng/mL), both separately and in combination. In other experiments, recombinant IGF-I (50 ng/mL; rIGF-I, R&D Systems, Minneapolis, MN, USA) was added. The culture was then maintained for 48 hours at 37°C in a humid atmosphere with 5% CO_2.

2.5. Parasite Load in Macrophages. Cover slips were removed from the plates, and the slides were stained with Giemsa dyes, mounted, and processed to evaluate parasitism under a light microscope (Carl Zeiss, Germany). 600 cells per group were counted. The data are presented as the number of parasites per 100 cells [(number of parasites/number of infected cells) × (number of infected cells/total number of cells) × 100]. The analysis was performed by two independent observers who were blinded to the experimental conditions.

2.6. NO Production. Nitrite (NO_2) accumulation in the cell culture supernatants was used as an indicator of NO production and was determined using the Griess reaction [30]. Fifty microliters of the culture supernatant was reacted with 50 μL of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid in bidistilled water) for 10 min at room temperature. The absorbance was measured at 540 nm using a Multiskan MCC/340 P version 2.20 plate reader (Labsystems), and the nitrite concentration was calculated using a standard curve for sodium nitrite ($NaNO_2$). The tests were run in triplicate.

2.7. Arginase Activity. Cells and infected cells were removed from the culture, lysed, and used to determine arginase activity [31]. Briefly, 50 μL of lysates was treated with the same volume of 10 mM $MnCl_2$ and 50 mM Tris-HCl, pH of 7.4 at 56°C for 10 min to activate arginase. Then, 25 μL of 0.5 M L-arginine at a pH of 9.7 was added to 25 μL of the activated lysate and incubated at 37°C for 60 min. The reaction was stopped with 400 μL of $H_2SO_4/H_3PO_4/H_2O$ (1/3/7, *v*/*v*/*v*). The urea concentration was measured at 540 nm using a spectrophotometer Multiskan MCC/340 P version 2.20 plate reader (Labsystems, Vantaa, Finland) after the addition of 25 μL of 9% α-isonitrosopropiophenone in 100% methanol and incubation 100°C for 45 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute.

2.8. RNA Extraction and Reverse Transcription. Total RNA was extracted from 2×10^6 cells using TRIzol (Invitrogen, USA), according to the manufacturer's protocol. RNA integrity was verified by agarose gel electrophoresis/ethidium bromide staining and spectrophotometry (OD260/280 absorption ratio greater than 1.8). Total RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) following the manufacturer's protocol and then stored at −20°C.

2.9. Quantitative qPCR. For real-time quantitative qPCR, we used the corresponding primer pairs for mouse sequences (*Igf-I*: 5′ TAC TTC AAC AAG CCC ACA GG 3′ and 5′ AGT CTT GGG CAT GTC AGT GT 3′; *Arg1*: 5′ AGC ACT GAG GAA AGC TGG TC 3′ and 5′ CAG ACC GTG GGT TCT TCA CA 3′; *Larg*: 5′ CAA CAC CAT GTC TGG TAC GGT CTC 3′ and 5′ CAC AGC ACG TAG ACC AAT GTA GGC 3′; *Nos2*: 5′ AGA GCC ACA GTC CTC TTT GC 3′ and 5′ GCT CCT CTT CCA AGG TGC TT 3′; *Cat-2b*: 5′ TAT GTT GTC TCG GCA GGC TC 3′ and 5′GAA AAG CAA CCC ATC CTC CG 3′; *β-actin*: 5′ GCC TTC CTT CTT GGG TAT GGA ATC 3′ and 5′ ACG GAT GTC AAC GTC ACA CTT CAT 3′; and *GAPDH*: 5′ AAC GAG AAG TTC GGC ATA GTC GAG 3′ and 5′ ACT ATC CAC CGT CTT CTG CTT TGC 3′). Reactions including master mix (SYBR® Green; Applied Biosystems, USA), 0.3 μM of each primer, and 1 μg of cDNA templates were run in triplicate on a PCR system (StepOne; Applied Biosystems, USA). The PCR conditions were the same for all primer combinations: 95°C for 10 min and 40 cycles of 92°C for 2 min, 57.5°C for 30 s, and 70°C for 30 s. After amplification, a melting curve was used to confirm the specificity of the target product. The relative expression data were quantified using the $2^{-\Delta\Delta Ct}$ method [32].

2.10. siRNA. Small interfering RNAs (siRNAs) targeting murine IGF-I were designed using GenBank (NM_010512) and IDT SciTools RNAi Design (Integrated DNA Technologies) (*sense* 5′ AAA GGA GAA GGA AAG GAA GUA CAT T 3′ and *antisense* 5′AAU GUA CUU CCU UUC CUU CUC CUU U 3′). Universal scrambled siRNAs were employed as controls (Invitrogen, USA). Macrophages were grown in DMEM supplemented with 0.5% bovine serum albumin (insulin-free BSA, Sigma, USA) in a 24-well plate and were transiently transfected with a 150 μM siRNA duplexes using Lipofectamine™ 2000, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells transfected with IGF-I siRNA were used after 48 h. The transfection efficiency was confirmed by real-time PCR and confocal microscopy. The inhibition percentage (% KD) for the IGF-I mRNA was calculated using the formula% $KD = \left(\left[1 - 2^{-\Delta\Delta Ct}\right] \times 100\right)$ [32].

2.11. Confocal Microscopy. For immunofluorescence staining, cells infected with *L. major* promastigotes were grown on glass slides. Twenty-four hours after infection, cells were fixed with 4% paraformaldehyde, washed with 0.001 M phosphate-buffered saline (PBS; pH 7.2), blocked for one hour with 2% BSA/PBS, and then incubated overnight with a monoclonal goat anti-mouse IGF-I antibody (1 : 75, R&D Systems, USA) and a polyclonal mouse anti-*Leishmania* antibody (1 : 400) produced in our laboratory. Alex Fluor 546-conjugated donkey anti-goat IgG (1 : 200, Invitrogen, USA) and Alexa Fluor 488-conjugated donkey anti-mouse IgG (1 : 400, Invitrogen, USA) were used as secondary antibodies. Fluorescence imaging analyses were performed using a Zeiss LSM 510 Meta laser-scanning confocal microscope. As a negative control, the primary antibodies were omitted from the reaction.

2.12. SDS-PAGE and Western Blot. Cell lysates (20 μg of protein in 20 μL) were separated on a denaturing gradient 4–12% Bis-Tris NuPAGE gel (Invitrogen, USA) according to the

manufacturer's instructions. Briefly, the separated proteins were blotted onto iBlot™ 2 Transfer Stack PVDF mini-membranes using an iBlot Dry Blotting System (Invitrogen, USA). Membranes were blocked with TBS-T buffer (150 mM NaCl, 20 mM Tris, 0.01% Tween 20, pH of 7.4) containing 5% fat-free milk for 1 h. Membranes were reacted with a 1 : 2000 dilution of a primary anti-phospho-Akt (Ser473) antibody, a 1 : 2000 dilution of a rabbit anti-p44/42 MAPK (137F5) mAb, and a 1 : 2000 dilution of a rabbit anti-p38 MAPK (D13E1) XP® mAb (Cell Signaling Technology, USA) overnight at 4°C and incubated with 1 : 1000 dilutions of a peroxidase-conjugated polyclonal anti-rabbit IgG and HRP-conjugated anti-biotin antibody (Cell Signaling Technology, USA) for 1 h at room temperature. Biotinylated protein molecular weight markers (Cell Signaling Technology, USA) were used. Bound antibodies were detected with an ECL chemiluminescence kit LumiGLO® Reagent (Cell Signaling Technology, USA) according to the manufacturer's instructions. An evaluation of the phosphorylation kinetics was conducted at different time points (0, 5, 10, 20, 30, and 60 minutes) after adding the stimulus (data not shown), and we chose the time of 30 minutes to present the results. Subsequently, protein bands were quantified by densitometry using AlphaEaseFC™ software 3.2 beta version (Alpha Innotech Corporation, San Leandro, CA, USA), and the results are expressed in arbitrary units, which were calculated by integrating the intensity of each pixel over the spot area and normalizing to the gel background.

2.13. Development of Footpad Lesions in Mice. Stationary-phase promastigotes (1×10^6) that had been preincubated with or without 50 ng/mL of recombinant human IGF-I (R&D Systems, USA) for 5 min and then washed were injected into the footpads of BALB/c and C57BL/6 mice. In the opposite footpad, we injected RPMI 1640 medium as a control. For six weeks, we measured the thickness of the foot to indicate the growth of the lesions using a micrometer (Mitsutoyo, Japan), and the difference between the infected and noninfected footpads in millimeters was considered the lesion size.

2.14. Statistical Analysis. Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Data were subjected to analysis of variance (ANOVA) and Tukey's posttest and were considered significant when $P < 0.05$.

3. Results

3.1. The Effect of IGF-I on L. major in Culture and within Macrophages. We first verified the effect of IGF-I on parasite growth *in vitro*. In the cultures stimulated with rIGF-I, we observed a larger number of parasites that reached the stationary phase earlier than nonstimulated cultures (Figure 1(a)).

When we evaluated the effect of IGF-I on the parasite burden in *L. major* promastigote- or amastigote-infected macrophages, we observed a significant increase in the number of parasites in macrophages stimulated with rIGF-I compared with that in nonstimulated macrophages (Figure 1(b)). Similar results were observed when amastigotes were used (Figure 1(c)).

3.2. The Effects of Leishmania Infection and Th1 and Th2 Cytokines on IGF-I Expression. We analyzed the effects of *Leishmania* infection and Th1 and Th2 cytokines on the amount of the *Igf-I* mRNA. Infection of RAW 264.7 cells and BALB/c mouse peritoneal macrophages with promastigotes decreased the amount of *Igf-I* mRNA compared with that of the uninfected control cells. When promastigote-infected RAW 264.7 cells were stimulated with IFN-γ, we observed a 15-fold decrease in the amount of *Igf-I* mRNA. When cells were simultaneously stimulated with IL-4 and IL-13, we observed a 2.45-fold increase in the amount of *Igf-I* mRNA compared to that in the nonstimulated controls. When cells were stimulated with either IL-4 or IL-13, we observed a 2.9- or 4.8-fold increase in the amount of *Igf-I* mRNA, respectively (Figure 2(a)).

Upon amastigote infection of RAW 264.7 cells, we observed different amounts of *Igf-I* mRNA that increased in all infected cells compared with that in uninfected controls. A 2.2-fold increase in the amount of *Igf-I* mRNA was observed upon amastigote infection, a 1.2-fold increase was observed following stimulation with IFN-γ, and an 8.7-fold increase was observed when cells were simultaneously stimulated with IL-4 and IL-13. When cells were stimulated with either IL-4 or IL-13, we observed a 9.2- or 2.5-fold increase in the amount of *Igf-I* mRNA, respectively (Figure 2(a)). A similar profile was observed in BALB/c peritoneal macrophages (Figure 2(b)).

We confirmed these results using confocal microscopy. In *Leishmania* promastigote-infected macrophages, we observed a decrease in IGF-I immunostaining upon stimulation with IFN-γ compared with that in control cells. When cells were simultaneously stimulated with IL-4 and IL-13 or with IL-4 alone, we observed an increase in immunostaining, indicating increased IGF-I expression (Figure 2(c)).

3.3. Inhibition of IGF-I Expression Using an IGF-I siRNA. Silencing IGF-I expression with siRNA resulted in an approximate 70% decrease in the mRNA amount in promastigote-infected RAW 264.7 cells (Figure 3(a)) and 78% in infected BALB/c peritoneal macrophages (Figure 3(b)). Using confocal microscopy, we observed a significant reduction in IGF-I immunostaining after siRNA transfection. Moreover, infection with *L. major* promastigotes alone inhibited IGF-I expression. A nonspecific double-stranded RNA (scrambled siRNA) was used as a negative control (Figure 3(c)). We observed similar results in cells infected with amastigotes (data not shown).

3.4. The Effect of IGF-I siRNA on Parasitism. Following infection with both promastigotes and amastigotes, we observed a significant increase in parasitism in cells exposed to Th2 cytokines ($P < 0.05$), as expected. In Th1 cytokine-stimulated cells, we noted a significant reduction in parasitism compared with that in the respective control ($P < 0.05$) (Figure 4).

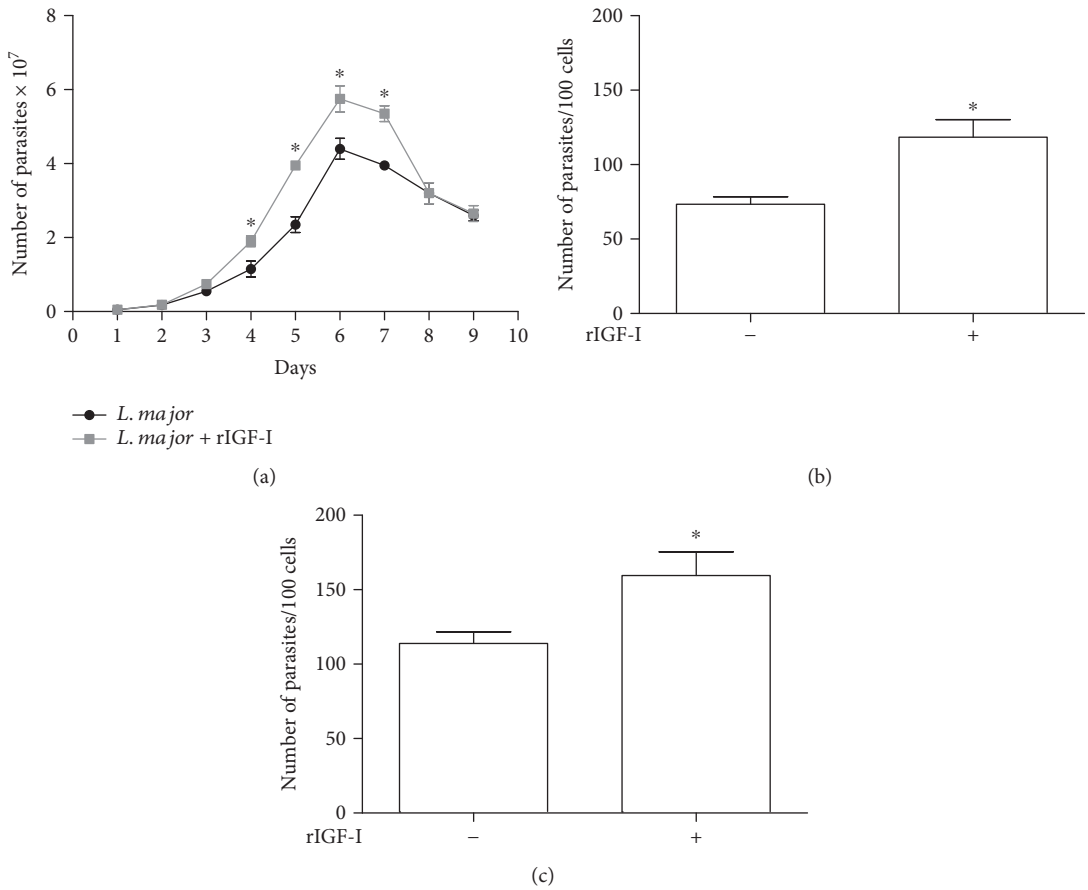


Figure 1: The effects of IGF-I on the growth of *L. major* in culture and within macrophages (mean ± standard deviation). (a) The effect of IGF-I on the growth of *Leishmania* promastigotes in culture maintained in 199 medium supplemented with 2% heat-inactivated FCS at 26°C is shown. *Leishmania* promastigotes were maintained with (gray line) or without (black line) 50 ng/mL rIGF-I. (b and c) The effect of IGF-I on the growth of *Leishmania* within macrophages is shown. RAW 264.7 cells were infected with *L. major* promastigotes (b) or amastigotes (c) treated with or without 50 ng/mL rIGF-I and incubated for 48 h. $^*P < 0.05$ (one-way ANOVA and Student's t test) compared to the control without rIGF-I.

Upon silencing IGF-I expression with an siRNA in *L. major* promastigote-infected RAW 264.7 cells, we observed a decrease in the parasite number from 79 parasites (median number in the control) to 57 parasites per 100 cells. In amastigote-infected cells, the parasite number was reduced from 139 to 91 parasites per 100 cells ($P < 0.05$). When we analyzed the involvement of Th1 and Th2 cytokines in parasitism following IGF-I silencing, all cells transfected with the siRNA showed a significant decrease in parasitism compared with that in the controls lacking siRNA following infection with both promastigotes and amastigotes (Figures 4(a) and 4(b)). The parasite load did not increase, even in Th2 cytokine-stimulated cells.

We observed similar results in *L. major*-infected BALB/c peritoneal macrophages to those obtained in *L. major*-infected RAW 264.7 cells, reinforcing the findings obtained from the RAW 264.7 cells (Figures 4(c) and 4(d)).

Experiments were performed to restore IGF-I activity after knockdown to ascertain the role of IGF-I. After transfection with siRNA, rIGF-I (50 ng/mL) was added to replace the loss of intracellular IGF-I, followed by an evaluation of parasitism. After the addition of rIGF-I to all cells transfected with siRNA, parasite numbers increased to the levels similar to the control cells lacking siRNA (Figure 4). We did not observe differences in parasitism in control cells treated with Lipofectamine or transfected with the scrambled siRNA compared with that in control cells. Infections with amastigotes or promastigotes produced similar results (Figures 4(e) and 4(f)).

3.5. The Effects of IGF-I siRNA on the mRNA Amount of Nos2, Arginase (Arg1), and Cationic Amino Acid Transporter 2 (Cat-2B) in Macrophages and Leishmania Arginase (Larg) mRNA Expression and Enzyme Activity. We analyzed the effects of IFN-γ and IL-4 and/or IL-13 in combination with levels of IGF-I on L-arginine metabolism. IL-4 stimulation produced a significant increase in both *Arg1* (Figure 5(a)) and *Larg* mRNAs (Figure 5(b)), as well as an increase in arginase activity (Figure 5(d)). However, IL-13 stimulation did not show an increase in *Arg1* mRNA or arginase activity (Figures 5(a) and 5(d)) but showed an increase in *Larg* mRNA (Figure 5(b)). A significant increase in the amount of *Cat-2B* mRNA (Figure 5(c)) was observed after treatment with IL-4 ($P < 0.05$) compared to that in the control group.

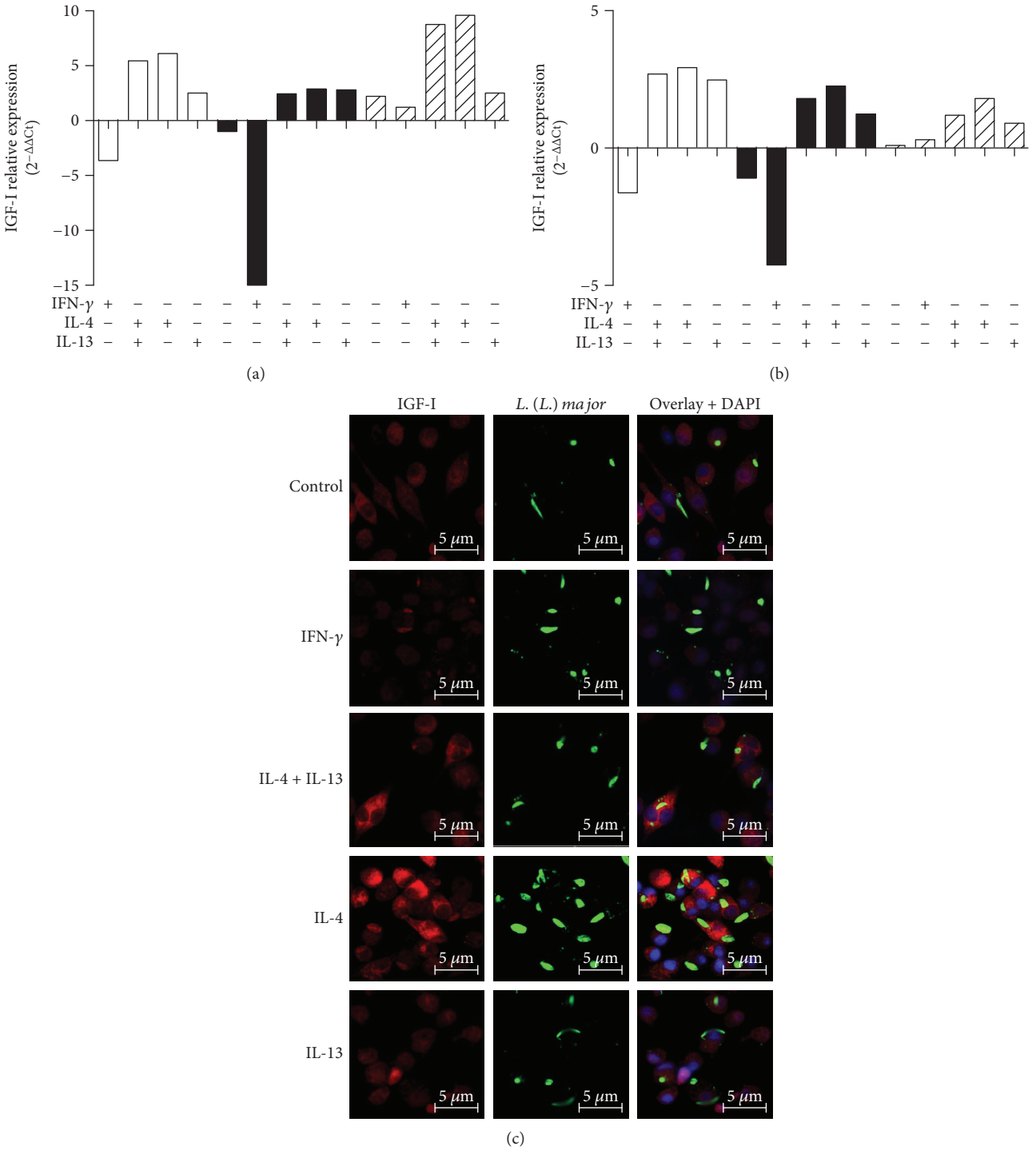


Figure 2: IGF-I expression in response to cytokine treatment. (a and b) Quantification of *Igf-I* mRNA in *L. major* promastigote- (black bars) or amastigote-infected (striped bars) or noninfected (white bars) RAW 264.7 cells (a) and BALB/c peritoneal macrophages (b) is shown. Cells were stimulated with IFN-γ (200 U/mL), IL-4 (2 ng/mL), and IL-13 (5 ng/mL) for 48 hours. One representative experiment from three independent assays is shown. (c) Detection of IGF-I expression using confocal microscopy of cells labeled with a 1 : 75 dilution of an anti-IGF-I antibody (using an Alexa Fluor 546-conjugated secondary antibody; red) and a 1 : 200 dilution of an anti-*Leishmania* antibody (using an Alexa Fluor 488-conjugated secondary antibody; green) in *L. major* promastigote-infected macrophages is shown. Nuclei were stained with DAPI (blue). Images were captured using a confocal Leica LSM510 microscope with a 63x oil immersion objective. The expressions are relative to the expression in untreated cells (the baseline).

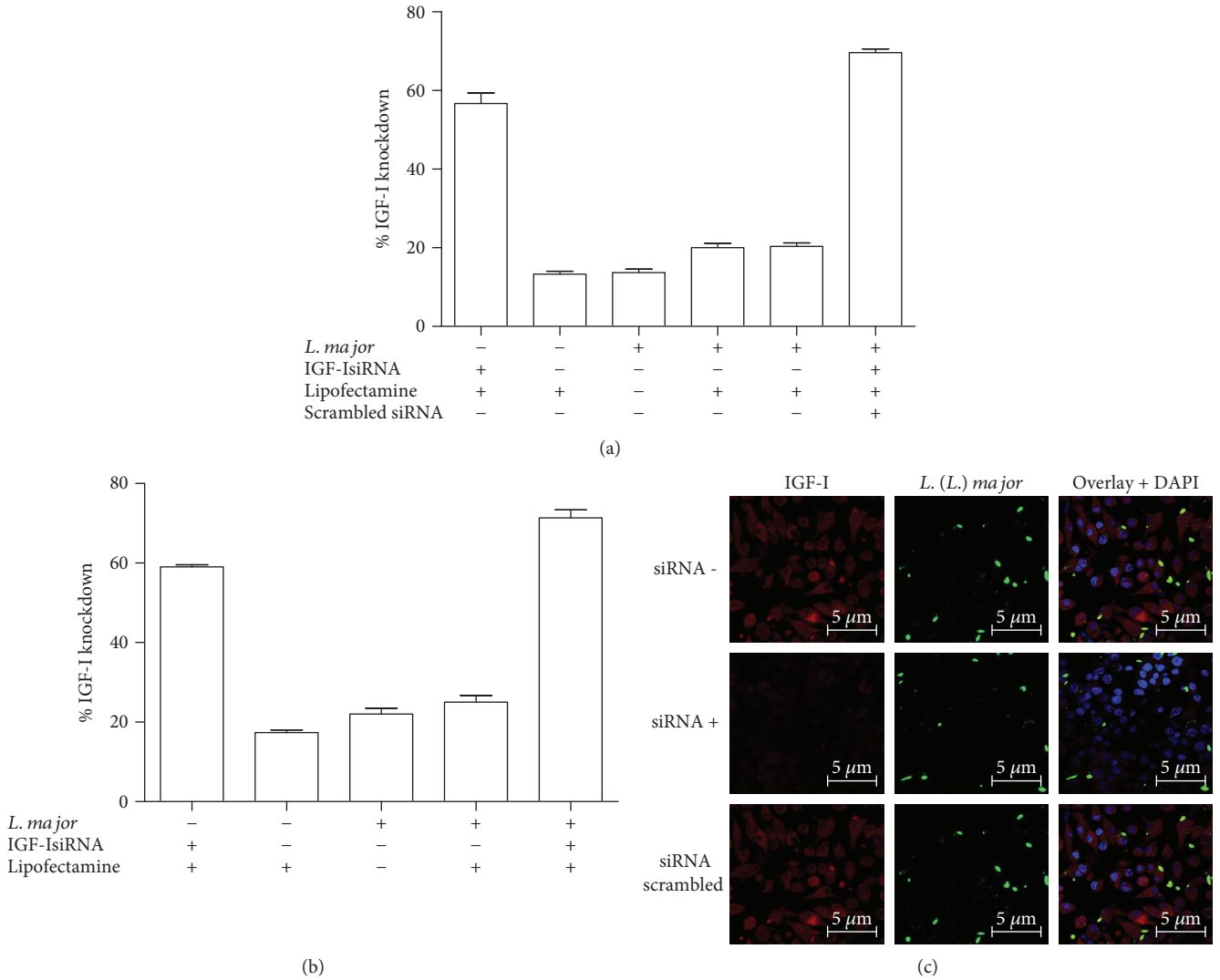


Figure 3: Expression of *Igf-I* mRNA upon IGF-I silencing with an siRNA. The percentage decrease in the amount of *Igf-I* mRNA in RAW 264.7 cells (a) or BALB/c peritoneal macrophages (b) infected with *L. major* promastigotes that were transfected with 150 μM siRNA, scrambled siRNA, or Lipofectamine alone 6 h after infection is shown. One representative experiment from three independent assays is shown. (c) The detection of IGF-I expression in *L. major* promastigote-infected RAW 264.7 cells transfected with (siRNA+) or without the IGF-I siRNA (siRNA−) or with a scrambled siRNA using confocal microscopy of immunostaining with a 1 : 75 dilution of an anti-IGF-I antibody (using an Alexa Fluor 546-conjugated secondary antibody; red) and a 1 : 200 dilution of an anti-*Leishmania* antibody (using an Alexa Fluor 488-conjugated secondary antibody; green) is shown. Nuclei were stained with DAPI (blue). Images were captured using a confocal Leica LSM510 microscope with a 63x oil immersion objective.

When cells were treated with siRNA, all groups showed a significant decrease in both the *Arg1* and *Larg* mRNA amounts and arginase activity and an increase in the *Cat-2B* mRNA amount (Figure 5(c)).

When IGF-I was restored by adding rIGF-I to cells transfected with siRNA, we observed increases in both the *Larg* mRNA amount and arginase activity. No differences in the *Arg1* mRNA amount were observed in macrophages.

In another branch of the L-arginine metabolic pathway, we observed a decrease in the *Nos2* mRNA amount in all groups treated with rIGF-I compared with the respective control group. In the group that was not stimulated with cytokines, we observed an increase in the *Nos2* mRNA amount after siRNA transfection. Upon the addition of Th2 cytokines, an alteration in NO production was not observed (Figures 5(e) and 5(f)). Similar results were obtained using amastigotes and BALB/c peritoneal macrophages (data not shown).

3.6. Evaluation of the Effects of siRNA and IL-4 on IGF-I Signaling Pathways. Because our results suggest that IGF-I is necessary for IL-4 to exert its effect on parasite growth in macrophages, we examined their intracellular signaling pathways. IGF-I and the cytokines IL-4 and IL-13 share common components in their signaling pathways. IGF-I triggers MAPK (ERK) and PI3K pathways [33, 34], and IL-4

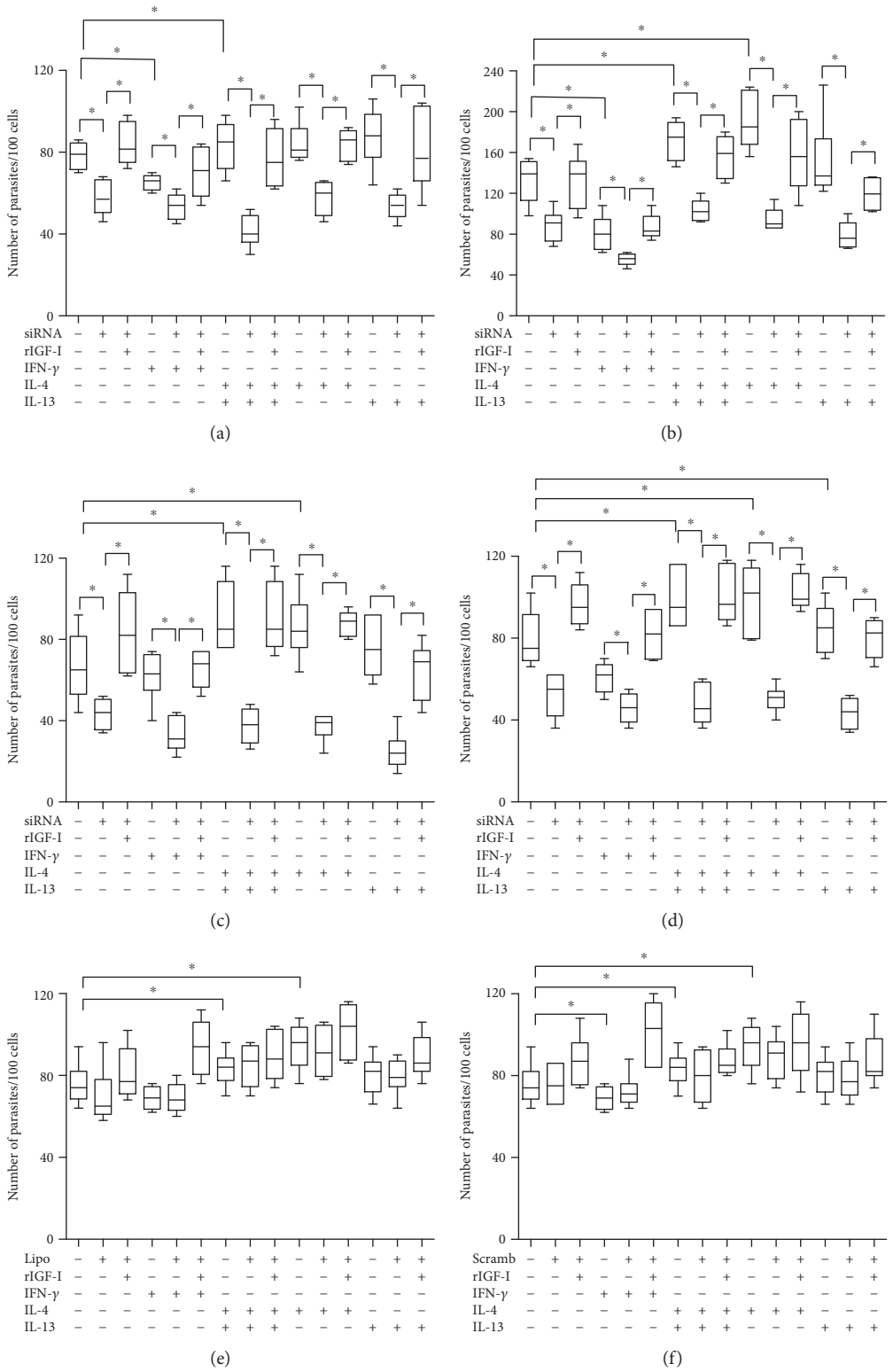


Figure 4: Parasitism in response to cytokine treatments and siRNA transfection. Parasitism (median number of parasites per 100 cells) in *L. major*-infected RAW 264.7 cells (a, b, e, and f) or BALB/c peritoneal macrophages (c and d) following transfection with IGF-I siRNA (a, b, c, and d), Lipofectamine alone (e), or scrambled siRNA (f) along with cytokine stimulation is shown. Promastigote- (a, c, e, and f) or amastigote-infected (b and d) cells transfected with or without siRNA or Lipofectamine were stimulated with IFN-γ (200 U/mL), IL-4 (2 ng/mL), IL-13 (5 ng/mL), and recombinant IGF-I (rIGF-I, 50 ng/mL) for 48 hours. One representative experiment from three independent assays is shown. $^*P < 0.05$ (ANOVA and Tukey's tests).

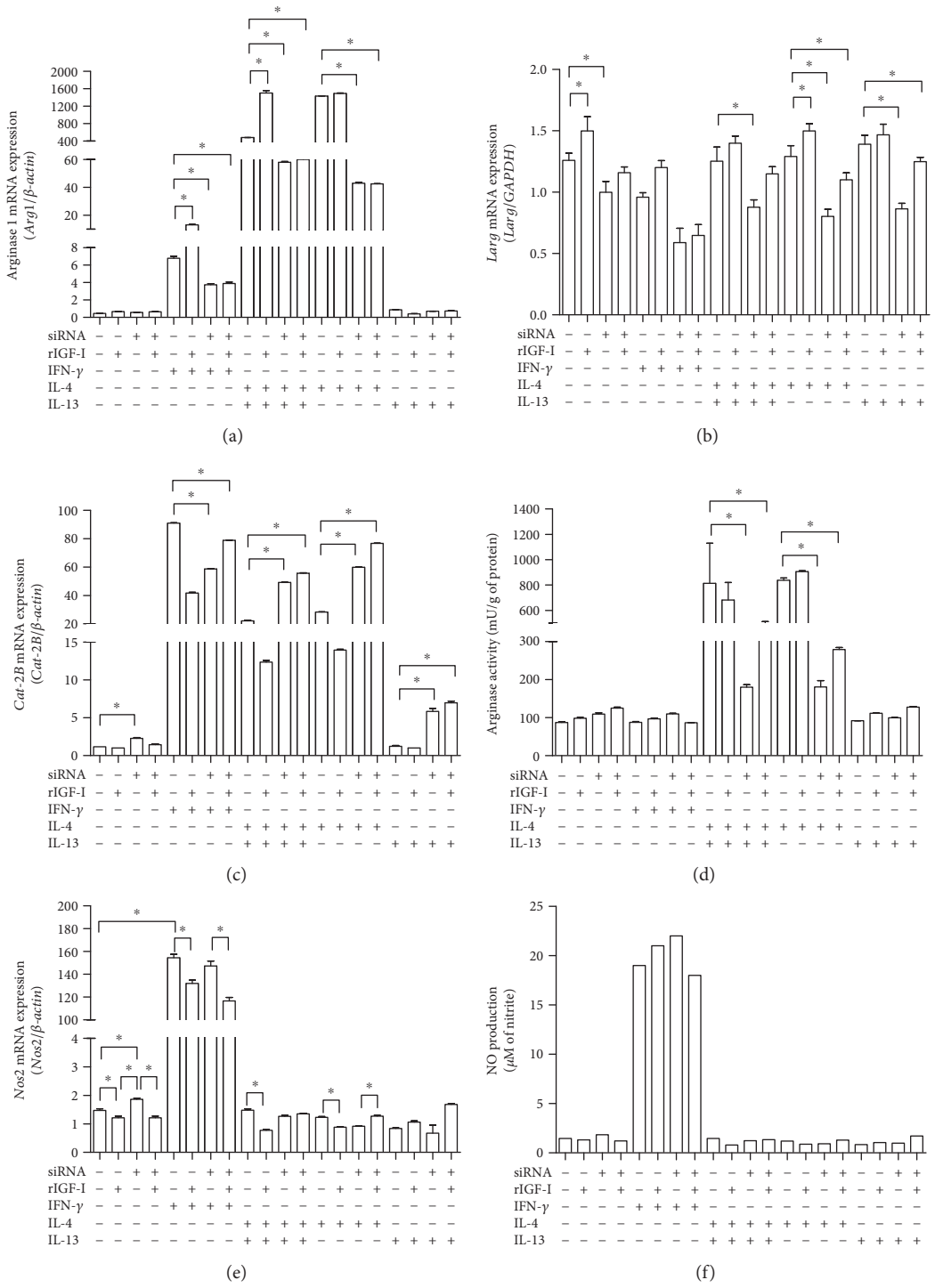


Figure 5: The effects of IGF-I siRNA transfection on the L-arginine metabolic pathway. The quantification of (a) *Arg1* mRNA, (b) *Larg* mRNA, (c) *Cat-2B* mRNA, (d) arginase activity, (e) *Nos2* mRNA, and (f) NO production in *L. major*-infected RAW 264.7 cells following transfection with IGF-I siRNA and cytokine stimulation is shown. Promastigote-infected cells transfected with or without IGF-I siRNA were stimulated with IFN-γ (200 U/mL), IL-4 (2 ng/mL), and IL-13 (5 ng/mL) for 48 hours. One representative experiment from three independent assays is shown. $^*P < 0.05$ (ANOVA and Tukey's tests) compared to the respective control without siRNA.

sequentially activates IRS-2 and the PI3K/Akt and Ras-MAPK pathways [35, 36] (Figure 6). The Jak-Stat pathway is also known to be triggered by IL-4.

We analyzed the key components of the IGF-I signaling pathway, that is, p44 (ERK), p38 (MAPK), and total and phosphorylated AKT, upon stimulation with IL-4 and IL-4 plus IL-13 after IGF-I silencing. We did not observe any difference in the total protein levels across all groups (data not shown); however, differences in the levels of phosphorylated proteins were observed.

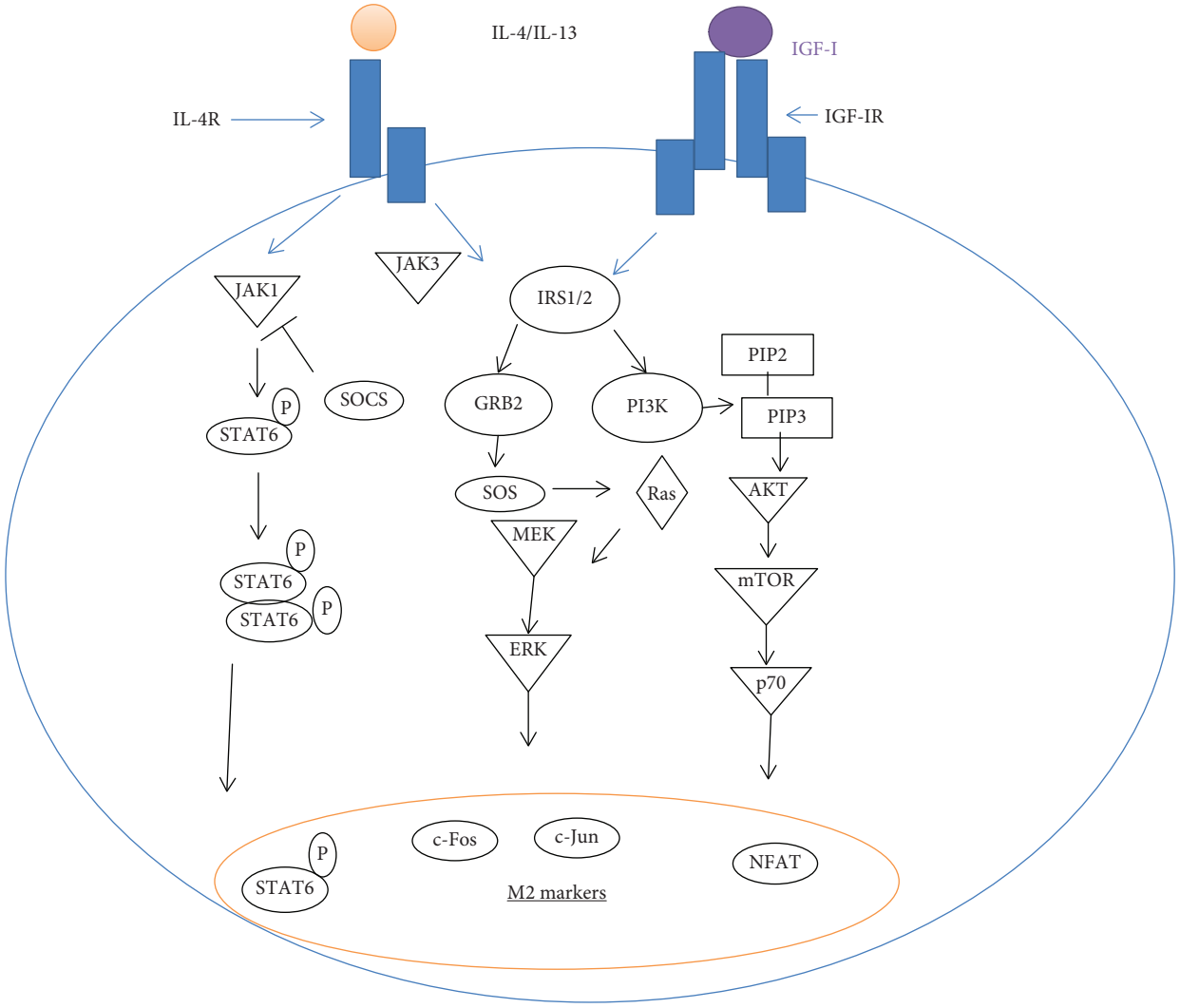


Figure 6: Scheme of the common components of the IGF-I and IL-4 signaling pathways.

Cells infected with *L. major* displayed a decrease in the levels of phospho-p44 (Figure 7(a)) and phospho-p38 (Figure 7(d)), but a difference in the phospho-AKT level was not observed (Figure 7(g)) compared with that in the noninfected RAW cells. We observed an increase in the levels of these phosphorylated proteins in all groups treated with rIGF-I compared with noninfected RAW cells. A similar increase was also observed with IL-4.

Upon silencing IGF-I expression using siRNA, all groups showed a decrease in the expression of all phosphoproteins. In IGF-I-silenced cells, Th2 cytokine stimulation did not restore the decreased expression of phospho-p44, phospho-p38, and phospho-AKT.

3.7. IGF-I Expression and the Effect of IGF-I on Lesion Development in L. major-Susceptible and -Resistant Mouse Strains. We initially evaluated the differences in the expression of IGF-I to determine whether the susceptibility of BALB/c mice and the resistance of C57BL/6 mice to *L. major* infection were related to IGF-I expression. The *Igf-I* mRNA was detected in higher levels in BALB/c cells than in C57BL/6 cells ($P < 0.05$) (Figure 8(a)). Confocal microscopy indicated a correlation between the amount of *Igf-I* mRNA and IGF-I expression, confirming that the C57BL/6 peritoneal macrophages showed less IGF-I immunostaining than BALB/c cells did (Figure 8(b)).

Then, we analyzed the effect of rIGF-I on lesion development in *L. major*-infected BALB/c and C57BL/6 mice. Parasites were preincubated with 50 ng/mL IGF-I for five minutes and then washed and injected into the footpads of the mice. In control BALB/c mice, the lesions progressed continuously, but when the parasites were preincubated with IGF-I, we observed a significantly greater lesion volume than that observed in the control mice (Figure 8(c)). In control C57BL/6 mice, the lesions progressed for three weeks and then stabilized and tended to diminish, but animals infected with parasites that were preincubated with IGF-I displayed significantly greater lesions, which, interestingly, progressed continuously, similar to that in the BALB/c mice (Figure 8(d)).

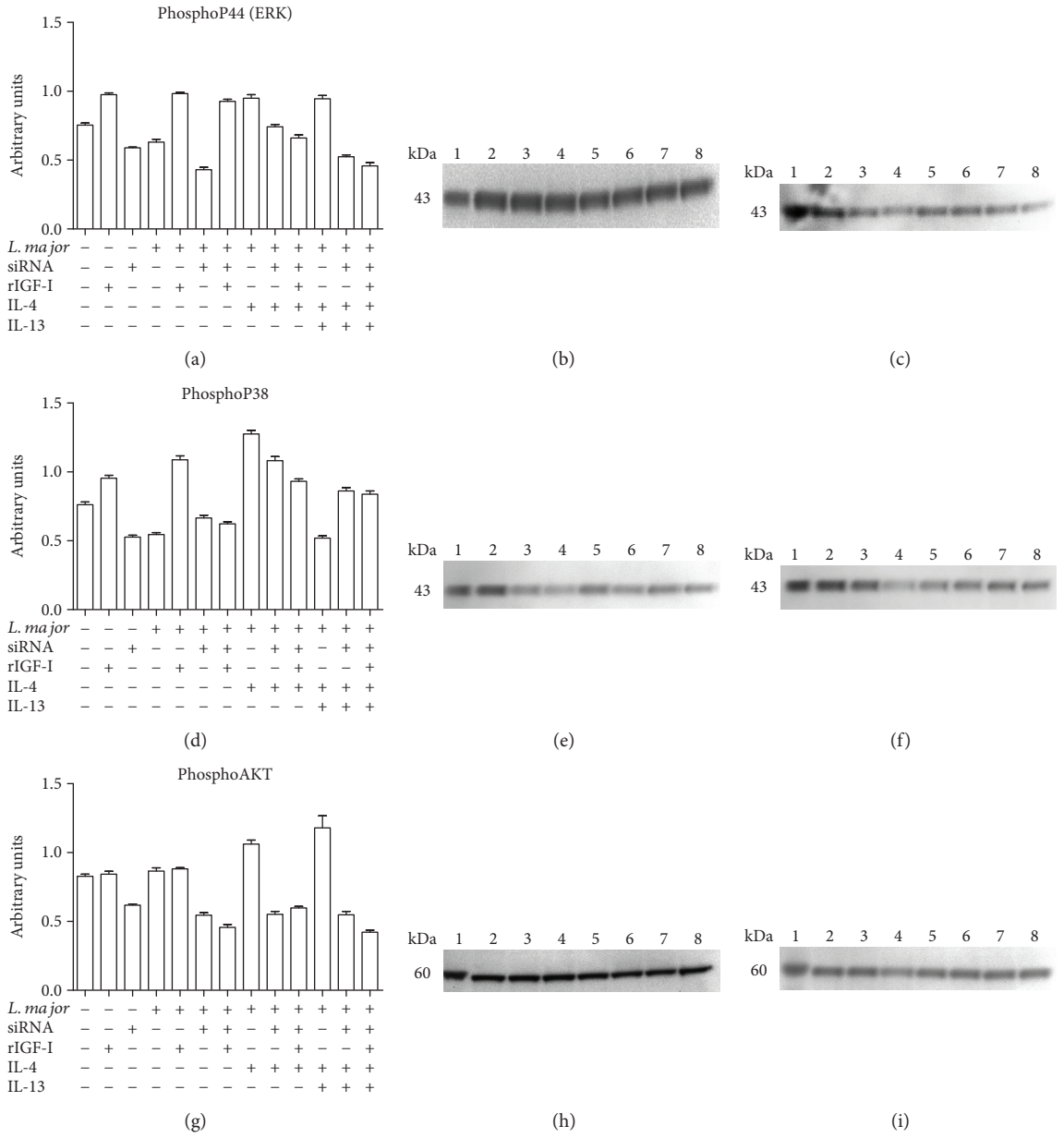


Figure 7: The effects of siRNA and IL-4 on components of the IGF-I signaling pathways: levels of phosphorylated p44 (ERK), p38 (MAPK), and AKT proteins. Promastigote-infected or noninfected cells transfected with or without IGF-I siRNA were stimulated for 30 minutes with IL-4 (2 ng/mL) and IL-13 (5 ng/mL). Cells were lysed, proteins were separated by 10% SDS-PAGE, and subsequently, Western blotting was performed using anti-phospho-p44 (a, b, and c), anti-phospho-p38 (d, e, and f), and anti-phospho-AKT (g, h, and i) antibodies. Protein bands corresponding to protein expression levels were subject to a densitometric analysis, and the data are expressed in arbitrary units (a, d, and g). A representative blot is shown. (b, e, h) The lanes represented the following: 1: control; 2: RAW; 3: RAW + rIGF; 4: RAW + siRNA; 5: RAW + Lm; 6: RAW + Lm + rIGF; 7: RAW + Lm + IL-4; and 8: RAW + Lm + IL-4 + IL-13. (c, f, i): 1: control; 2: RAW; 3: RAW + Lm + siRNA; 4: RAW + Lm + siRNA + rIGF; 5: RAW + Lm + siRNA + IL-4; 6: RAW + Lm + siRNA + IL-4 + rIGF; 7: RAW + Lm + siRNA + IL-4 + IL-13; and 8: RAW + Lm + siRNA + IL-4 + IL-13 + rIGF. See Materials and Methods for additional details.

4. Discussion

Due to the known roles of Th1 and Th2 cytokines in the resistance and susceptibility of certain inbred mouse strains to *L. major* infection and their effects on the expression of the hormone IGF-I, which has an important impact on *Leishmania* growth within host macrophages, we examined the effect of interference in the hormone IGF-I expression on the adaptive immune response. We initially analyzed the effects of Th1 and Th2 cytokines on the amount of *Igf-I* mRNA and confirmed that IGF-I expression was decreased by IFN-γ and increased by IL-4 and IL-13, which was consistent with previous results [4, 6]. Furthermore, when we concomitantly analyzed IGF-I expression and the parasitism of *L. major* in RAW 264.7 cells or BALB/c peritoneal macrophages *in vitro*, we observed that the macrophages stimulated

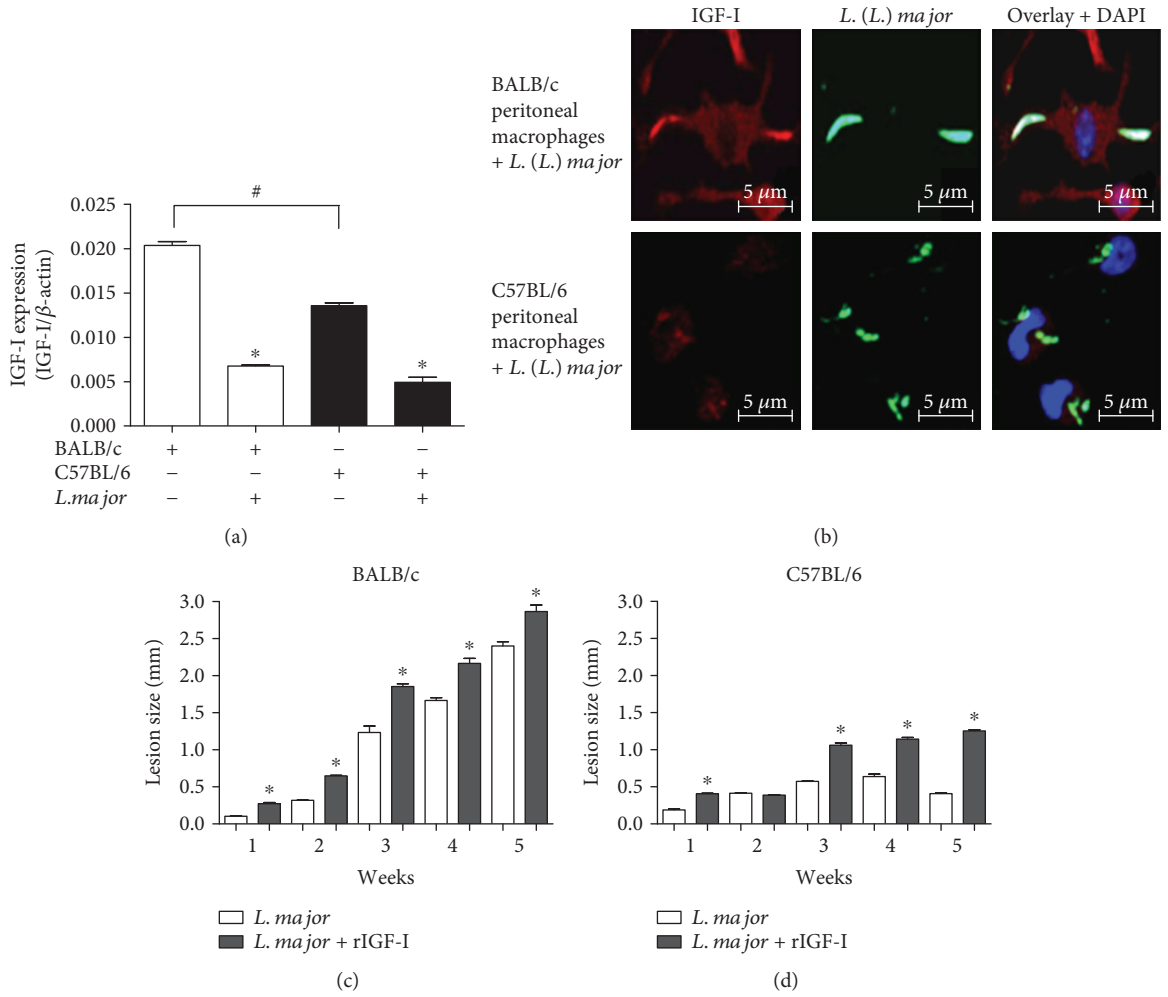


Figure 8: IGF-I expression and the effect of IGF-I on lesion development in *L. major*-susceptible and -resistant mouse strains. (a) The ratio of the *Igf-I* mRNA amount to *β-actin* mRNA amount in *L. major*-infected BALB/c (white bars) and C57BL/6 peritoneal macrophages (black bars) is shown. (b) Confocal microscopy was used to detect IGF-I expression via immunostaining with a 1 : 75 dilution of an anti-IGF-I antibody (using an Alexa Fluor 546-conjugated secondary antibody; red) and a 1 : 200 dilution of an anti-*Leishmania* antibody (using an Alexa Fluor 488-conjugated secondary antibody; green). DAPI (blue) was used to stain the nuclei. Images were captured using a confocal Leica LSM510 microscope with a 63x oil immersion objective. (c and d) Stationary-phase promastigotes (10^6) that were preincubated with or without recombinant IGF-I (50 ng/mL) for 5 min were injected into the footpads of BALB/c and C57BL/6 mice, and lesion development was measured for six weeks. One representative experiment from three independent assays is shown. $^*P < 0.05$ (ANOVA and Tukey's tests) compared to the respective controls. $^\#P < 0.05$ (ANOVA and Tukey's tests) between BALB/c and C57BL/6.

with IFN-γ exhibited a reduction in the parasite load, accompanied by a parallel reduction in IGF-I expression and ARG1 activity and an increase in NO production. Furthermore, IL-4 and IL-13 stimulation increased parasitism, accompanied by a parallel increase in IGF-I expression and arginase activity and a reduction in NO production.

These data showing the parallel effects of those cytokines and the expression of IGF-I on parasitism compelled us to explore the interference/participation of this hormone in the actions of cytokines and other factors during *Leishmania* infection.

The idea of immune-endocrine cross talk is not new and has been described in studies examining the roles of prolactin [37], growth hormone (GH) [38, 39], IGF-I, and thyroid-stimulating hormone in the development, maintenance, and function of the immune system, which in turn cause reciprocal changes in the endocrine system [5, 40].

IGF-I exhibits pleiotropic properties, including the ability to promote cellular proliferation, differentiation, nutrient transport, energy storage, gene transcription, protein synthesis, and activation of the immune response and inflammation [12, 41]. However, its specific role in the adaptive immune response is not known. Thus, we addressed this aspect in the present study using a knockdown strategy in RAW 264.7 and mouse peritoneal cells infected with *L. major* knowing that a significant amount of IGF-I colocalizes with *L. major* in the cytoplasm of *Leishmania*-infected RAW 264.7 cells.

Using an IGF-I siRNA, we silenced the *Igf-I* mRNA in macrophages and evaluated parasitism. We observed a

significant decrease in parasitism in the siRNA-transfected group compared with that in the control group without siRNA transfection in response to both promastigote and amastigote infections. This effect was reversed by the addition of rIGF-I, which induced an increase in the number of parasites, even when siRNA-mediated knockdown was maintained. This restoration observed upon the addition of recombinant IGF-I was likely induced by an increase in the levels of *Larg* mRNA and *Larg* activity, accompanied by a slight decrease in the *Nos-2* mRNA amount and NO production. Similar results were previously reported in which the addition of rIGF-I induced alternative activation of macrophages and arginase activation in *L. amazonensis*-infected macrophages [16, 17]. The present data definitively confirm the role of intrinsic IGF-I in intracellular parasite growth. Preliminary data further showed that the IGF-I siRNA decreased the parasite load in *Leishmania major*-infected BALB/c mouse footpad lesions (data not shown).

After observing such a clear-cut effect of IGF-I on parasite growth in macrophages, we proceeded to explore its role in infection development in *L. major*-infected macrophages stimulated with cytokines. After silencing *Igf-I* mRNA expression, the parasitism observed upon stimulation of the cells with cytokines did not follow the expected profile. IL-4 and IL-13 should have increased parasitism; however, they were completely ineffective when the *Igf-I* mRNA was silenced. Analyzing mRNA for components of L-arginine metabolism, we observed that the effect of IL-4 on infection progression was via the increase of *Arg1* and *Larg* mRNAs, while the effect of IL-13 on infection progression was only via the increase in *Larg* mRNA.

The effects of IL-4 and IL-13 were restored by the administration of rIGF-I to IGF-I-silenced cells in a mechanism dependent on *Leishmania* arginase production, but not by ARG1. In another study using *L. amazonensis*-infected macrophages, a novel L-arginine usage pathway independent of macrophage NOS2 and ARG1 activation was involved in the response, suggesting that *Leishmania* may have the ability to obtain access to the intracellular L-arginine pool while residing within the host macrophage [42, 43]. This evidence was confirmed in the present study, as we observed an increase in parasitism upon the addition of rIGF-I in silenced cells without changes in the amount of *Arg1* mRNA but an increase in the amount of *L*arg mRNA. Other studies have supported the importance of arginase production by the parasite since in vitro cultured *L. donovani* is capable of directly acquiring L-arginine from the medium and producing urea [44] or the deficiency in the infection and replication capabilities in *L. amazonensis* arginase knockout [45].

L-Arginine is supplied from the extracellular milieu by the cationic amino acid transporter 2 (CAT-2B), a member of the classical amino acid cationic transporter system y+ (SLC7) [42]. The observed increase in NOS2 expression may be related to an increase in CAT-2B expression since CAT-2B is required for the regulation of NOS2 activity that conversely may modulate CAT2-B expression through NO production [46–49].

The production of NO does not always follow the effect of the cytokines on parasitism, as the control of *L. major* parasitic infections does not appear to be related to NO production but rather to the presence or absence of IGF-I. Based on these results, cytokines alone are not sufficient to induce or control parasite growth, and IGF-I plays a crucial role in this process. We postulate that IGF-I may be an effector element of IL-4, which to the best of our knowledge constitutes the first evidence of cross talk between this hormone and the adaptive immune system in *L. major* infection.

Immune-endocrine cross talk involving IGF-I has been observed by others to mainly involve inflammatory cytokines IL-1 and TNF-α, which may inhibit both the expression of IGF-I or IGF-IR and the IGF-I-induced tyrosine phosphorylation of IRS-1 and IRS-2. This is an effect that is mediated by receptor cross talk and leads to intracellularly mediated IGF resistance [5]. Thus, these findings fundamentally differ from the findings of the present study showing that IGF-I plays a fundamental role as an effector element of Th2 cytokines.

The interaction of the endocrine and immune systems is somewhat expected, as they share several ligands and receptors in their signaling pathways. By analyzing the signaling pathways of IL-4/IL-13, which are related to the susceptibility to infection, we noticed shared components with the IGF-I pathway.

The biological effects of IGF-I occur through its binding to its receptor (IGF-IR), which is present in several cell types and tissues, mainly in macrophages. IGF-I signaling consists of two main pathways, the PI3K/Akt and mitogen-activated protein kinase (Ras/MAPK/ERK) pathways, when IRS-1 associates with the GRB2/SOS complex. IGF-I can also bind to insulin receptor (IR) when some excess of free IGF-I is present in the system that could have occurred in the present study but with lower affinity. IGF-IR and IR are highly homologous tyrosine kinase receptors sharing many common steps, inducing IRS1/2 phosphorylation and also AKT and MAPK [34, 50, 51]. Thus, if some IGF-I binds to IR, the resulted signaling will be nearly the same generating similar biological effects.

IL-4 mediates its effects through two receptors: the type I IL-4 receptor (IL-4Rα and IL-2Rγ) and the type II receptor (IL-4Rα and IL-13Rα1). Activation of these receptors results in STAT-6 phosphorylation that is necessary for the induction of the alternative macrophage pathway (M2). The ligation of the type I IL-4R activates JAK3 that participates in the activation of Akt, resulting in the induction of the M2 macrophage phenotype characterized by upregulation of molecules such as the mannose receptor, ARG1, and chitinase 3-like 3 [52]. IGF-I may also increase STAT-6 activity through IL-4, which is required to activate the expression of this cytokine [53].

In the present work, our analysis of the components of the IGF-I signaling pathway revealed increases in the amount of arginase mRNA, the phosphorylation of p44, p38, and Akt and parasitism in all groups treated either with rIGF-I or with IL-4. These results corroborated previous studies showing that IL-4 upregulates Akt activation and subsequently activates the alternative macrophage profile [54].

In the present study of *L. major* infection, we observed that all groups in which IGF-I expression was silenced using an siRNA, even IL-4-stimulated cells, showed decreases in

both macrophage and parasite arginase mRNA amounts and decreases in the levels of all evaluated phosphorylated proteins. A decrease in parasitism accompanied these results, reinforcing the importance of IGF-I in the signaling induced by IL-4. In the literature, a similar study using an anti-IGF-I antibody or PI3K inhibitor in bone marrow-derived macrophages showed that IGF-I inhibition attenuated the IL-4-induced increases in the mRNA amount of M2 markers, such as mannose receptor, *Arg1* mRNA and chitinase 3-like 3, as well as ARG1 protein expression, suggesting that IGF-I is required for IL-4-induced Akt phosphorylation and M2 activation [52]. Based on this evidence, we postulate that IGF-I is the element that actually activates the PI3K/Akt pathway, which IL-4 uses. In IFN-γ-treated cells, the application of the IGF-I siRNA strengthened the effect of decreasing the parasite load, and the addition of rIGF-I almost nullified this effect, returning it to levels similar to the control group. Reinforcing our finding, the inhibition of PI3K or treatment with an anti-IGF-I antibody exacerbates the effects of IFN-γ [52].

Our data are original in that they clearly show the interference of IGF-I on the IL-4 effect. In experimental visceral leishmaniasis, IGF-I increases the expression of arginase, while the deletion of IGF-IR leads to decreased arginase in a STAT-6-dependent mechanism and restriction of *L. donovani* growth [55]. These authors explored the role of extrinsic IGF-I but not of intrinsic IGF-I present within the cell, and they did not explore the interference of IGF-I on the IL-4 effect. Furthermore, it is known that the immune responses to *Leishmania* infection vary considerably according to the species of the parasite. In particular, the immune response to *L. donovani* and *L. infantum*, which are strains leading to visceral leishmaniasis, is quite different from the immune response established in the *L. major* model. In experimental visceral leishmaniasis, the role of IL-4 in the susceptibility to visceral disease is not clearly defined [55, 56]. Moreover, L-arginine metabolism of *L. donovani* is shown to be different from that of other species [57].

Regarding *Leishmania* infections in experimental models, we hypothesized that the profiles of mouse resistance and susceptibility to *L. major* infection are not only due to the effects of Th1 and Th2 cytokines, but that IGF-I may also play an important role in infection development due to constitutive differences in IGF-I expression. The BALB/c and C57BL/6 mouse strains did not exhibit similar expression profiles of IGF-I, with the latter displaying lower expression. Similarly, lower serum IGF-I levels have been detected in C57BL/6 mice compared to that in C3H/HeJ mice [29]. To address this issue, we analyzed the effect of IGF-I in vivo and found that lesion development in mice was increased when *L. major* promastigotes were preincubated with recombinant IGF-I and applied to susceptible and resistant mouse strains. The lesions in BALB/c mice increased in size and were progressive, but the profile of lesion development was more striking in C57BL/6 mice, as the lesions in mice injected with IGF-I-preincubated parasites not only increased in size but also became progressive, even in the late phase when the lesions in the control animals were diminishing. This resistant-to-susceptible profile reversion was not observed in certain previous studies [25, 28]. Although the lesions in C57BL/6 mice increased progressively following treatment with rIGF-I, the lesion size was smaller than lesions in BALB/c mice. This difference might be due to the IL-4 production pattern in these mouse strains. IL-4 production is similar in the initial phase of infection in both susceptible and resistant mice; however, the production of this cytokine in resistant mice is transient [26]. Another study also showed a nonsustained IL-4 level. In resistant mice, treatment with rIL-4 at the beginning of the infection did not induce the susceptible profile due to the noncontinuous production of IL-4 during the infection [25]. Thus, the main characteristics and differences in terms of susceptibility and resistance observed in certain *L. major*-infected mouse strains may be due to cytokines to some extent, but the susceptibility essentially depends on the presence of IGF-I.

5. Conclusions

The present study provides new insights into the immunology of leishmaniasis, reinforcing the importance of IGF-I in *Leishmania* infection by revealing a significant immune-endocrine interaction in this context. Our data strongly suggest that IGF-I is an effector element of IL-4 actions, leading to M2 macrophage differentiation, involving the PI3K/Akt pathway during *L. major* infection.

Our findings raise questions about pathogenic processes in other diseases in which Th2 cytokines play an important role. In relation to the pathogenesis of leishmaniasis, a polymorphism in the IGF-I gene has been detected in the human population [58, 59]; thus, we speculate that individuals in endemic areas will be susceptible or resistant or will exhibit exacerbation or modulation of the pathogenic process, depending on the expression of basal IGF-I.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (fellowships 2008/04106-0 to Luiza C. Reis and 2005/52271-1 to Fabricio Petitto-Assis), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant 484499/2006 and research fellowship to Hiro Goto), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/SIU (Grant 001/2013 and fellowships 11613/13-0 to Luiza C. Reis and 11615/13-3 to Eduardo Milton Ramos-Sanchez), the Programa Nacional de Pós Doutorado (PNPD)/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (fellowship 1476173 to Luiza C. Reis) and LIM 38 (Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo). The authors acknowledge Ana Lúcia Garippo for providing

technical assistance with confocal microscopy, Celio Xavier da Costa dos Santos for providing initial assistance with the siRNA methodology, and Sandra Muxel for providing technical assistance with the arginase expression methodology.

References

[1] J. Frystyk, "Free insulin-like growth factors – measurements and relationships to growth hormone secretion and glucose homeostasis," *Growth Hormone & IGF Research*, vol. 14, no. 5, pp. 337–375, 2004.

[2] T. Hehlgans and K. Pfeffer, "The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games," *Immunology*, vol. 115, no. 1, pp. 1–20, 2005.

[3] A. M. Valverde, M. Benito, and M. Lorenzo, "The brown adipose cell: a model for understanding the molecular mechanisms of insulin resistance," *Acta Physiologica Scandinavica*, vol. 183, no. 1, pp. 59–73, 2005.

[4] S. Arkins, N. Rebeiz, D. L. Brunke-Reese, A. Biragyn, and K. W. Kelley, "Interferon-gamma inhibits macrophage insulin-like growth factor-I synthesis at the transcriptional level," *Molecular Endocrinology*, vol. 9, no. 3, pp. 350–360, 1995.

[5] J. C. O'Connor, R. H. McCusker, K. Strle, R. W. Johnson, R. Dantzer, and K. W. Kelley, "Regulation of IGF-I function by proinflammatory cytokines: at the interface of immunology and endocrinology," *Cellular Immunology*, vol. 252, no. 1-2, pp. 91–110, 2008.

[6] M. W. Wynes and D. W. H. Riches, "Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13," *Journal of Immunology*, vol. 171, no. 7, pp. 3550–3559, 2003.

[7] P. Scott and F. O. Novais, "Cutaneous leishmaniasis: immune responses in protection and pathogenesis," *Nature Reviews Immunology*, vol. 16, no. 9, pp. 581–592, 2016.

[8] World Health Organization (WHO), "Control of leishmaniases," *World Health Organ Technical Report Series*, no. 949, pp. xii–xiii, 2010.

[9] F. Y. Liew, S. Millott, C. Parkinson, R. M. Palmer, and S. Moncada, "Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine," *The Journal of Immunology*, vol. 144, no. 12, pp. 4794–4797, 1990.

[10] S. C. Roberts, M. J. Tancer, M. R. Polinsky, K. M. Gibson, O. Heby, and B. Ullman, "Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants," *The Journal of Biological Chemistry*, vol. 279, no. 22, pp. 23668–23678, 2004.

[11] M. F. L. da Silva and L. M. Floeter-Winter, "Arginase in *Leishmania*," *Sub-Cellular Biochemistry*, vol. 74, pp. 103–117, 2014.

[12] J. I. Jones and D. R. Clemmons, "Insulin-like growth factors and their binding proteins: biological actions," *Endocrine Reviews*, vol. 16, no. 1, pp. 3–34, 1995.

[13] C. M. C. Gomes, H. Goto, C. E. P. Corbett, and M. Gidlund, "Insulin-like growth factor-1 is a growth promoting factor for *Leishmania* promastigotes," *Acta Tropica*, vol. 64, no. 3-4, pp. 225–228, 1997.

[14] C. M. C. Gomes, H. P. Monteiro, M. Gidlund, C. E. P. Corbett, and H. Goto, "Insulin-like growth factor-I induces phosphorylation in Leishmania (Leishmania) mexicana promastigotes and amastigotes," *The Journal of Eukaryotic Microbiology*, vol. 45, no. 3, pp. 352–355, 1998.

[15] H. Goto, C. M. C. Gomes, C. E. P. Corbett, H. P. Monteiro, and M. Gidlund, "Insulin-like growth factor I is a growth-promoting factor for *Leishmania* promastigotes and amastigotes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 22, pp. 13211–13216, 1998.

[16] C. M. V. Vendrame, M. D. T. Carvalho, F. J. O. Rios, E. R. Manuli, F. Petitto-Assis, and H. Goto, "Effect of insulin-like growth factor-I on *Leishmania amazonensis* promastigote arginase activation and reciprocal inhibition of NOS2 pathway in macrophage *in vitro*," *Scandinavian Journal of Immunology*, vol. 66, no. 2-3, pp. 287–296, 2007.

[17] C. M. V. Vendrame, M. D. T. Carvalho, A. G. Tempone, and H. Goto, "Insulin-like growth factor-i induces arginase activity in *leishmania amazonensis* amastigote-infected macrophages through a cytokine-independent mechanism," *Mediators of Inflammation*, vol. 2014, 13 pages, 2014.

[18] C. M. Gomes, H. Goto, V. L. Ribeiro da Matta, M. D. Laurenti, M. Gidlund, and C. E. Corbett, "Insulin-like growth factor (IGF)-I affects parasite growth and host cell migration in experimental cutaneous leishmaniasis," *International Journal of Experimental Pathology*, vol. 81, no. 4, pp. 249–255, 2000.

[19] L. C. Reis, E. M. Ramos-Sanchez, and H. Goto, "The interactions and essential effects of intrinsic insulin-like growth factor-I on *Leishmania* (*Leishmania*) major growth within macrophages," *Parasite Immunology*, vol. 35, no. 7-8, pp. 239–244, 2013.

[20] P. Scott and J. P. Farrell, "Experimental cutaneous leishmaniasis: induction and regulation of T cells following infection of mice with *Leishmania major*," *Chemical Immunology*, vol. 70, pp. 60–80, 1998.

[21] F. P. Heinzel, M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley, "Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets," *Journal of Experimental Medicine*, vol. 169, no. 1, pp. 59–72, 1989.

[22] R. M. Locksley, F. P. Heinzel, M. D. Sadick, B. J. Holaday, and K. D. Gardner Jr, "Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets," *Annales de l'Institut Pasteur. Immunologie*, vol. 138, no. 5, pp. 744–749, 1987.

[23] D. J. Matthews, C. L. Emson, G. J. McKenzie, H. E. Jolin, J. M. Blackwell, and A. N. J. McKenzie, "IL-13 is a susceptibility factor for *Leishmania major* infection," *Journal of Immunology*, vol. 164, no. 3, pp. 1458–1462, 2000.

[24] S. L. Reiner and R. M. Locksley, "The regulation of immunity to *Leishmania major*," *Annual Review of Immunology*, vol. 13, no. 1, pp. 151–177, 1995.

[25] B. D. Hondowicz, T. M. Scharton-Kersten, D. E. Jones, and P. Scott, "Leishmania major-infected C3H mice treated with anti-IL-12 mAb develop but do not maintain a Th2 response," *Journal of Immunology*, vol. 159, no. 10, pp. 5024–5031, 1997.

[26] D. B. Stetson, M. Mohrs, V. Mallet-Designe, L. Teyton, and R. M. Locksley, "Rapid expansion and IL-4 expression by *Leishmania*-specific naive helper T cells in vivo," *Immunity*, vol. 17, no. 2, pp. 191–200, 2002.

[27] P. Scott, A. Eaton, W. C. Gause, X. di Zhou, and B. Hondowicz, "Early IL-4 production does not predict susceptibility to

Leishmania major," *Experimental Parasitology*, vol. 84, no. 2, pp. 178–187, 1996.

[28] A. H. Shankar and R. G. Titus, "T cell and non-T cell compartments can independently determine resistance to Leishmania major," *The Journal of Experimental Medicine*, vol. 181, no. 3, pp. 845–855, 1995.

[29] K. Iida, C. J. Rosen, C. Ackert-Bicknell, and M. O. Thorner, "Genetic differences in the IGF-I gene among inbred strains of mice with different serum IGF-I levels," *The Journal of Endocrinology*, vol. 186, no. 3, pp. 481–489, 2005.

[30] L. C. Green, D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum, "Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids," *Analytical Biochemistry*, vol. 126, no. 1, pp. 131–138, 1982.

[31] I. M. Corraliza, M. L. Campo, G. Soler, and M. Modolell, "Determination of arginase activity in macrophages: a micromethod," *Journal of Immunological Methods*, vol. 174, no. 1-2, pp. 231–235, 1994.

[32] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, article e45, 2001.

[33] J. B. C. Carvalheira, H. G. Zecchin, and M. J. A. Saad, "Vias de sinalização da insulina," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 46, no. 4, pp. 419–425, 2002.

[34] L. Laviola, A. Natalicchio, S. Perrini, and F. Giorgino, "Abnormalities of IGF-I signaling in the pathogenesis of diseases of the bone, brain, and fetoplacental unit in humans," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 295, no. 5, pp. E991–E999, 2008.

[35] N. M. Heller, S. Matsukura, S. N. Georas et al., "Interferon-γ inhibits STAT6 signal transduction and gene expression in human airway epithelial cells," *American Journal of Respiratory Cell and Molecular Biology*, vol. 31, no. 5, pp. 573–582, 2004.

[36] S. Gordon and F. O. Martinez, "Alternative activation of macrophages: mechanism and functions," *Immunity*, vol. 32, no. 5, pp. 593–604, 2010.

[37] R. R. Gala, "Prolactin and growth hormone in the regulation of the immune system," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 198, no. 1, pp. 513–527, 1991.

[38] R. Kooijman, E. L. Hooghe-Peters, and R. Hooghe, "Prolactin, growth hormone, and insulin-like growth factor-I in the immune system," *Advances in Immunology*, vol. 63, pp. 377–454, 1996.

[39] D. A. Weigent, "Immunoregulatory properties of growth hormone and prolactin," *Pharmacology & Therapeutics*, vol. 69, no. 3, pp. 237–257, 1996.

[40] K. W. Kelley, D. A. Weigent, and R. Kooijman, "Protein hormones and immunity," *Brain, Behavior, and Immunity*, vol. 21, no. 4, pp. 384–392, 2007.

[41] W. S. Cohick and D. R. Clemmons, "The insulin-like growth factors," *Annual Review of Physiology*, vol. 55, no. 1, pp. 131–153, 1993.

[42] N. Wanasen, C. L. MacLeod, L. G. Ellies, and L. Soong, "L-Arginine and cationic amino acid transporter 2B regulate growth and survival of Leishmania amazonensis amastigotes in macrophages," *Infection and Immunity*, vol. 75, no. 6, pp. 2802–2810, 2007.

[43] E. A. Castilho-Martins, M. F. Laranjeira da Silva, M. G. dos Santos, S. M. Muxel, and L. M. Floeter-Winter, "Axenic *Leishmania amazonensis* promastigotes sense both the external and internal arginine pool distinctly regulating the two transporter-coding genes," *PLoS One*, vol. 6, no. 11, article e27818, 2011.

[44] M. Kandpal, R. B. Fouce, A. Pal, P. Y. Guru, and B. L. Tekwani, "Kinetics and molecular characteristics of arginine transport by *Leishmania donovani* promastigotes," *Molecular and Biochemical Parasitology*, vol. 71, no. 2, pp. 193–201, 1995.

[45] M. F. L. da Silva, R. A. Zampieri, S. M. Muxel, S. M. Beverley, and L. M. Floeter-Winter, "*Leishmania amazonensis* arginase compartmentalization in the glycosome is important for parasite infectivity," *PLoS One*, vol. 7, no. 3, article e34022, 2012.

[46] B. Nicholson, C. K. Manner, J. Kleeman, and C. L. MacLeod, "Sustained nitric oxide production in macrophages requires the arginine transporter CAT2," *The Journal of Biological Chemistry*, vol. 276, no. 19, pp. 15881–15885, 2001.

[47] C. J. Huang, P. S. Tsai, C. H. Yang et al., "Pulmonary transcription of CAT-2 and CAT-2B but not CAT-1 and CAT-2A were upregulated in hemorrhagic shock rats," *Resuscitation*, vol. 63, no. 2, pp. 203–212, 2004.

[48] M. C. Liu, P. S. Tsai, C. H. Yang, C. H. Liu, C. C. Chen, and C. J. Huang, "Propofol significantly attenuates iNOS, CAT-2, and CAT-2B transcription in lipopolysaccharide-stimulated murine macrophages," *Acta Anaesthesiologica Taiwania*, vol. 44, no. 2, pp. 73–81, 2006.

[49] M. Rath, I. Muller, P. Kropf, E. I. Closs, and M. Munder, "Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages," *Frontiers in Immunology*, vol. 5, p. 532, 2014.

[50] M. F. White, "The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action," *Recent Progress in Hormone Research*, vol. 53, pp. 119–138, 1998.

[51] L. Soon, L. Flechner, J. S. Gutkind et al., "Insulin-like growth factor I synergizes with interleukin 4 for hematopoietic cell proliferation independent of insulin receptor substrate expression," *Molecular and Cellular Biology*, vol. 19, no. 5, pp. 3816–3828, 1999.

[52] J. P. Barrett, A. M. Minogue, A. Falvey, and M. A. Lynch, "Involvement of IGF-1 and Akt in M1/M2 activation state in bone marrow-derived macrophages," *Experimental Cell Research*, vol. 335, no. 2, pp. 258–268, 2015.

[53] J. H. Kim, H. H. Park, and C. E. Lee, "IGF-1 potentiation of IL-4-induced CD23/FcεRII expression in human B cells," *Molecules and Cells*, vol. 15, no. 3, pp. 307–312, 2003.

[54] D. Ruckerl, S. J. Jenkins, N. N. Laqtom et al., "Induction of IL-4Rα-dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo," *Blood*, vol. 120, no. 11, pp. 2307–2316, 2012.

[55] E. Y. Osorio, B. L. Travi, A. M. da Cruz, O. A. Saldarriaga, A. A. Medina, and P. C. Melby, "Growth factor and Th2 cytokine signaling pathways converge at STAT6 to promote arginase expression in progressive experimental visceral leishmaniasis," *PLoS Pathogens*, vol. 10, no. 6, article e1004165, 2014.

[56] R. Kumar and S. Nylén, "Immunobiology of visceral leishmaniasis," *Frontiers in Immunology*, vol. 3, 2012.

[57] J. M. Boitz, C. A. Gilroy, T. D. Olenyik et al., "Arginase is essential for survival of *Leishmania donovani* promastigotes but not intracellular amastigotes," *Infection and Immunity*, vol. 85, no. 1, pp. e00554–e00516, 2016.

[58] N. Vaessen, P. Heutink, J. A. Janssen et al., "A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction," *Diabetes*, vol. 50, no. 3, pp. 637–642, 2001.

[59] T. M. Frayling, A. T. Hattersley, A. McCarthy et al., "A putative functional polymorphism in the IGF-I gene: association studies with type 2 diabetes, adult height, glucose tolerance, and fetal growth in U.K. populations," *Diabetes*, vol. 51, no. 7, pp. 2313–2316, 2002.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 8597361, 11 pages
https://doi.org/10.1155/2018/8597361

Research Article

Intrauterine Malnutrition Reduced Long Leptin Receptor Isoform Expression and Proinflammatory Cytokine Production in Male Rat Pulmonary Endothelial Cells Stimulated by Lipopolysaccharide

Aleksandro M. Balbino,[1] Marina M. Silva,[1] Gabriela A. Azevedo,[1] Noemi L. Gil,[1] Renaide R. Ferreira,[2] Leila A. dos Santos,[1] Rebéca M. Gasparin,[1] Liliam Fernandes,[1] Maristella A. Landgraf,[1,3] and Richardt G. Landgraf[1]

[1]*Department of Pharmaceuticals Sciences, Universidade Federal de São Paulo-Campus Diadema, Diadema, SP, Brazil*
[2]*Biotério Central, Universidade de São Paulo, São Paulo, SP, Brazil*
[3]*Department of Pharmacology, Universidade de São Paulo, São Paulo, SP, Brazil*

Correspondence should be addressed to Maristella A. Landgraf; mvitta@usp.br
and Richardt G. Landgraf; richardt.landgraf@gmail.com

Received 8 February 2018; Revised 8 May 2018; Accepted 27 May 2018; Published 9 July 2018

Academic Editor: Naïma Moustaïd-Moussa

Background/Aims. We have previously shown that low birth weight (LBW) rats exposed to intrauterine malnutrition have an impaired lung inflammatory response and reduced levels of inflammatory mediators; however, circulating leptin levels were not increased. We evaluated long leptin receptor isoform (ObRb) expression in lung endothelial cells from low birth weight rats and examined its role in the production of lipid mediators and cytokines. *Methods*. Lung endothelial cells were obtained from normal birth weight (NBW) rats or LBW rats subjected to intrauterine malnutrition. These cells were stimulated with leptin (10 ng/mL), LPS (lipopolysaccharide, 1 μg/mL), or leptin plus LPS. Six hours after stimulation, the production of inflammatory mediators (PGE_2, LTB_4, IL-1β, and IL-6) was evaluated using commercial ELISA kits, and Western blotting was performed to investigate p38MAPK, NF-κB, and ObRb expression. *Results*. Leptin increased IL-1β levels in only cells from the NBW group, whereas LPS increased PGE_2 and LTB_4 levels in cells from both groups; leptin addition potentiated lipid mediator production induced by LPS in the NBW group. LPS enhanced the production of IL-1β and IL-6 in only endothelial cells from NBW rats. Leptin receptor expression was decreased (63%) in endothelial cells from LBW rats. None of the stimuli increased NF-κB or p38 signaling pathway expression in cells from LBW rats. *Conclusion*. These results suggest that intrauterine malnutrition compromises leptin receptor expression and cytokine production in pulmonary endothelial cells stimulated by LPS; these effects seem to involve the NF-κB and p38MAPK signaling pathways.

1. Introduction

Leptin (derived from the Greek word "leptos," meaning thin) is a nonglycosylated peptide hormone with a molecular weight of 16 kDa; in murine animals, leptin is encoded by the obese gene (Ob), which shows 84% homology with the human leptin gene [1]. Leptin, primarily synthesized by adipocytes in white adipose tissue, is a pleiotropic molecule involved in promoting energy expenditure and satiety, as well as homeostasis, immunity, and reproductive neuroendocrine and neuroprotective functions [2–4]. These effects depend on binding to its receptor, long leptin receptor isoform (ObRb), a transmembrane protein with a cytoplasmic domain that activates signal transduction pathways,

including the Janus kinase-signal transducer and activator of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) signaling pathways [5, 6].

In a recent review, Kaczyńska et al. demonstrated the involvement of leptin in pulmonary physiology by stimulating the lung ventilation process and in different respiratory diseases; in asthma, leptin increases airway hyperresponsiveness and induces eosinophil accumulation, and in obstructive sleep apnea syndrome, leptin appears to be involved in the apnea-hypopnea index and the incidence of hypercapnia episodes [7]. Other authors have demonstrated that leptin negatively regulates LPS-induced lung injury and modulates corticosterone and insulin levels [8].

Acute malnutrition is associated with reduced leptin levels and immunosuppression [9, 10], cytokine expression deregulation [11], and decreased leukotriene synthesis in alveolar macrophages [12]. In a previous study, we observed that rats with a normal birth weight had increased circulating leptin levels after allergic stimuli in the lung, whereas rats with a low birth weight did not have increased circulating leptin levels or leukotriene production in the lungs after allergic stimulation; these results suggest that leptin plays a role in the reduced inflammatory responses of these animals [13].

The endothelium plays an important role in maintaining vascular tone and laminar blood flow and in cellular and molecular events related to immune reactions and responses to tissue injury [14, 15]. In vitro models of endothelial cells from lung tissue are important tools for understanding the interactions between the endothelium and leukocyte trafficking [16]. In lung tissue, a variety of cells, including bronchial epithelial cells, alveolar type II pneumocytes, macrophages, and mast cells, has been shown to produce leptin [17–19]; in addition, the lungs are the peripheral tissues with the highest ObRb expression levels [17, 20], and numerous cell types, including airway smooth muscle cells [21], bronchial and pulmonary epithelial cells [19], and endothelial cells [22], display high leptin receptor levels.

Proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), stimulate the endothelium to express adhesion molecules (selectins, vascular cell adhesion protein 1 (VCAM-1), platelet endothelial cell adhesion molecule 1 (PECAM-1), and intercellular adhesion molecule-1 (ICAM-1)) that mediate the rolling, adhesion, and migration of leukocytes through the vessels [23].

Low birth weight rats exposed to intrauterine malnutrition have an impaired lung inflammatory response that is associated with reduced leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4) [11], and interleukin-6 (IL-6) [24] levels in the lung tissue; in addition, circulating leptin levels are not increased. Based on this information, we proposed that leptin might play an important role in the reduced production of inflammatory mediators in these animals. Therefore, we investigated whether leptin can modulate the production of inflammatory mediators in LPS-stimulated primary cultured lung endothelial cells from low birth weight rats exposed to intrauterine malnutrition. We also evaluated the participation of the NF-κB and p38MAPK signaling pathways in this process.

2. Materials and Methods

2.1. Animals. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA), and this project was approved by the Ethical Committee for the Animal Research of the Federal University of São Paulo (CEUA 1038/11). Seven male and twenty female Wistar rats from the CEDEME colony (Federal University of São Paulo) were used to obtain offspring. The rats were housed in a $22 \pm 1^{\circ}$C environment at 60% humidity and were maintained on a 12 h light-dark cycle. The male offspring of dams that were nourished and malnourished during the entire gestational period were used at 12 weeks of age. We avoided using female rats because of the fluctuation in their hormone levels (estrogen/progesterone). Males and females have different catabolism, which can significantly alter the regulation and production of several hormones [25]. These differences could modulate the inflammatory response and influence our results.

2.2. Protocol for the Induction of Intrauterine Undernutrition. Timed mating was carried out in age-matched (12 to 16 weeks old) female and male Wistar rats. To assess the estrus stage of the females, vaginal smears were checked before the males were introduced. Day 1 of the pregnancy was determined as the day when spermatozoa were detected in the vaginal smear. After confirmation that mating occurred, the rats were housed individually in standard rat cages. The female rats were divided randomly into two groups: nourished ad libitum and undernourished. The nourished female rats were fed with a standard commercial rat diet (Nuvital, Nuvital Nutrientes S/A, PR, Brazil) containing protein (minimum 22%), carbohydrates (maximum 54%), fat (minimum 4.5%), cellulose (maximum 8%), minerals (maximum 10%), water (maximum 12.5%), and vitamins. The undernourished female rats were fed with the same diet at 50% of the nourished female rat intake; this rate was determined according to the amount of food consumed by the control group from day 1 of the pregnancy until day 23 (parturition). All rats were fed daily in the morning, and consumption was determined 24 h later. After parturition, the dams received food ad libitum; therefore, the pups differed in only prenatal dietary experience. In a previous study, we demonstrated that the litter size and male to female ratio did not differ between the offspring from malnourished and nourished rats [26]. To prevent variation in neonatal growth due to the availability of milk during suckling, the litter sizes were standardized to eight pups on day 1. We did not observe a significant difference in the lung weight/birth weight ratio between the normal birth weight (NBW = 0.00597) and low birth weight (LBW = 0.00588) groups.

2.3. Endothelial Cell Isolation and Culture. Cell cultures were established according to procedures previously described by Chen et al. [27]. Randomly selected male rats from different litters were euthanized at 12 weeks of age by an overdose of anesthesia (ketamine/xylazine); then, their lungs were excised, washed with phosphate-buffered saline (PBS), cut

into $1 \times 1 \times 1\,mm^3$ pieces, and placed in six-well (35 mm) dishes. The tissues were covered with Dulbecco's modified Eagle's medium (DMEM-low glucose) supplemented with fetal bovine serum (FBS, 20%) and gentamicin (40 mg/L), pH 7.4 and placed in a CO_2 incubator (Sheldon Mfg. Inc., USA) (37°C). Lung explants were discarded after 60 h, and the medium was changed every 2-3 days. The cells were grown to confluence and further propagated at a 1 : 4 ratio using trypsin (0.1%). The cells were maintained in culture until the fourth passage when all assays were performed.

2.4. Identification and Characterization of Endothelial Cells. Cells were seeded on sterile glass coverslips (13 mm) and fixed in paraformaldehyde (PFA, 4%) at room temperature for 30 min. The cells were permeabilized with Nonidet P40 (1%) and blocked with FBS (5%) in PBS for 30 min at 37°C. The samples were incubated overnight at 4°C with primary rabbit antibodies against von Willebrand factor (vWF) and *Ulex europaeus* lectin agglutinin I (UEA-1), which binds specifically to L-fucose residues on the endothelium (1 : 50 dilution). In all experiments, cellular staining was detected using bovine anti-rabbit IgG—Texas red-conjugated or goat anti-mouse IgG—FITC-conjugated secondary antibody at 1 : 100 dilution for 2 h at 37°C. Controls were obtained using coverslips incubated with only FBS, followed by secondary antibody. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) at 1 : 400 dilution for 5 min at 37°C. The coverslips were observed, and images were obtained by a fluorescence microscope (Axiovert 100M, Carl Zeiss SMT, Germany).

2.5. Inflammatory Stimulus (In Vitro). Cells were seeded in six-well dishes (500,000 cells/well), and semiconfluent cultures were incubated with DMEM supplemented with fetal bovine serum for 24 h in an incubator (37°C/5% CO_2). After 24 h, the cells were washed with ice-cold PBS; DMEM was added, and the cells were stimulated with LPS (1 μg/mL) [28–30] and/or leptin (10 ng/mL) [31, 32] for 6 h. Then, the cells were collected in cold PBS and centrifuged at 1500 rpm for 5 min at 4°C. For the negative control group, only DMEM was added to the cells. Finally, the cells and the supernatant from the cells were collected and stored in a freezer at −80°C for all subsequent analyses.

2.6. Quantification of Lipid Mediators in the Cell Supernatant. PGE_2 and LTB_4 concentrations were determined by using EIA kits (Cayman Chemical Co., MI, USA) according to the method of Pradelles et al. [33]. The sensitivity of the LTB_4 assay was 4.0 pg/mL, and that of the PGE_2 assay was 15 pg/mL. The CV% values were as follows: LTB_4: intra-assay < 8.37%, interassay < 24.41%; PGE_2: intra-assay < 3.7%, interassay < 20.9%.

2.7. ObRb Receptor, NF-κB p65, and Phospho-p38 MAPK Quantification by Western Blotting. Cells were stimulated with LPS (1 μg/mL) and/or leptin (10 ng/mL) for 6 h; then, they were collected in cold PBS and centrifuged at 1500 rpm for 5 min at 4°C. The cell pellet was suspended in lysis buffer (Tris-HCl 50 mmol/L, pH 7.4, NaCl 100 mmol/L, and NP40 0.5%) with a protease/phosphatase inhibitor (Halth TM, Thermo Scientific, USA). Protein concentrations were determined by a BCA protein assay kit (Thermo Scientific, USA). Equal amounts of protein (55 μg) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were transferred onto nitrocellulose membranes (0.45 μm) and blocked for 60 min with 5% (wt/vol) nonfat dry milk diluted in TTBS (Tris base 0.2 mmol/L, NaCl 1.4 mmol/L, and Tween 20 0.1%), pH 7.6. The membranes were incubated overnight with polyclonal antibodies against ObRb (sc-8325 (H-300) rabbit polyclonal IgG, Santa Cruz Biotechnology Inc., EUA), NF-κB p65 (number 3987, Cell Signaling; rabbit polyclonal IgG Tech, USA), or phospho-p38 MAPK (number 9215, Cell Signaling; rabbit polyclonal IgG Tech, USA) at 1 : 1000 dilutions. The blots were washed with TTBS (3×5 min) and incubated with a secondary horseradish peroxidase- (HRP-) conjugated goat anti-rabbit antibody (number 70745, Cell Signaling Tech, USA) at a 1 : 2000 dilution for 60 min at room temperature. ObRb receptor, NF-κB p65, and phospho-p38 MAPK expression was detected by chemiluminescence (GeneGnome System, Syngene, UK) and quantified by densitometry (Gene Tools Software, UK). β-Actin expression was used as an internal control (1 : 2000 dilution, number 4970, Cell Signaling).

2.8. Quantification of Cytokines in the Cell Supernatant. Milliplex® map Kit-Rat Cytokine chemokine magnetic bead panels (EMD Millipore Corporation, Darmstadt, Germany) were used to measure IL-1β, IL-6, and leptin levels in the cell supernatant. The kits were used according to the manufacturer's instructions (MAGPIX™, Luminex®, MiraiBio, Alameda, CA). The data were analyzed using xPONENT® software (MAGPIX, Luminex, MiraiBio, Alameda, CA). The standard curves ranged from 1.95 to 32,000 pg/mL. The lower limits of detection for each cytokine were as follows: IL-1β (2.0 pg/mL), IL-6 (0.6 pg/mL), and leptin (10 pg/mL). The CV% values were as follows: IL-1β: intra-assay < 15%, interassay < 20%; IL-6: intra-assay < 10%, interassay < 20%; and leptin: intra-assay < 20%, interassay < 25%.

2.9. Statistical Analysis. Statistical analyses were carried out using GraphPad Prism software (v.6; GraphPad Software, San Diego, CA, USA). The body weight gain is presented as the mean percentage, and the other results are presented as the means ± SEM. Statistical evaluations of the data were determined by two-way analysis of variance followed by the Tukey-Kramer multiple comparison test. Student's t-test was used when necessary. A P value lower than 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the Offspring. Litter size did not differ between the NBW and LBW groups, indicating that food restriction during the gestation period did not affect reproductive ability. These data agree with the results found by Landgraf et al. [26, 34]. The offspring from the undernourished dams throughout gestation had significantly lower birth weights than the nourished offspring (Figure 1(a)).

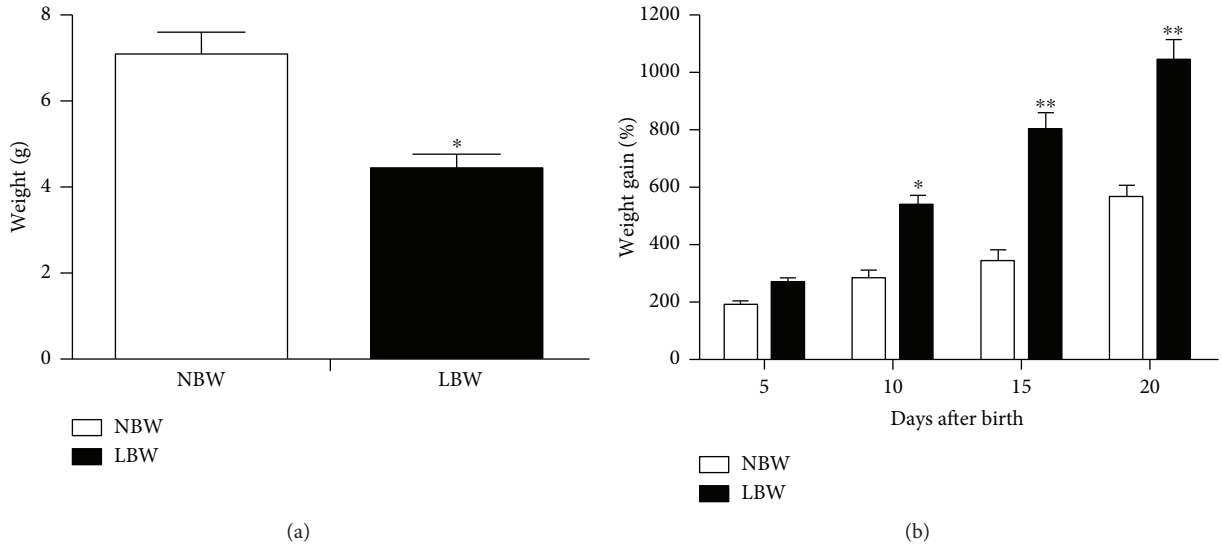


Figure 1: Offspring weight. (a) Offspring weight on the day of birth. The results are presented as the means ± SEM of 12–15 animals/group, $^{*}P < 0.05$ compared to the NBW group. (b) Mean percentage of weight gain from day 0 until day 20. The results are presented as the mean percentage of weight gain ± SEM of 12–15 animals/group, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the NBW group.

After 10 days, the low birth weight (LBW) offspring had higher percentages of body weight gain than the normal birth weight offspring (NBW), and this difference remained until the twentieth day (Figure 1(b)).

3.2. Primary Cultured Endothelial Cells. Primary cultured endothelial cells obtained from the lung explants grew in a monolayer of polygonal cells, exhibited strong contact inhibition, and were characterized morphologically by a cobblestone appearance similar to that observed in a previous study [35]. Positive staining for UEA-1 (Figure 2(a)) and vWF (Figure 2(b)) was detected in more than 90% of the cells in culture.

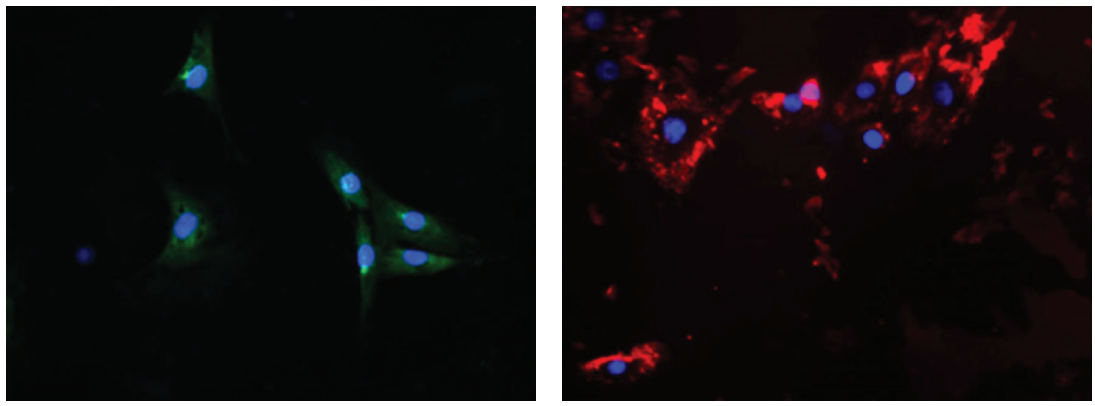
3.3. Leptin Potentiated the LPS-Induced Secretion of Lipid Mediators in Only the Lung Endothelial Cell Supernatants from NBW Rats. Two-way ANOVA showed no significant interaction effect of intrauterine growth restriction and treatment on PGE_2 ($P = 0.26$) and LTB_4 secretion ($P = 0.86$), but there was a significant main effect for treatment ($P < 0.001$). A significant increase in LPS-induced PGE_2 and LTB_4 secretion in lung endothelial cells cultured from both NBW and LBW was observed. The addition of leptin potentiated the LPS-induced production of PGE_2 (86.33 ± 7.8 to 110.7 ± 2.1 ng/mL) and LTB_4 (460.8 ± 19.9 to 545.0 ± 25.5 pg/mL) in the supernatants of lung endothelial cells from NBW rats but not from LBW rats (Figures 3(a) and 3(b)).

3.4. LPS Induced Cytokine Secretion from Lung Endothelial Cells from Only NBW Rats. Lung endothelial cells from NBW rats showed increased IL-1β and IL-6 production after LPS stimulation. The addition of leptin did not alter the production of these cytokines in cells from NBW rats (Figures 4(a) and 4(b)). Lung endothelial cells from LBW rats produced neither IL-1β nor IL-6 leptin after stimulation. A significant interaction effect of intrauterine growth restriction and treatment on IL-1β ($P = 0.002$) and IL-6 ($P = 0.02$) production was confirmed by two-way ANOVA, indicating that these factors acted dependently. Moreover, the effects of intrauterine growth restriction ($P < 0.001$) and treatment ($P < 0.001$) on IL-1β secretion were also significant. Although two-way ANOVA revealed a significant effect of intrauterine growth restriction on IL-6 production, no significant effect was observed for treatment ($P = 0.10$).

3.5. Leptin Secretion from Lung Endothelial Cells. We observed increases in leptin levels after treatment with only leptin and leptin plus LPS (Figure 5), and this effect was confirmed by two-way ANOVA, which indicated a significant effect of treatment on leptin levels ($P < 0.001$).

3.6. Lung Endothelial Cells from LBW Rats Showed Decreased ObRb Receptor Expression. In cells from NBW rats, both leptin and LPS enhanced ObRb expression, and LPS induced a significantly higher increase in ObRb expression than leptin; in addition, leptin did not potentiate the ObRb expression induced by LPS (Figure 6). ObRb receptor expression did not differ between the two groups under basal conditions, and the addition of leptin, LPS, or leptin plus LPS did not increase ObRb receptor expression in LBW rats (Figure 6).

3.7. Lung Endothelial Cells from LBW Rats Showed Decreased p38 MAPK and NF-κB Pathway Expression. We evaluated the p38 MAPK and NF-κB pathway expression in endothelial cells obtained from lung tissues. Both LPS and leptin administration increased the NF-κB and p38 pathway expression in cells from NBW rats (Figures 7(a) and 7(b)); in addition, leptin did not increase the p38 MAPK and NF-κB pathway expression induced by LPS. In cells from LBW rats, none of



(a) (b)

Figure 2: Immunostaining of specific endothelial cell markers. Staining for (a) *Ulex europeaus* lectin agglutinin I (UEA-1), green, and (b) von Willebrand factor (vWF), red. The nuclei were counterstained with DAPI solution for cellular localization. 400-fold increase.

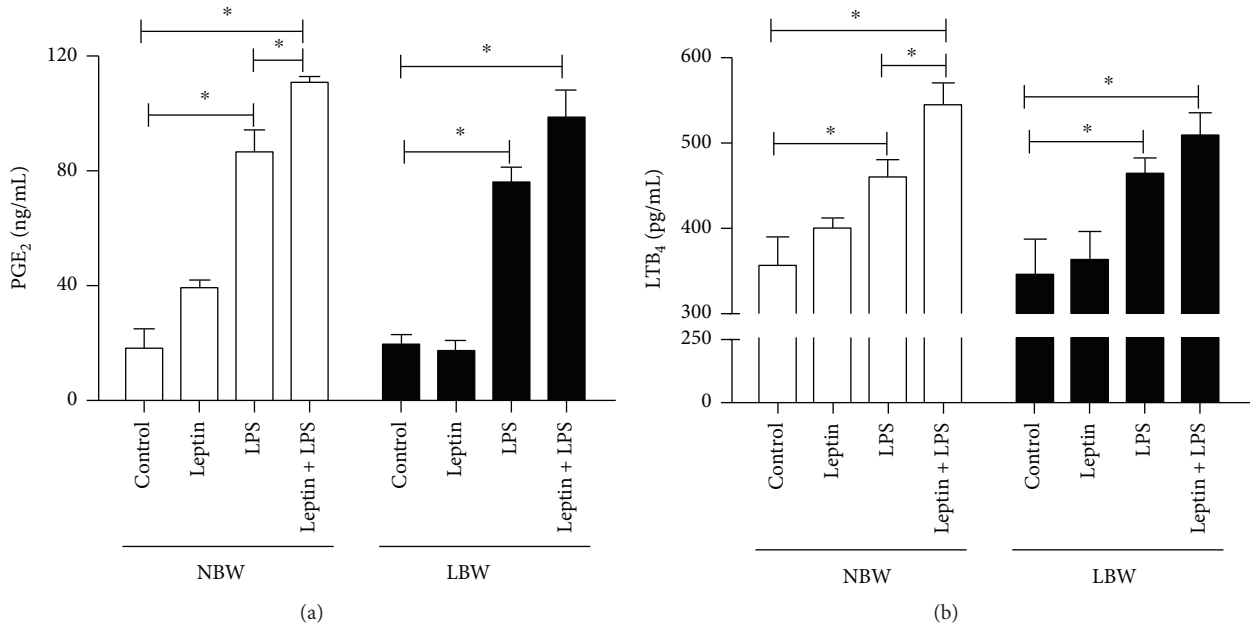


Figure 3: Effect of leptin on LPS-induced PGE_2 and LTB_4 secretion into the supernatants of pulmonary endothelial cells. PGE_2 and LTB_4 were measured in the supernatants of pulmonary endothelial cells using an EIA kit 6 h after stimulus with LPS and/or leptin. Cells were obtained from 8 male Wistar rats selected randomly from 6 different litters per group. The results are presented as the means ± SEM, $^*P < 0.05$.

the stimuli mediated changes in the p38 MAPK and NF-κB pathways (Figures 7(a) and 7(b)).

4. Discussion

In the present study, we demonstrated that lung endothelial cells from intrauterine undernourished rats with a low birth weight had deficient IL-1β and IL-6 production after inflammatory stimuli. This deficiency could be associated with the lack of ObRb receptor expression.

Maternal prenatal undernutrition could result in important alterations in offspring development [36, 37]. Consistent with previous studies, we observed that the offspring of prenatally undernourished mothers presented with a 30% reduction in birth weight, followed by accelerated growth characterized by rapid weight gain [22]. Frisancho [38] suggested that this phenomenon might be a compensatory mechanism associated with reduced fat oxidation and increased carbohydrate metabolism.

In this study, we used a technique described by Chen et al. [27] and Loiola et al. [35]; lung endothelial cells derived from lung tissue explants were grown in FBS- (fetal bovine serum-) enriched medium, and no substances that could damage the structure and function of the pulmonary endothelial cells were added. Here, the lung endothelial cells were characterized using selective markers for these cells, namely, ULEX, which binds specifically to endothelial cell-specific glycoproteins and glycolipids [39], and the von Willebrand factor, which is a glycoprotein produced exclusively by endothelial cells and platelets [40].

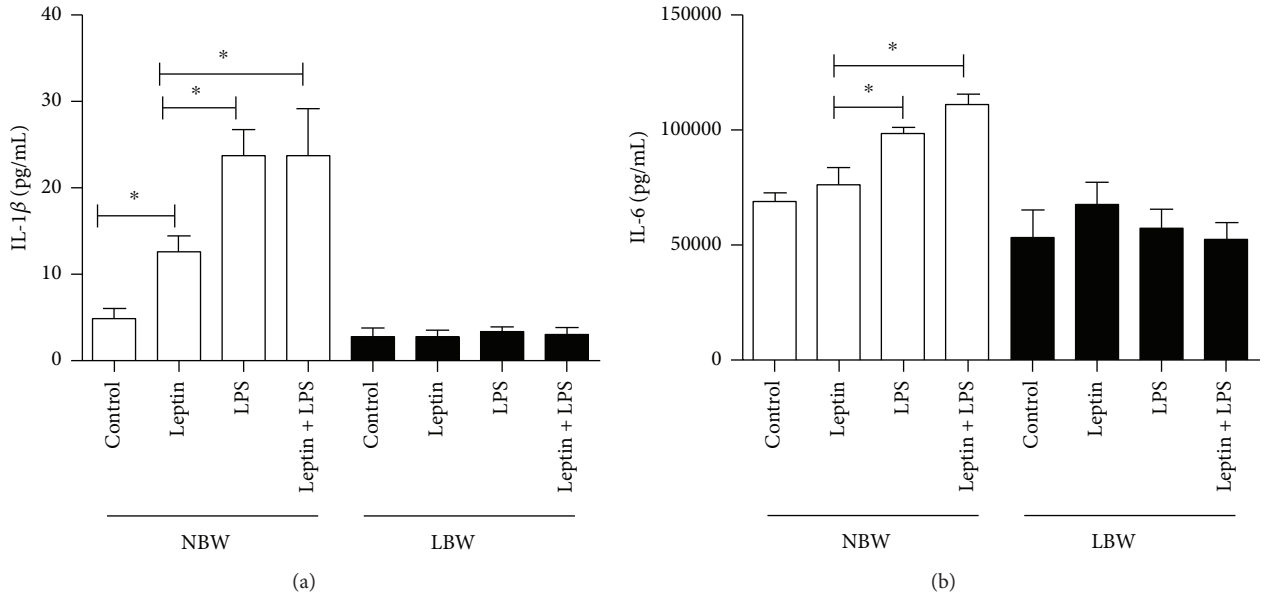


Figure 4: Effect of leptin on LPS-induced cytokine secretion into the supernatants of pulmonary endothelial cells. (a) Interleukin-1β (IL-1β) and (b) interleukin-6 (IL-6) were quantified in the supernatants of pulmonary endothelial cells 6 h after stimulus with LPS and/or leptin by multiplex assays as described in Materials and Methods. Cells were obtained from 8 male Wistar rats selected randomly from 6 different litters per group. The results are presented as the means ± SEM, $^*P < 0.05$.

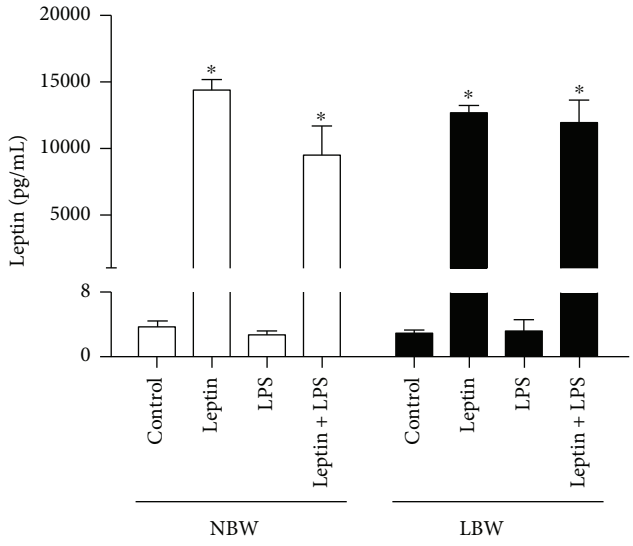


Figure 5: Effect of leptin on LPS-induced leptin secretion into the supernatants of pulmonary endothelial cells. Leptin was quantified in the supernatants of pulmonary endothelial cells 6 h after stimulus with LPS and/or leptin by multiplex assays as described in Materials and Methods. Cells were obtained from 8 male Wistar rats selected randomly from 6 different litters per group. The results are presented as the means ± SEM, $^*P < 0.05$.

Leptin is an adipocyte-derived protein encoded by the ob gene; although its plasma levels are directly correlated with body fat mass, this protein can be synthesized to a lesser extent by other tissues, such as brown adipose tissue, placenta [41], liver, stomach, intestine [42], hypothalamus, pituitary [43], skeletal muscle [44], and immune cells [45]. Leptin can modulate not only the endocrine system but also homeostasis, hematopoiesis, energy metabolism, innate and adaptive immune responses, and endothelial cell activation [31, 46].

Lipopolysaccharide (LPS), also termed endotoxin, is the major component of the outer membrane of Gram-negative bacteria [47]. It is considered a potent inducer of inflammation, and it directly activates the vascular endothelium, leading to the production of cytokines and inflammatory mediators and the expression of adhesion molecules, which contribute to diapedesis [48]. In the present study, LPS induced the production of lipid mediators and cytokines in endothelial cells from NBW rats, thus confirming the data from previous studies.

According to Rola-Pleszczynski and Stankova [49], two classes of soluble mediators act as important agents in the orchestration of the inflammatory response: lipid mediators and cytokines, which are synthesized from phagocytes and parenchymal cells. A loop involving eicosanoids and cytokine production has been previously demonstrated [49, 50]. The release of PGE_2 and LTB_4 augments the response to a variety of inflammatory stimuli, such as IL-1, IL-6, and TNF-α; on the other hand, leukotrienes could also modulate the release of these cytokines. However, in studying eicosanoid metabolism in porcine endothelial cells, Bustus et al. [51] demonstrated that the addition of anti-IL-1β antibodies did not alter cyclooxygenase-2 (COX-2) expression in stimulated endothelial cells, indicating that IL-1β was not responsible for the increases in COX-2 and PGE_2 in that system. Our data demonstrated that the LPS stimulation of endothelial cells from LBW rats increased PGE_2 and LTB_4 but not IL-1β levels, indicating that the production of lipid mediators was not dependent on IL-1β in our model; these results contrast those observed by Bustus et al. [51].

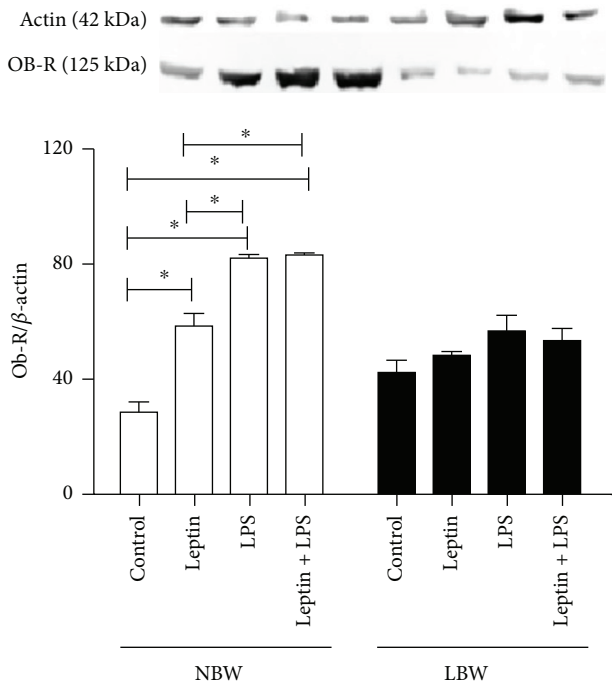


Figure 6: Effect of leptin on LPS-induced Ob-R expression in pulmonary endothelial cells. Endothelial cells were harvested 6 h after stimulus with LPS and/or leptin to quantify the expression levels of Ob-R using Western blotting. The graphs represent the band intensities determined by densitometric analyses and normalized to the total amount of β-actin present in each lane. Cells were obtained from 8 male Wistar rats selected randomly from 6 different litters per group. The results are presented as the means ± SEM, $^*P < 0.05$.

The production of IL-1β during cell injury, infection, or inflammation occurs primarily in monocytes and macrophages, in addition to nonimmune cells, such as fibroblasts and endothelial cells [52]. In the present study, we found an important interaction effect of treatment with LPS and leptin and intrauterine growth restriction (and consequently low birth weight) on cytokine secretion. Leptin significantly increased IL-1β production and potentiated IL-1β production induced by LPS in cells from NBW rats, but the same effects were not observed in endothelial cells from LBW rats; regardless of the stimulus, IL-1β production remained at basal levels and did not change. Studies concerning the role of leptin in the production of inflammatory mediators are conflicting. It has been suggested that leptin has anti-inflammatory effects and that the administration of high doses of leptin could result in neutrophil inhibition in the lungs of rats with acute lung injury induced by acute pancreatitis [53]. Recently, we demonstrated that leptin downregulates LPS-induced acute lung injury by modulating corticosterone and insulin levels [8]. However, it has already been shown that leptin potentiates the effects of LPS on proliferation, monocyte activation, and proinflammatory cytokine production *in vitro* [54–56]. IL-1β and IL-6 stimulate the expression of adhesion molecules on the vascular endothelium and contribute to leukocyte migration [57]. It has already been demonstrated that LBW rats have reduced expression levels of adhesion molecules, such as L-selectin, P-selectin, and ICAM-1; these reductions can attenuate leukocyte migration [26]. Based on these data, we suggest that the failure of endothelial cells from LBW rats to produce IL-1β and IL-6 after LPS stimulation could be an important factor associated with reduced adhesion molecule expression and the consequently decreased leukocyte migration.

In endothelial cells, leptin receptor activation is associated with oxidative stress, chemokine and cytokine production, and adhesion molecule expression [57]. Mice lacking leptin receptors (db/db) are obese and present with a series of dysfunctions, such as hyperinsulinemia, increased cortisol levels, and impaired immune function [58, 59]. Leptin acts on target cells through interacting with its receptor, Ob-R, which is widely expressed in different parts of the body [60]. The expression of short (ObRa) and long (ObRb) leptin receptors has already been demonstrated in bronchial and alveolar epithelial cells in the lung [19, 61]. The ability of the short leptin isoform to activate the JAK signaling pathway is low, and it cannot activate the STAT pathway at all [62]. In addition, it has been demonstrated in adipocyte culture that the activating effects of leptin on the STAT3 and MAPK pathways are exerted through its long receptor [63]. Another study demonstrated that pulmonary macrophages express high levels of the long form leptin receptor; this isoform is the only one capable of inducing STAT3 signaling and is one of the main targets of leptin action in the lung [64]. Our results demonstrated that lung endothelial cells could not produce leptin after LPS stimulation. Both leptin and LPS additions significantly increased the expression of ObRb in endothelial cells from NBW rats, unlike what was observed in cells from LBW rats. Considering these data, we suggest that the reduced Ob-R response to the inflammatory stimuli might compromise the production of cytokines and lipid mediators in endothelial cells from LBW rats.

LPS can initiate several intracellular signaling events, such as stimulating pathways to activate NF-κB and p38 MAPK, inducing cytokine expression [65, 66], and reducing proinflammatory cytokine expression, including IL-1β and TNF-α, to decrease NF-κB and p38 MAPK pathway activation [67]. In addition, HUVEC exposure to leptin upregulates ObRb receptor expression and enhances NF-κB activation and proinflammatory cytokine secretion [68]. Our data agree well with the literature data because we demonstrated that LPS and leptin-treated endothelial cells from NBW rats presented with increased proinflammatory cytokine expression and NF-κB and p38 MAPK activation. In endothelial cells from LBW rats, increases in proinflammatory cytokine expression and NF-κB and p38 MAPK activation were not observed, even after stimulation with LPS and/or leptin, suggesting that these pathways may be compromised in these animals and thus contribute to the decreased inflammatory response observed in our previous results [8, 13, 26].

In a previous study, our group demonstrated that LBW rats, despite normal basal leptin levels, did not have increased leptin levels after an inflammatory stimulus, and this inability to upregulate leptin levels was accompanied by an attenuated inflammatory response [13]. Here, the deficiency in ObRb expression likely reflects the ability of endothelial cells to

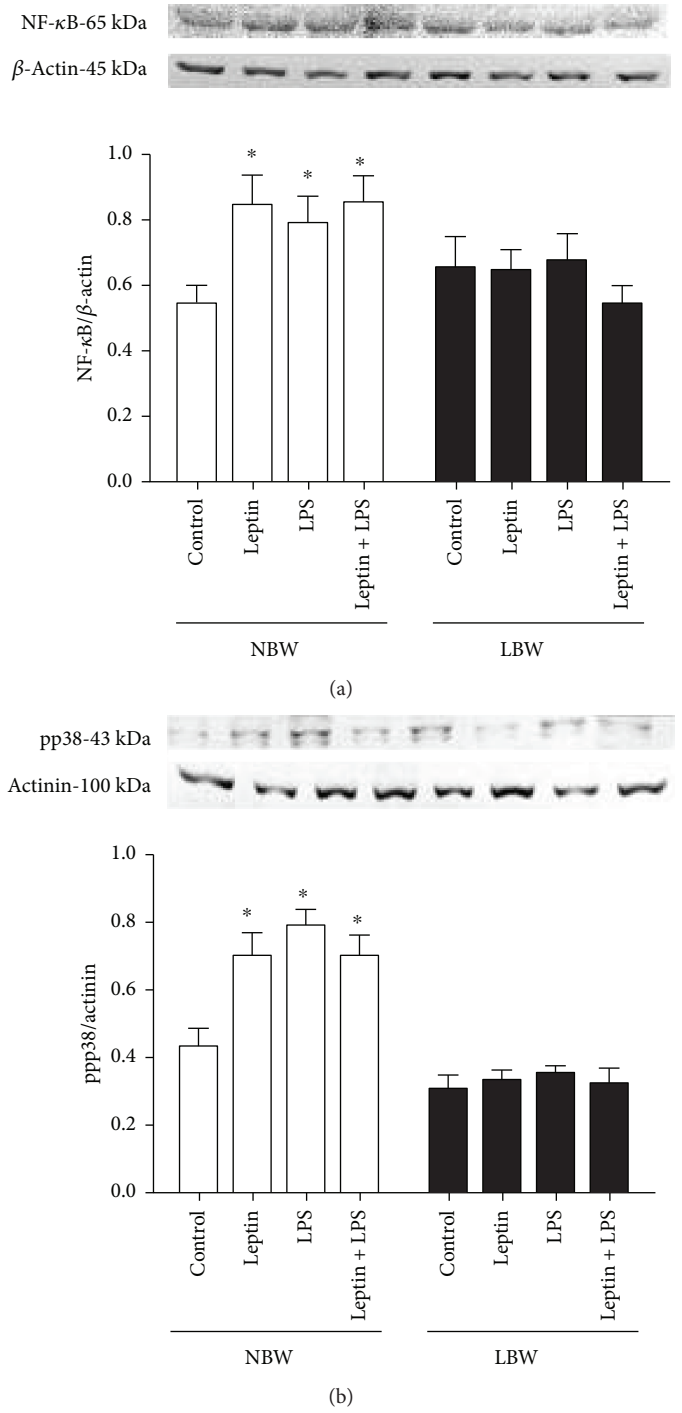


Figure 7: Involvement of the p38 kinase and NF-κB pathways in the regulation of Ob-R expression on LPS-induced Ob-R expression in pulmonary endothelial cells. Endothelial cells were harvested 6 h after stimulus with LPS and/or leptin to quantify the activation of the p38 MAPK and NF-κB pathways using Western blotting. The graphs represent the band intensities determined by densitometric analyses and normalized to the total amount of β-actin (NF-κB) and β-actinin (pp38) present in each lane. Cells were obtained from 8 male Wistar rats selected randomly from 5 different litters per group. The results are presented as the means ± SEM, $^*P < 0.05$.

adequately respond to LPS and leptin itself and contributes to the decreased production of inflammatory mediators.

Therefore, we suggest that the low birth weight induced by intrauterine malnutrition could induce molecular changes to the physiological responses of pulmonary endothelial cells to compromise the expression of ObRb and reduce the expression of inflammatory mediators and cytokines; our data also indicate the participation of the NF-κB and p38 MAPK pathways in this process. These events could contribute to the attenuation of the inflammatory response observed

in intrauterine malnourished rats with a low birth weight. Taken together, these data indicate a key role of leptin in the reduced inflammatory response of LBW rats.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Some of the data presented in this article were included in a poster presentation at the 39th ESPEN (The European Society for Clinical Nutrition and Metabolism), The Hague, Netherlands, 2017 [69].

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Authors' Contributions

Maristella A. Landgraf and Richardt G. Landgraf contributed equally to this work.

Acknowledgments

This work was financially supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2010/01404-0, 2012/51104-8, 2014/18760-4, and 2017/02042-3).

References

[1] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, and J. M. Friedman, "Positional cloning of the mouse obese gene and its human homologue," *Nature*, vol. 372, no. 6505, pp. 425–432, 1994.

[2] S. Cinti, R. C. Frederich, M. C. Zingaretti, R. De Matteis, J. S. Flier, and B. B. Lowell, "Immunohistochemical localization of leptin and uncoupling protein in white and brown adipose tissue," *Endocrinology*, vol. 138, no. 2, pp. 797–804, 1997.

[3] M. J. De Blasio, M. Boije, O. R. Vaughan et al., "Developmental expression and glucocorticoid control of the leptin receptor in fetal ovine lung," *PLoS One*, vol. 10, no. 8, article e0136115, 2015.

[4] Z. Zhang, S. Sun, C. Du et al., "Effects of leptin on Na^+/Ca^{2+} exchanger in PC12 cells," *Cellular Physiology and Biochemistry*, vol. 40, no. 6, pp. 1529–1537, 2016.

[5] M. Vadacca, D. P. Margiotta, L. Navarini, and A. Afeltra, "Leptin in immuno-rheumatological diseases," *Cellular & Molecular Immunology*, vol. 8, no. 3, pp. 203–212, 2011.

[6] T. Kelesidis, I. Kelesidis, S. Chou, and C. S. Mantzoros, "Narrative review: the role of leptin in human physiology: emerging clinical applications," *Annals of Internal Medicine*, vol. 152, no. 2, pp. 93–100, 2010.

[7] K. Kaczyńska, D. Zając, P. Wojciechowski, E. Kogut, and M. Szereda-Przestaszewska, "Neuropeptides and breathing in health and disease," *Pulmonary Pharmacology & Therapeutics*, vol. 48, pp. 217–224, 2018.

[8] M. A. Landgraf, R. C. Silva, M. Corrêa-Costa et al., "Leptin downregulates LPS-induced lung injury: role of corticosterone and insulin," *Cellular Physiology and Biochemistry*, vol. 33, no. 3, pp. 835–846, 2014.

[9] C. D. Morrison, "Leptin signaling in brain: a link between nutrition and cognition?," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1792, no. 5, pp. 401–408, 2009.

[10] G. M. Lord, G. Matarese, J. K. Howard, R. J. Baker, S. R. Bloom, and R. I. Lechler, "Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression," *Nature*, vol. 394, no. 6696, pp. 897–901, 1998.

[11] R. Faggioni, K. R. Feingold, and C. Grunfeld, "Leptin regulation of the immune response and the immunodeficiency of malnutrition," *The FASEB Journal*, vol. 15, no. 14, pp. 2565–2571, 2001.

[12] P. Mancuso, C. Canetti, A. Gottschalk, P. K. Tithof, and M. Peters-Golden, "Leptin augments alveolar macrophage leukotriene synthesis by increasing phospholipase activity and enhancing group IVC iPLA2 (cPLA2gamma) protein expression," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 287, no. 3, pp. L497–L502, 2004.

[13] M. A. Landgraf, R. G. Landgraf, S. Jancar, and Z. B. Fortes, "Influence of age on the development of immunological lung response in intrauterine undernourishment," *Nutrition*, vol. 24, no. 3, pp. 262–269, 2008.

[14] M. A. Gimbrone Jr., "Vascular endothelium: nature's blood-compatible container," *Annals of the New York Academy of Sciences*, vol. 516, no. 1, pp. 5–11, 1987.

[15] K. Nakao, K. Kuwahara, T. Nishikini et al., "Endothelium-derived C-type natriuretic peptide contributes to blood pressure regulation by maintaining endothelial integrity," *Hypertension*, vol. 69, no. 2, pp. 286–296, 2017.

[16] J. C. B. B. Essone, N. N'Dilimabaka, J. Ondzaga et al., "Comparison of apoptosis in human primary pulmonary endothelial cells and a brain microvascular endothelial cell line co-cultured with *Plasmodium falciparum* field isolates," *BMC Infectious Diseases*, vol. 17, no. 1, pp. 454–463, 2017.

[17] T. C. Theoharides, "Neuroendocrinology of mast cells: challenges and controversies," *Experimental Dermatology*, vol. 26, no. 9, pp. 751–759, 2017.

[18] J. H. J. Vernooy, N. E. A. Drummen, R. J. Van Suylen et al., "Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers," *Thorax*, vol. 64, no. 1, pp. 26–32, 2009.

[19] A. Bruno, E. Pace, P. Chanez et al., "Leptin and leptin receptor expression in asthma," *The Journal of Allergy and Clinical Immunology*, vol. 124, no. 2, pp. 230–237.e4, 2009.

[20] B. Lollmann, S. Grüninger, A. Stricker-Krongrad, and M. Chiesi, "Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues," *Biochemical and Biophysical Research Communications*, vol. 238, no. 2, pp. 648–652, 1997.

[21] P. Nair, K. Radford, A. Fanat, L. J. Janssen, M. Peters-Golden, and P. G. Cox, "The effects of leptin on airway smooth muscle responses," *American Journal of Respiratory Cell and Molecular Biology*, vol. 39, no. 4, pp. 475–481, 2008.

[22] M. R. Sierra-Honigmann, A. K. Nath, C. Murakami et al., "Biological action of leptin as an angiogenic factor," *Science*, vol. 281, no. 5383, pp. 1683–1686, 1998.

[23] P. Turowski, P. Adamson, and J. Greenwood, "Pharmacological targeting of ICAM-1 signaling in brain endothelial cells: potential for treating neuroinflammation," *Cellular and Molecular Neurobiology*, vol. 25, no. 1, pp. 153–170, 2005.

[24] M. A. Landgraf, R. G. Landgraf, R. C. Silva, P. Semedo, N. O. Câmara, and Z. B. Fortes, "Intrauterine undernourishment alters TH1/TH2 cytokine balance and attenuates lung allergic inflammation in Wistar rats," *Cellular Physiology and Biochemistry*, vol. 30, no. 3, pp. 552–562, 2012.

[25] G. Rando and W. Wahli, "Sex differences in nuclear receptor-regulated liver metabolic pathways," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1812, no. 8, pp. 964–973, 2011.

[26] M. A. Landgraf, L. L. Martinez, V. M. F. Rastelli et al., "Intrauterine undernutrition in rats interferes with leukocyte migration, decreasing adhesion molecule expression in leukocytes and endothelial cells," *The Journal of Nutrition*, vol. 135, no. 6, pp. 1480–1485, 2005.

[27] S. F. Chen, X. Fei, and S. H. Li, "A new simple method for isolation of microvascular endothelial cells avoiding both chemical and mechanical injuries," *Microvascular Research*, vol. 50, no. 1, pp. 119–128, 1995.

[28] X. Chen, M. Xiu, J. Xing, S. Yu, D. Min, and F. Guo, "Lanthanum chloride inhibits LPS mediated expressions of pro-inflammatory cytokines and adhesion molecules in HUVECs: involvement of NF-κB-Jmjd3 signaling," *Cellular Physiology and Biochemistry*, vol. 42, no. 5, pp. 1713–1724, 2017.

[29] Y. Zhou, T. Zhang, X. Wang et al., "Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways," *Cellular Physiology and Biochemistry*, vol. 36, no. 2, pp. 631–641, 2015.

[30] L. E. Prestes-Carneiro, M. T. Shio, P. D. Fernandes, and S. Jancar, "Cross-regulation of iNOS and COX-2 by its products in murine macrophages under stress conditions," *Cellular Physiology and Biochemistry*, vol. 20, no. 5, pp. 283–292, 2007.

[31] Y. Nozhenko, A. M. Rodríguez, and A. Palou, "Leptin rapidly induces the expression of metabolic and myokine genes in C2C12 muscle cells to regulate nutrient partition and oxidation," *Cellular Physiology and Biochemistry*, vol. 35, no. 1, pp. 92–103, 2015.

[32] A. V. Sirotkin and R. Grossmann, "Leptin directly controls proliferation, apoptosis and secretory activity of cultured chicken ovarian cells," *Comparative Biochemistry and Physiology Part A, Molecular & Integrative Physiology*, vol. 148, no. 2, pp. 422–429, 2007.

[33] P. J. Pradelles, J. Grassi, and J. Maclouf, "Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay," *Analytical Chemistry*, vol. 57, no. 7, pp. 1170–1173, 1985.

[34] M. A. Landgraf, C. Tostes Rde, P. Borelli et al., "Mechanisms involved in the reduced leukocyte migration in intrauterine undernourishment," *Nutrition*, vol. 23, no. 2, pp. 145–156, 2007.

[35] R. A. Loiola, T. C. Torres, C. M. Aburaya et al., "Generation and characterization of a spontaneously immortalized endothelial cell line from mice microcirculation," *Experimental Cell Research*, vol. 319, no. 8, pp. 1102–1110, 2013.

[36] C. Breton, M. A. Lukaszewski, P. Y. Risold et al., "Maternal prenatal undernutrition alters the response of POMC neurons to energy status variation in adult male rat offspring," *American Journal of Physiology Endocrinology and Metabolism*, vol. 296, no. 3, pp. E462–E472, 2009.

[37] B. Coupe, V. Amarger, I. Grit, A. Benani, and P. Parnet, "Nutritional programming affects hypothalamic organization and early response to leptin," *Endocrinology*, vol. 151, no. 2, pp. 702–713, 2010.

[38] A. R. Frisancho, "Reduced rate of fat oxidation: a metabolic pathway to obesity in the developing nations," *American Journal of Human Biology*, vol. 15, no. 4, pp. 522–532, 2003.

[39] C. J. Jackson, P. K. Garbett, B. Nissen, and L. Schrieber, "Binding of human endothelium to Ulex europaeus I-coated dynabeads: application to the isolation of microvascular endothelium," *Journal of Cell Science*, vol. 96, pp. 257–262, 1990.

[40] Z. M. Ruggeri and J. Ware, "von Willebrand factor," *The FASEB Journal*, vol. 7, no. 2, pp. 308–316, 1993.

[41] R. Señarís, T. Garcia-Caballero, X. Casabiell et al., "Synthesis of leptin in human placenta," *Endocrinology*, vol. 138, no. 10, pp. 4501–4504, 1997.

[42] A. Bado, S. Levasseur, S. Attoub et al., "The stomach is a source of leptin," *Nature*, vol. 394, no. 6695, pp. 790–793, 1998.

[43] L. Jin, B. G. Burguera, M. E. Couce et al., "Leptin and leptin receptor expression in normal and neoplastic human pituitary: evidence of a regulatory role for leptin on pituitary cell proliferation," *Journal of Clinical Endocrinology and Metabolism*, vol. 84, no. 8, pp. 2903–2911, 1999.

[44] J. Wang, R. Liu, M. Hawkins, N. Barzilai, and L. Rossetti, "A nutrient-sensing pathway regulates leptin gene expression in muscle and fat," *Nature*, vol. 393, no. 6686, pp. 684–688, 1998.

[45] V. De Rosa, C. Procaccini, G. Calì et al., "A key role of leptin in the control of regulatory T cell proliferation," *Immunity*, vol. 26, no. 2, pp. 241–255, 2007.

[46] W. W. Pan and M. G. Myers Jr., "Leptin and the maintenance of elevated body weight," *Nature Reviews Neuroscience*, vol. 19, no. 2, pp. 95–105, 2018.

[47] R. S. Berman, J. D. Frew, and W. Martin, "Endotoxin-induced arterial endothelial barrier dysfunction assessed by an in vitro model," *British Journal of Pharmacology*, vol. 110, no. 4, pp. 1282–1284, 1993.

[48] A. Bierhaus, J. Chen, B. Liliensiek, and P. P. Nawroth, "LPS and cytokine-activated endothelium," *Seminars in Thrombosis and Hemostasis*, vol. 26, no. 05, pp. 571–588, 2000.

[49] M. Rola-Pleszczynski and J. Stankova, "Cytokine gene regulation by PGE(2), LTB(4) and PAF," *Mediators of Inflammation*, vol. 1, no. 1, 8 pages, 1992.

[50] S. K. Juhn, M. K. Jung, M. D. Hoffman et al., "The role of inflammatory mediators in the pathogenesis of otitis media and sequelae," *Clinical and Experimental Otorhinolaryngology*, vol. 1, no. 3, pp. 117–138, 2008.

[51] M. Bustus, T. M. Coffman, S. Saadi, and P. Jeffrey, "Modulation of eicosanoid metabolism in endothelial cells in a xenograft model. Role of cyclooxygenase," *The Journal of Clinical Investigation*, vol. 100, no. 5, pp. 1150–1158, 1997.

[52] G. A. Duque and A. Descoteaux, "Macrophage cytokines: involvement in immunity and infectious diseases," *Frontiers in Immunology*, vol. 5, p. 491, 2014.

[53] F. A. Gultekin, M. Kerem, E. Tatlicioglu, A. Aricioglu, C. Unsal, and N. Bukan, "Leptin treatment ameliorates acute lung injury in rats with cerulein-induced acute pancreatitis," *World Journal of Gastroenterology*, vol. 13, no. 21, pp. 2932–2938, 2007.

[54] S. Loffreda, S. Q. Yang, H. Z. Lin et al., "Leptin regulates proinflammatory immune responses," *The FASEB Journal*, vol. 12, no. 1, pp. 57–65, 1998.

[55] J. Santos-Alvarez, R. Goberna, and V. Sanchez-Margalet, "Human leptin stimulates proliferation and activation of human circulating monocytes," *Cellular Immunology*, vol. 194, no. 1, pp. 6–11, 1999.

[56] N. Kiguchi, T. Maeda, Y. Kobayashi, Y. Fukazawa, and S. Kishioka, "Leptin enhances CC-chemokine ligand expression in cultured murine macrophage," *Biochemical and Biophysical Research Communications*, vol. 384, no. 3, pp. 311–315, 2009.

[57] G. Ramesh, A. G. MacLean, and M. T. Philipp, "Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain," *Mediators of Inflammation*, vol. 2013, Article ID 480739, 20 pages, 2013.

[58] C. T. Montague, I. S. Farooqi, J. P. Whitehead et al., "Congenital leptin deficiency is associated with severe early-onset obesity in humans," *Nature*, vol. 387, no. 6636, pp. 903–908, 1997.

[59] I. S. Farooqi, G. Matarese, G. M. Lord et al., "Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency," *The Journal of Clinical Investigation*, vol. 110, no. 8, pp. 1093–1103, 2002.

[60] L. A. Tartaglia, M. Dembski, X. Weng et al., "Identification and expression cloning of a leptin receptor, OB-R," *Cell*, vol. 83, no. 7, pp. 1263–1271, 1995.

[61] A. Bellmeyer, J. M. Martino, N. S. Chandel, G. R. Scott Budinger, D. A. Dean, and G. M. Mutlu, "Leptin resistance protects mice from hyperoxia-induced acute lung injury," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 6, pp. 587–594, 2007.

[62] C. Bjorbaek, S. Uotani, B. da Silva, and J. S. Flier, "Divergent signaling capacities of the long and short isoforms of the leptin receptor," *The Journal of Biological Chemistry*, vol. 272, no. 51, pp. 32686–32695, 1997.

[63] Y. B. Kim, S. Uotani, D. D. Pierroz, J. S. Flier, and B. B. Kahn, "In vivo administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin," *Endocrinology*, vol. 141, no. 7, pp. 2328–2339, 2000.

[64] M. G. Myers Jr., "Leptin receptor signaling and the regulation of mammalian physiology," *Recent Progress in Hormone Research*, vol. 59, no. 1, pp. 287–304, 2004.

[65] M. J. Sweet and D. A. Hume, "Endotoxin signal transduction in macrophages," *Journal of Leukocyte Biology*, vol. 60, no. 1, pp. 8–26, 1996.

[66] H. Yang, D. W. Young, F. Gusovsky, and J. C. Chow, "Cellular events mediated by lipopolysaccharide-stimulated Toll-like receptor 4," *The Journal of Biological Chemistry*, vol. 275, no. 27, pp. 20861–20866, 2000.

[67] L. F. Sun, D. Q. An, G. L. Niyazi, W. H. Ma, Z. W. Xu, and Y. Xie, "Effects of Tianxiangdan Granule treatment on atherosclerosis via NF-κB and p38 MAPK signaling pathways," *Molecular Medicine Reports*, vol. 17, no. 1, pp. 1642–1650, 2018.

[68] M. R. Indra, S. Karyono, R. Ratnawati, and S. G. Malik, "Quercetin suppresses inflammation by reducing ERK1/2 phosphorylation and NF kappa B activation in leptin-induced human umbilical vein endothelial cells (HUVECs)," *BMC Research Notes*, vol. 6, no. 1, p. 275, 2013.

[69] A. M. Balbino, M. M. Silva, G. A. Azevedo, N. L. Gil, M. A. Landgraf, and R. G. Landgraf, "SUN-LB337: Lung endothelial cells from intrauterine malnutrition-induced low-birth-weight rats fail to express the leptin receptor (OBRB) and down regulate inflammatory responses," *Clinical Nutrition*, vol. 36, no. 1, article S179, Supplement 1, 2017.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3062319, 12 pages
https://doi.org/10.1155/2018/3062319

Research Article

Protective Effect of Sex Hormone-Binding Globulin against Metabolic Syndrome: *In Vitro* Evidence Showing Anti-Inflammatory and Lipolytic Effects on Adipocytes and Macrophages

Hiroki Yamazaki,[1] **Akifumi Kushiyama**,[2] **Hideyuki Sakoda**,[3] **Midori Fujishiro**,[4] **Takeshi Yamamotoya**,[5] **Yusuke Nakatsu**,[5] **Takako Kikuchi**,[2] **Sunao Kaneko**,[6] **Hirotoshi Tanaka**,[1] **and Tomoichiro Asano**[5]

[1]*Department of Rheumatology and Allergy, IMSUT Hospital, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[2]*Division of Diabetes and Metabolism, Institute for Adult Diseases, Asahi Life Foundation, 2-2-6, Bakuro-cho, Chuo-ku, Tokyo 103-0002, Japan*
[3]*Division of Neurology, Respirology, Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake-cho, Miyazaki, Miyazaki 889-1692, Japan*
[4]*Division of Diabetes and Metabolic Diseases, Nihon University School of Medicine, 30-1, Oyaguchi Kami-cho, Itabashi-ku, Tokyo 173-8610, Japan*
[5]*Department of Medical Science, Graduate School of Medicine, University of Hiroshima, 1-2-3 Kasumi, Minami-ku, Hiroshima, Hiroshima 734-8551, Japan*
[6]*Department of Diabetes and Metabolic Diseases, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Correspondence should be addressed to Tomoichiro Asano; tasano@hiroshima-u.ac.jp

Received 20 February 2018; Revised 14 May 2018; Accepted 16 May 2018; Published 25 June 2018

Academic Editor: Joilson O. Martins

Sex hormone-binding globulin (SHBG) is a serum protein released mainly by the liver, and a low serum level correlates with a risk for metabolic syndrome including diabetes, obesity, and cardiovascular events. However, the underlying molecular mechanism(s) linking SHBG and metabolic syndrome remains unknown. In this study, using adipocytes and macrophages, we focused on the *in vitro* effects of SHBG on inflammation as well as lipid metabolism. Incubation with 20 nM SHBG markedly suppressed lipopolysaccharide- (LPS-) induced inflammatory cytokines, such as MCP-1, TNFα, and IL-6 in adipocytes and macrophages, along with phosphorylations of JNK and ERK. Anti-inflammatory effects were also observed in 3T3-L1 adipocytes cocultured with LPS-stimulated macrophages. In addition, SHBG treatment for 18 hrs or longer significantly induced the lipid degradation of differentiated 3T3-L1 cells, with alterations in its corresponding gene and protein levels. Notably, these effects of SHBG were not altered by coaddition of large amounts of testosterone or estradiol. In conclusion, SHBG suppresses inflammation and lipid accumulation in macrophages and adipocytes, which might be among the mechanisms underlying the protective effect of SHBG, that is, its actions which reduce the incidence of metabolic syndrome.

1. Introduction

SHBG is a 40–50 kDa protein mainly synthesized in the liver and secreted into the bloodstream. This protein is comprised of two laminin G-like (LG) domains [1], and the molecular weights of serum SHBG proteins are partially dependent on their glycosylation status [2]. The conventional roles of SHBG involve transporting sex hormones and the regulation

of hormone dynamics [3]. Numerous studies have confirmed the relationship between the serum SHBG concentration and metabolic syndrome. Low SHBG concentrations correlate with higher levels of serum inflammatory markers [4, 5]. Relatively low levels of SHBG are also a risk factor for obesity, metabolic syndrome, and diabetes [6–10]. Thus, the serum SHBG concentration has been regarded as a biomarker for metabolic syndrome.

On the other hand, interestingly, db/db mice overexpressing human SHBG reportedly show resistance to the development of obesity and hepatosteatosis [11, 12]. In addition, a single nucleotide polymorphism related to an elevated plasma SHBG concentration reportedly correlates with a reduced risk of diabetes [13]. The hormone-like effect of SHBG has also been demonstrated in experiments using prostatic cells [14, 15], MCF-7 breast cancer cells [16], cytotrophoblasts [17], proximal tubule epithelial cells [18, 19], and hepatocytes [12]. The results of these previous studies led us to speculate that the serum SHBG level is not only simply a consequence of altered metabolic conditions but also exerts favorable effects protecting against the development of metabolic disorders.

In this study, using adipocytes and macrophages, we focused on the *in vitro* effects of SHBG in inflammation as well as lipid metabolism, since lipid accumulation and inflammation are both necessary for the development of metabolic syndrome. Herein, we present evidence of the favorable actions of SHBG in adipocytes and macrophages.

2. Materials and Methods

2.1. Chemicals and Reagents. SHBG protein was purchased from two companies, Abcam (ab151275) and Fitzgerald Industries (30-AS40). According to the explanations provided by these manufacturers, SHBG protein was purified from human sera, and its purity exceeded 90%. While we confirmed the effects of SHBG from these two companies to be the same, the data presented herein were those obtained with the SHBG from Abcam. In addition, we measured the amounts of testosterone and estradiol contaminating the SHBG protein, since no information was given regarding this issue in the materials provided by the manufacturers. Sex hormones were measured using the ELISA kits for testosterone and estradiol (Cayman) according to the manufacturer's instructions. Diethyl ether was added to the SHBG protein samples and mixed thoroughly with a vortex. The upper ether layer was collected using a pasteur pipette and transferred into a clean tube. This extraction procedure was repeated four times. After evaporating the combined ether extracts, the samples were dissolved in the buffer and subjected to analysis with the ELISA kits.

Lipopolysaccharide (LPS) (from *Escherichia coli* 0111:B4) was purchased from Sigma. Recombinant murine TNFα was purchased from Genzyme (3410T). Anti-β-actin antibody (sc-1616), anti-CCAAT/enhancer binding protein α (CEBPα) antibody (sc-61), and horseradish peroxidase- (HRP-) labeled anti-goat IgG antibody (sc-2020) were from Santa Cruz Biotechnology. Anti-TNFα antibody (#11948), anti-stress-activated c-Jun amino-terminal kinase (JNK)1/2 antibody (#9252), anti-phospho-extracellular signal-related kinase (ERK)1/2 (Thr202/Tyr204) antibody (#9101), anti-ERK1/2 antibody (#9102), anti-adipose triglyceride lipase (ATGL) antibody (#2439), HRP-labeled anti-rabbit (#7074), and anti-mouse IgG antibody (#7076) were all from Cell Signaling Technology. Anti-phospho-JNK1/2 (Thr183/Tyr185) antibody was obtained from BD Biosciences (#562480).

2.2. 3T3-L1 Cell Culture and Differentiation. 3T3-L1 cells were differentiated as previously described [20, 21] with some modifications. Briefly, 3T3-L1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Wako) containing 10% donor calf serum (Invitrogen) in a 5–10% CO_2 incubator. For the experiments, cells were spread onto collagen type I coated plates (Iwaki) and induced to differentiate with DMEM containing 10% fetal calf serum (FCS) (Biowest), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 4 μg/ml dexamethasone (Sigma), and 167 nM insulin (Sigma). Two days later, the media were replaced with DMEM containing 10% FCS and 167 nM insulin. After another two days, the media were replaced with DMEM containing 10% FCS and the media were then changed every other day. Penicillin-streptomycin (Invitrogen) was added to all media at a 0.5% concentration.

For experiments on mature 3T3-L1 cells, we used cells that had been differentiating for more than 6 days [22]. Mature adipocytes were treated with SHBG proteins in phenol red-free DMEM (Wako) containing 0.2% fatty acid-free bovine serum albumin (BSA) (Wako). The concentration of BSA was much higher than that of SHBG protein. We used phenol red-free media to eliminate estrogen-like effects of phenol red [23].

For experiments evaluating inflammatory cytokine levels in adipocytes, 3T3-L1 cells were pretreated overnight with 20 nM SHBG, followed by stimulation with 1 ng/ml LPS or 1 ng/ml TNFα for 12–24 hrs. In some experiments, 1 or 20 μM testosterone (T) (Wako, 208-08341) or 17β-estradiol (E_2) (Sigma, E8875) was coadded with 0–20 nM of SHBG protein. Considering the amounts and the reported association constants of T and E_2 with SHBG ($1.6 \times 10^9\,M^{-1}$ and $6.8 \times 10^8\,M^{-1}$, resp. [24]), it was assumed that more than 99% of SHBG would form a complex with T or E_2.

2.3. Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using Transcriptor Universal cDNA Master (Roche) followed by RT-PCR employing LightCycler 480 SYBR Green I Master (Roche). Sequences of the primers used in this study are listed in Table 1. The 36B4 mRNA level served as the internal control.

2.4. Preparation of Mouse Peritoneal Macrophages. The isolation protocol was reported previously [39, 40] and achieved a final cell population comprised of more than 90% macrophages. We employed this protocol, with a slight modification. Peritoneal macrophages were collected from C57BL/6N mice. RPMI 1640 media without phenol red (Gibco) was used, and the cells were incubated at 37°C in a

Table 1: Primer sequences used for this study.

Gene	Forward primer ($5' \rightarrow 3'$)	Reverse primer ($5' \rightarrow 3'$)	Reference
36B4	GCTCCAAGCAGATGCAGCA	CCGGATGTGAGGCAGCAG	[25]
Mcp-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG	[26]
Tnfa	GAACTGGCAGAAGAGGCACT	AGGGTCTGGGCCATAGAACT	[26]
Il-6	TCGTGGAAATGAGAAAAGAGTTG	AGTGCATCATCGTTGTTCATACA	[27]
Cebpa	TGAGCCGTGAACTGGACACG	CAGCCTAGAGATCCAGCGAC	[28]
Pparg	TCTTCCATCACGGAGAGGTC	GATGCACTGCCTATGAGCAC	[28]
Srebp1	AAGCAAATCACTGAAGGACCTGG	AAAGACAAGGGGCTACTCTGGGAG	[29]
Fas	ATCCTGGAACGAGAACACGATCT	AGAGACGTGTCACTCCTGGACTT	[30]
Acsl1	GACGACCTCAAGGTGCTTCA	ACCCAGGCTCGACTGTATCT	—
Pepck	CTAACTTGGCCATGATGAACC	CTTCACTGAGGTGCCAGGAG	[31]
Pgc1b	GCTCTGACGCTCTGAAGGAC	CACCGAAGTGAGGTGCTTATG	[30]
Hsl	CAGTGCCTATTCAGGGACAGAG	CACTCCTGCGCATAGACTCC	—
Mgl	AGGCGAACTCCACAGAATGTT	AGCCAGCTCATCATAACGGC	—
Acrp30	GCTCCTGCTTTGGTCCCTCCAC	GCCCTTCAGCTCCTGTCATTCC	[32]
Glut4	CAGCTCTCAGGCATCAAT	TCTACTAAGAGCACCGAG	[33]
Fabp4	TGGGAACCTGGAAGCTTGTC	CTTTCCTTGTGGCAAAGCCC	—
Atgl	AACACCAGCATCCAGTTCAA	GGTTCAGTAGGCCATTCCTC	[34]
Ucp2	CTACAAGACCATTGCACGAGAGG	AGCTGCTCATAGGTGACAAACAT	[35]
Agt	AGGTTGGCGCTGAAGGATAC	GATGTATACGCGGTCCCCAG	[36]
Pgc1a	GCCCGGTACAGTGAGTGTTC	CTGGGCCGTTTAGTCTTCCT	[30]
Cebpb	CAAGCTGAGCGACGAGTACA	AGCTGCTCCACCTTCTTCTG	[37]
Ppara	CGGGAAAGACCAGCAACAAC	TGGCAGCAGTGGAAGAATCG	[30]
Chrebp	GATGGTGCGAACAGCTCTTCT	CTGGGCTGTGTCATGGTGAA	[30]
Ucp1	GATGGTGAACCCGACAACTT	CTGAAACTCCGGCTGAGAAG	[38]
Cpt1a	GACTCCGCTCGCTCATTCC	ACCAGTGATGATGCCATTCTTG	—

5% CO_2 incubator. Macrophages were collected by injection of 5 ml of RPMI 1640 media containing 10% FCS intraperitoneally under diethyl ether anesthesia and then left on ice until centrifugation. After centrifugation at 1500 rpm for 2 minutes at room temperature, the supernatant was removed, and hemolysis buffer (BD PharmLyse) was added to remove the red blood cells. After 2 minutes, we centrifuged the samples at 1500 rpm for 2 minutes at room temperature and the supernatant was removed. Cells were seeded at a density of 1.5×10^6 cells/well in a 12-well plate in RPMI 1640 media containing 10% FCS. Two hours later, the cells were gently washed twice with RPMI 1640 media to remove nonadherent cells and the medium was then replaced with RPMI 1640 containing 0.2% fatty acid-free BSA for the experiments. For experiments evaluating inflammatory cytokine levels in macrophages, cells were pretreated with 20 nM SHBG overnight, followed by 1 ng/ml LPS for 0–8 hrs.

2.5. Immunoblotting Analysis. The cells were solubilized with Laemmli buffer (0.2 M Tris·HCl, 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.1% bromophenol blue) containing 100 mM dithiothreitol. Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE. Then, the proteins were transferred to Immobilon (Millipore), blocked with 1% BSA (Intergen), reacted with the primary antibodies and subsequently with the HRP-labeled secondary antibodies. Chemiluminescence was detected using an ImageQuant LAS 4000 mini (Fuji Film).

2.6. Coculture System of 3T3-L1 Adipocytes and Murine Macrophages. The 3T3-L1 adipocytes and murine macrophages were cocultured in a transwell system (Corning, Acton, MA) with a 0.4 μm porous membrane to separate the upper and lower chambers. Mouse peritoneal macrophages were harvested and seeded at a density of 1.5×10^6 cells/well in the upper chamber, while differentiated 3T3-L1 cells were in the lower chamber. Both macrophages and differentiated 3T3-L1 cells were washed with RPMI 1640 medium, and the culture medium was then replaced with RPMI 1640 containing 0.2% fatty acid-free BSA for the experiments. SHBG at the 20 nM concentration was added to both the upper and the lower chambers, and coculture was then started. After incubation overnight, 100 pg/ml LPS was added and the cells were collected 12 hrs later.

2.7. Lipid Staining. Differentiated 3T3-L1 cells were treated with 0–100 nM SHBG protein in serum-free media and incubated for 3 days, followed by Oil Red O staining or Nile Red staining.

Oil Red O (Sigma) was dissolved in isopropanol to assure that the concentration would be 0.3%. This stock solution was mixed with distilled water (3 : 2), followed by incubation

for 30 minutes, and filtered with 0.45 μm before use. The cells were washed twice with PBS and fixed with 10% neutral buffered formalin for 10 minutes. After the cells were washed twice with PBS, Oil Red O working solution was added followed by another 10-minute incubation and then washed with PBS. Images were taken using a light microscope FSX100 (Olympus).

Nile Red (AdipoRed, Lonza) becomes fluorescent when it is partitioned in a hydrophobic environment and shows selective fluorescence for intracellular lipid droplets [41]. The staining protocol was carried out according to the manufacturer's instructions. Fluorescence with excitation at 485 nm and emission at 535 nM was measured using ARVO MX-fla (PerkinElmer).

2.8. Measurement of Glycerol in the Culture Medium. Differentiated 3T3-L1 cells were treated with 20 nM SHBG protein for 18 or 35 hrs. Glycerol concentrations in culture media were measured employing a Glycerol Assay Kit (Sigma). With this kit, the glycerol concentration is determined by a coupled enzyme assay involving glycerol kinase and glycerol phosphate oxidase, resulting in a colorimetric product.

2.9. cAMP Measurement. Differentiated 3T3-L1 cells were treated with 20 nM SHBG protein or 10 μM isoproterenol for 1 or 18 hrs. Intracellular cAMP concentrations were measured using a cAMP EIA kit (Cayman). This assay is based on the competition between free cAMP and a cAMP tracer. Measurements were carried out according to the manufacturer's instructions. Stimulation with isoproterenol (Sigma) was used to confirm the production of cAMP.

2.10. Data Analysis. All data are presented as the means ± standard deviation (S.D.). The differences between two groups were evaluated by t-test. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. SHBG Suppressed LPS- or TNFα-Induced Inflammatory Cytokine Levels in Mouse Peritoneal Macrophages and Differentiated 3T3-L1 Cells. First of all, we measured the concentrations of testosterone and 17β-estradiol contaminating the SHBG protein purchased from Abcam, to exclude the possibility of its functions being attributable to these sex hormones. The results obtained with the ELISA assay kits revealed that Abcam's SHBG protein contained molar ratios of 1 : 5600 and 1 : 10000 for testosterone and estradiol, respectively, to SHBG. Therefore, it is unlikely that the contaminant testosterone and 17β-estradiol contributed to the results obtained using Abcam's SHBG protein in this study.

Murine macrophages were stimulated with or without 1 ng/ml LPS for 8 hrs, and the effects of 20 nM SHBG were examined. While LPS markedly raised mRNA levels of monocyte chemoattractant protein-1 (MCP-1), TNFα, and IL-6, SHBG suppressed them under both basal and LPS-stimulated conditions in the approximate range of 50–90% (Figure 1(a)).

Next, the effects of SHBG on the signal transductions leading to inflammatory cytokine levels were investigated. Maximal phosphorylations of JNK1/2 and ERK1/2 occurred at 30 min after the addition of 1 ng/ml LPS, while the intracellular TNFα content peaked around 2 hrs. In the presence of 20 nM SHBG, LPS-induced phosphorylations of JNK1/2 and ERK1/2 as well as TNFα production were reduced. The band intensity of TNFα normalized by β-actin at 2 hrs, phosphorylation of JNK normalized by JNK at 1 hr, and phosphorylation of ERK normalized by ERK at 1 hr were significantly decreased (Figure 1(b)).

Similarly, the effects of SHBG on MCP-1 and IL-6 levels in 3T3-L1 adipocytes were investigated, by stimulating these cells with LPS or TNFα and comparing the results to those in adipocytes without stimulation. It was revealed that 20 nM SHBG markedly suppressed LPS-induced MCP-1 and IL-6 mRNA upregulations as well as TNFα-induced MCP-1 levels (Figure 1(c)). These results indicate that SHBG exerts anti-inflammatory effects directly on macrophages and adipocytes.

3.2. Inflammatory Cytokine Levels Were Also Suppressed in the Coculture System of Peritoneal Macrophages and 3T3-L1 Adipocytes. We cocultured 3T3-L1 adipocytes and murine macrophages using a transwell system. Then, LPS was added, and the resulting cytokine levels in both 3T3-L1 adipocytes and macrophages were compared between the presence and the absence of 20 nM SHBG. In this experiment, SHBG exerted inhibitory effects on basal cytokine levels in 3T3-L1 adipocytes. Notably, the addition of 20 nM SHBG markedly suppressed LPS-induced MCP-1 and IL-6 levels in 3T3-L1 adipocytes (Figure 2(a)), as well as MCP-1, TNFα, and IL-6 levels in murine macrophages (Figure 2(b)). Although the optimal medium for 3T3-L1 cells is DMEM, coculturing in RPMI did not apparently impair the functions of 3T3-L1 cells.

3.3. SHBG Reduced the Lipid Accumulation in 3T3-L1 Adipocytes. Differentiated 3T3-L1 cells were treated with SHBG proteins at the indicated concentrations in serum-free media and incubated for 3 days and followed by Oil Red O staining (Figure 3(a)). It was revealed that SHBG protein reduced lipid accumulation in 3T3-L1 adipocytes in a concentration-dependent manner. Glycerol concentrations in the culture media were increased in the presence of 20 nM SHBG for 18 or 35 hrs (Figure 3(b)), which suggests lipolysis to be enhanced by SHBG. It was found that SHBG did not alter the intracellular cAMP concentration, in contrast to the marked cAMP increase induced by isoproterenol (Figure 3(c)). Interestingly, treatment with 20 nM SHBG proteins for 3 days markedly reduced CEBPα and increased ATGL proteins (Figure 3(d)).

3.4. SHBG Altered the mRNA Levels Related to Lipid Metabolism in Differentiated 3T3-L1 Adipocytes. Differentiated 3T3-L1 cells were treated with 20 nM SHBG protein for 18 hrs, and mRNA levels were measured by RT-PCR. Importantly, mRNA levels of CEBPα, peroxisome proliferator-activated receptor γ (PPARγ), and sterol regulatory element-binding protein 1 (SREBP1), gene encoding key transcriptional factors for adipogenic differentiation

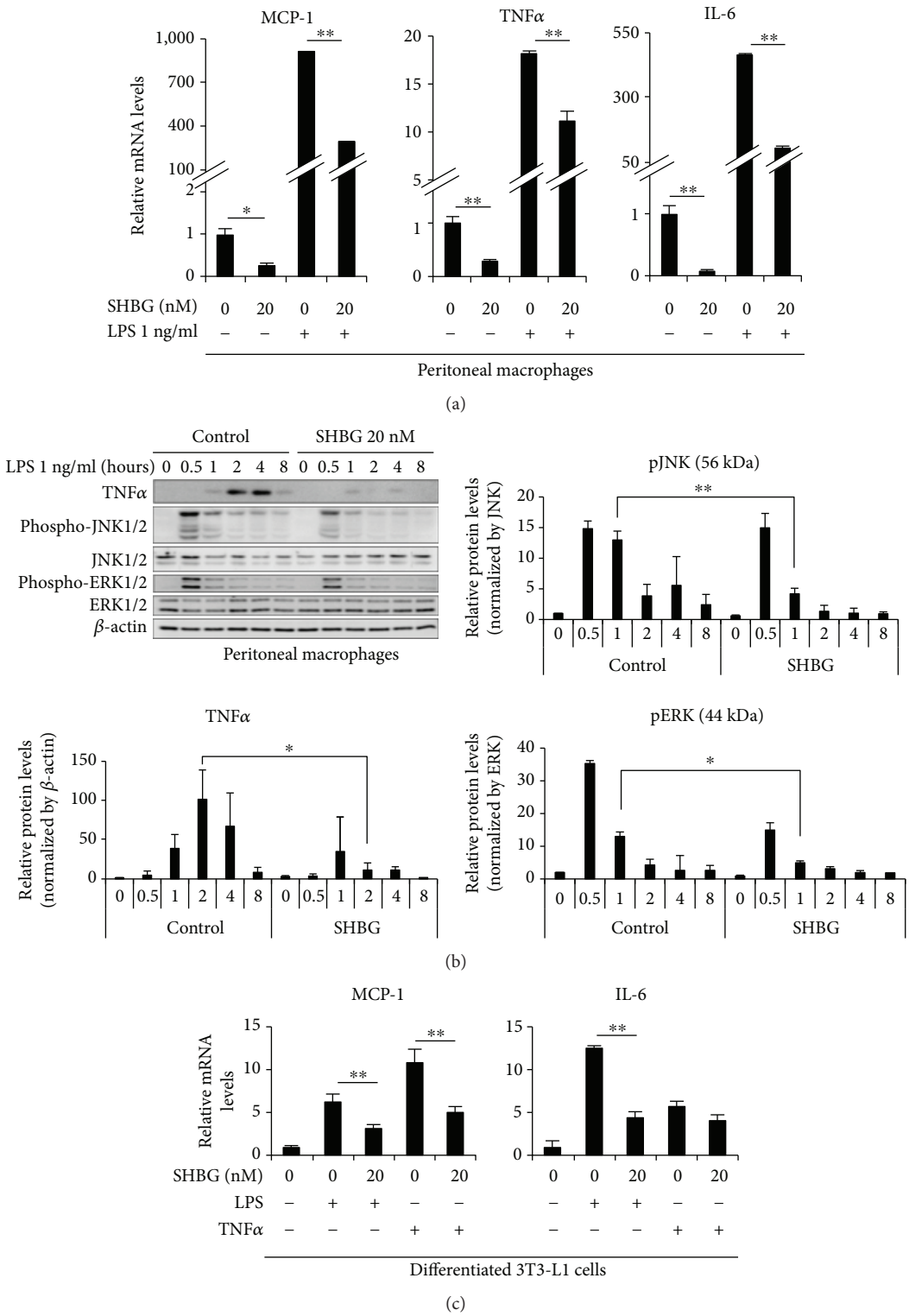


Figure 1: SHBG inhibits inflammatory cytokine levels in peritoneal macrophages and differentiated 3T3-L1 cells. (a) Peritoneal macrophages from C57BL/6 mice were treated with SHBG overnight, followed by 1 ng/ml LPS stimulation for 8 hrs. mRNA levels of inflammatory cytokines were measured by RT-PCR. Student's *t*-test was performed. Data are the means ± S.D. ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$). (b) Peritoneal macrophages from C57BL/6 mice were treated with SHBG protein overnight, followed by 1 ng/ml LPS stimulation for the indicated times. Inflammatory signaling was evaluated by Western blotting. Each band was quantified using ImageJ. Relative intensities are shown. Data are the means ± S.D. ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (c) Differentiated 3T3-L1 cells were treated with SHBG proteins overnight, followed by 1 ng/ml LPS or 1 ng/ml TNFα stimulation for 24 hrs. mRNA levels of MCP-1 and IL-6 were measured by RT-PCR. Student's *t*-test was performed. Data are the means ± S.D. ($n = 3$, $^{**}p < 0.01$).

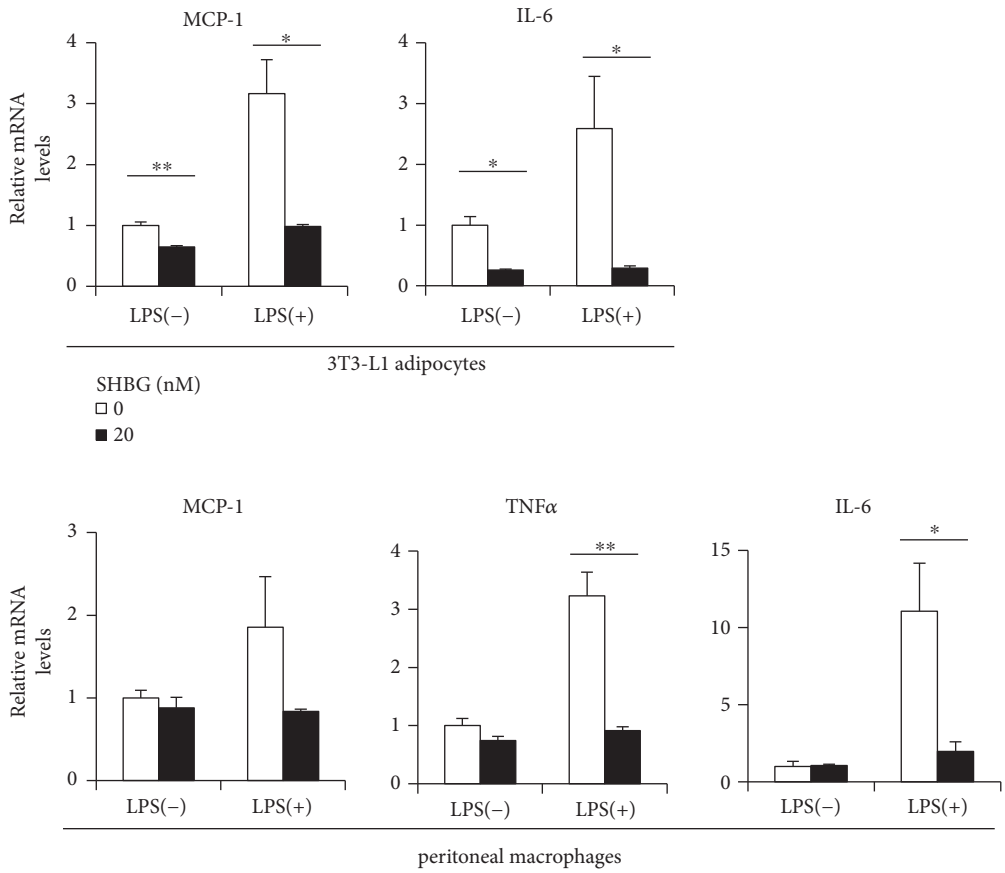


Figure 2: SHBG inhibits inflammatory cytokine levels in 3T3-L1 cells cocultured with peritoneal macrophages. Differentiated 3T3-L1 cells and peritoneal macrophages from C57BL/6 were cocultured using a transwell system overnight, in culture media with or without SHBG protein. Thereafter, we added 100 pg/ml LPS to the culture media and cells were collected 12 hrs later. mRNA levels of inflammatory cytokines in each cell were measured by RT-PCR. Student's t-test was performed. Data are the means ± S.D. ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

and triglyceride synthesis, are significantly downregulated by 18 hrs of incubation with 20 nM SHBG. The genes downregulated by SHBG included fatty acid synthase (FAS), acyl-CoA synthetase 1 (ACSL1), phosphoenolpyruvate carboxykinase (PEPCK), PPARγ co-activator-1β (PGC1β), hormone-sensitive lipase (HSL), monoacylglycerol lipase (MGL), adipocyte complement-related protein of 30 kDa (ACRP30), glucose transporter type 4 (GLUT4), and fatty acid binding protein 4 (FABP4), while ATGL, uncoupling protein-2 (UCP2), and angiotensinogen (AGT) were all upregulated. PGC1α, CEBPβ, PPARα, carbohydrate response element binding protein (ChREBP), UCP1, and carnitine palmitoyltransferase 1A (CPT1A) were not significantly changed. Taken together, these observations raise the possibility that SHBG induces dedifferentiation via downregulation of its key transcription factors and lipid metabolism genes (Figure 4).

3.5. Coincubations with an Excess of Testosterone or 17β-Estradiol Did Not Affect the Function of SHBG. Differentiated 3T3-L1 cells were treated with 20 nM SHBG in the presence or absence of 1 μM testosterone or 17β-estradiol overnight and then stimulated with 1 ng/ml LPS for 12 hrs. The suppressive effects of SHBG on MCP-1 and IL-6 levels were unaffected by testosterone or 17β-estradiol (Figure 5(a)). Similarly, no significant effect of testosterone or 17β-estradiol on the reduced lipid content by SHBG was observed in 3T3-L1 adipocytes (Figure 5(b)).

4. Discussion

In the present study, it was clearly demonstrated that SHBG exhibits anti-inflammatory effects involving macrophages and adipocytes, as evidenced by suppressed mRNA levels for inflammatory cytokines such as MCP-1, TNFα, and IL-6. MCP-1, which is known to be highly expressed in adipocytes, is related to the induction of chronic inflammation [42]. Chronic inflammation in adipose tissues is reportedly exacerbated by LPS from the intestinal tract accompanied by obesity or high-fat diets [43–45]. In addition, it is very likely that SHBG enhances lipolysis or induces dedifferentiation of mature adipocytes, based on the effects on a series of mRNA level data. Under conditions of obesity, macrophages reportedly infiltrate adipose tissue, and interactions between macrophages and adipocytes occur via a paracrine mechanism [46], which exacerbates the metabolic syndrome phenotype. Our experiments

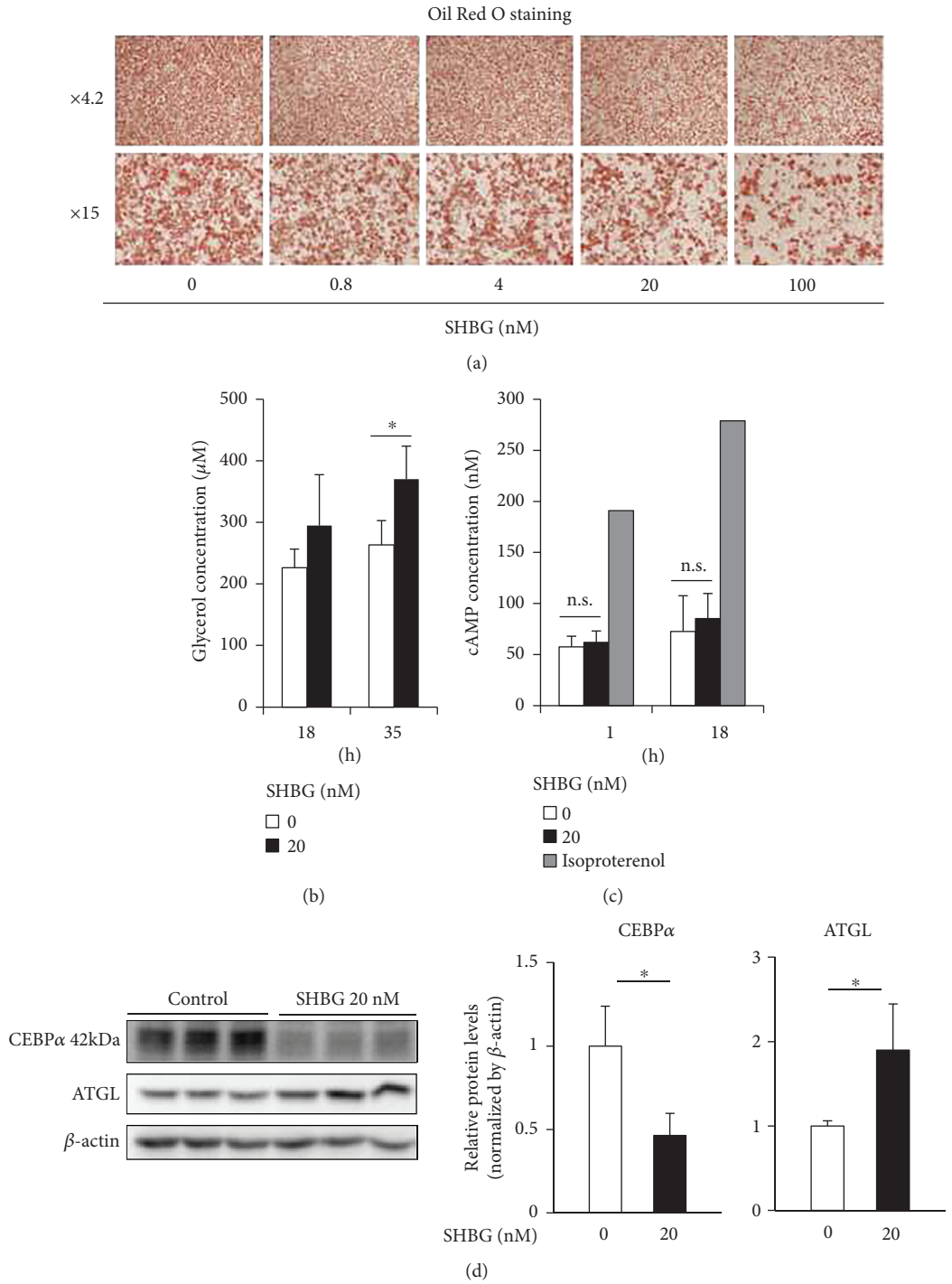


Figure 3: SHBG reduces lipid contents of differentiated 3T3-L1 cells with alterations in corresponding protein levels. (a) Differentiated 3T3-L1 cells were treated with SHBG proteins at the indicated concentrations in serum-free media and incubated for 3 days. Oil Red O staining was performed. Representative fluorescent microscopy images are shown. (b) Differentiated 3T3-L1 cells were treated with 20 nM SHBG proteins for 18 or 35 hrs. Glycerol concentrations in culture media were measured by ELISA. Student's *t*-test was performed. Data are the means ± S.D. ($n = 3$, $^*p < 0.05$). (c) Differentiated 3T3-L1 cells were treated with 20 nM SHBG proteins or 10 μM isoproterenol for 1 or 18 hrs. Intracellular cAMP concentrations were measured. Student's *t*-test was performed. Data are the means ± S.D. (SHBG 0 and 20 nM: $n = 4$). (d) Differentiated 3T3-L1 cells were treated with 20 nM SHBG proteins for 3 days. Protein levels of CEBPα and ATGL were evaluated by Western blotting. Each band was quantified using ImageJ. Relative intensities normalized by β-actin are shown. Data are means ± S.D. ($n = 3$, $^*p < 0.05$).

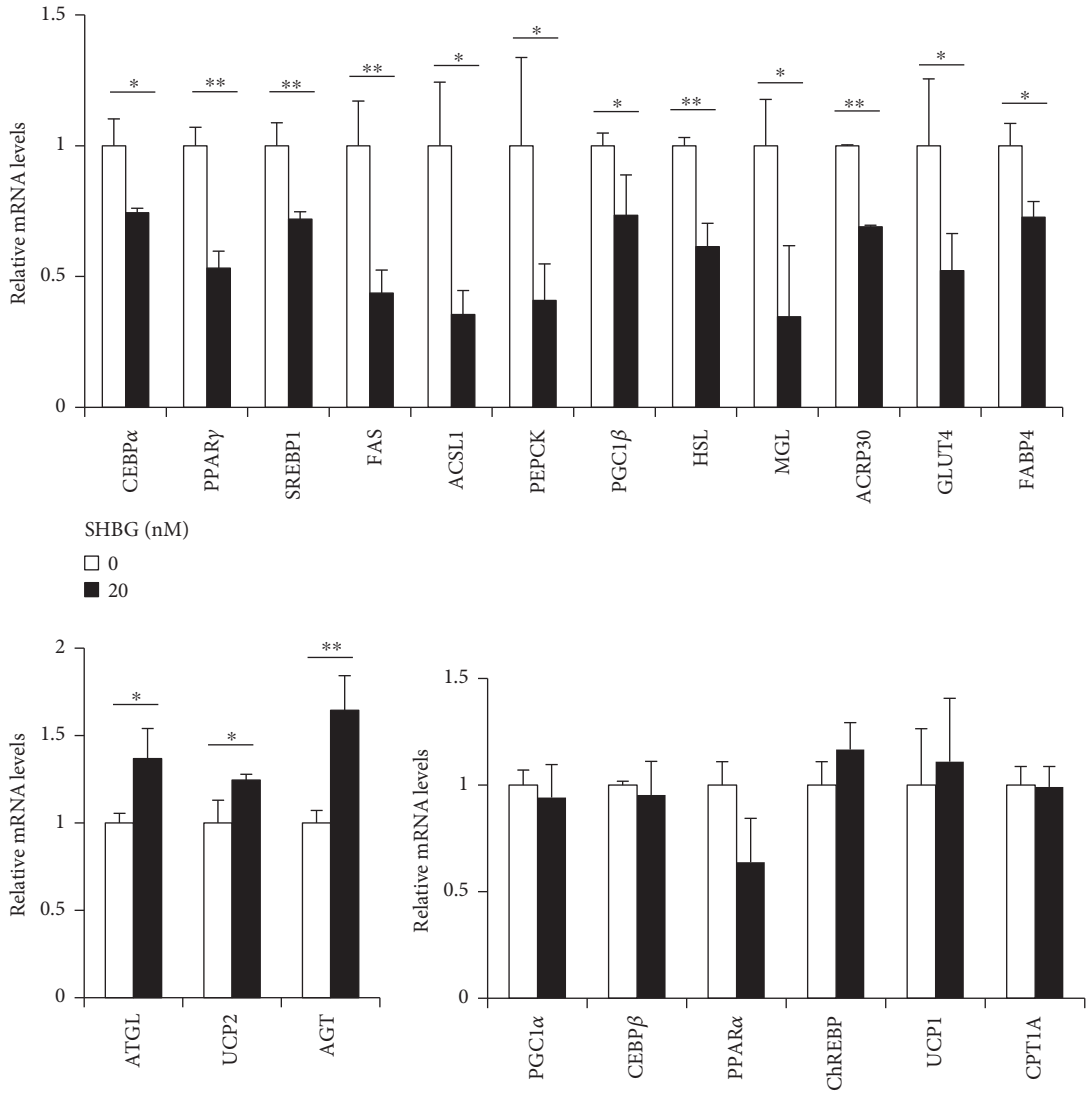


Figure 4: SHBG alters some of the mRNA levels related to lipid metabolism in differentiated 3T3-L1 cells. Differentiated 3T3-L1 cells were treated with 20 nM SHBG proteins for 18 hrs. mRNA levels were measured by RT-PCR. Relative levels normalized by the 36B4 level are shown. Student's t-test was performed. Data are the means ± S.D. ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

using a coculture system yielded results supporting the anti-inflammatory effects of SHBG.

It should be noted that the SHBG concentration used in this study is physiological. The median serum SHBG concentration is 20.8 nM in young adult men, increasing to 44.5 nM with aging [47]. Women have serum SHBG concentrations several times higher than those in men, reaching approximately 100 nM [47, 48]. Thus, the 20 nM mainly used in our experiments is the approximate normal lower limit. Thus, it may be reasonable to regard SHBG as contributing to protection from metabolic syndrome accompanying inflammation and obesity.

Assuming the presence of a specific receptor for SHBG, signal transduction from the SHBG receptor reportedly suppresses the phosphorylations of JNK and ERK, possibly inhibiting the activation of transcriptional factors such as AP-1 [49, 50]. AP-1 regulates MCP-1, a key chemokine for monocyte/macrophage migration and infiltration [51]. Lipolytic actions of SHBG were observed to be accompanied by changes in various mRNA and protein levels. Key transcription factors such as CEBPα, PPARγ, and SREBP1 controlling adipogenesis and lipogenesis were suppressed by SHBG. SHBG might influence the metabolic processes in adipocytes by modulating nutrient usage or hormonal cascades including growth factor signaling. There are many other documented mechanisms of action of SHBG. The increased intracellular cAMP levels in several cells [14, 16, 17] suggest the involvement of G protein and adenylate cyclase, though neither of these responses was observed in our present experiments. SHBG protein itself might not exert activity inducing signaling cascades. For example, SHBG reportedly competes with osteocalcin-induced signaling by binding to GPRC6A [52]. Such chronic and low-grade inhibition or modulation of other protein receptor-mediated processes might be important. Although SHBG is certainly a trace protein in serum, the local concentrations in tissues can be high,

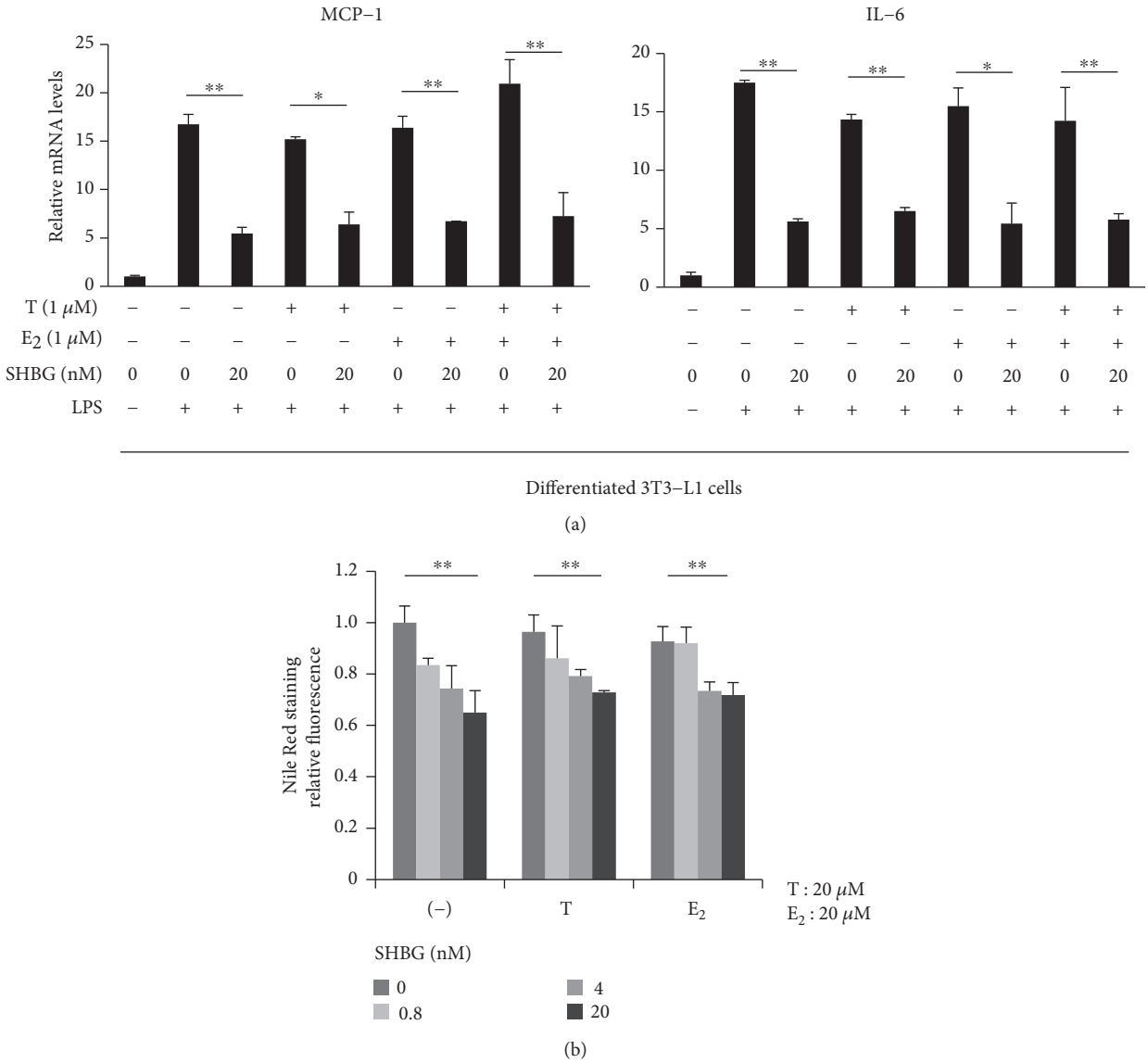


Figure 5: Coincubation with testosterone or 17β-estradiol did not affect the function of SHBG. (a) Differentiated 3T3-L1 cells were treated with SHBG protein in the presence or absence of 1 μM testosterone (T) or 17β-estradiol (E_2) overnight, followed by stimulation with 1 ng/ml LPS for 12 hrs. mRNA levels of MCP-1 and IL-6 were measured by RT-PCR. Student's t-test was performed. Mean ± S.D. ($n = 3$, $^*p < 0.05$, $^{**}p < 0.01$). (b) Differentiated 3T3-L1 cells were treated with SHBG protein at concentrations ranging from 0–20 nM in serum-free media containing 20 μM of testosterone (T) or 17β-estradiol (E_2). Three days later, Nile Red staining was performed and fluorescence was quantified. The Jonckheere test was performed. Mean ± S.D. ($n = 4$, $^{**}p < 0.01$).

considering the finding that the fibulin family sequesters SHBG and possibly controls access of some molecules to target cells [53, 54]. Furthermore, the internalization of SHBG and actions within cells, reportedly enhancing or inhibiting sex hormone actions [15, 18, 19], might be physiologically meaningful. The modes of SHBG action might differ depending on the targeted cell or phenotype, although the relevant SHBG receptor(s) has not yet been identified. Further investigations are necessary to unravel these mechanisms.

SHBG exists as a complex with sex hormones to some degree in sera. In human sera, the proportion of unbound SHBG to total SHBG is 50% in men and 80% in women [55]. Considering the reported association constants [24], the coincubations with an excess of testosterone or 17β-estradiol in our experiments were postulated to have saturated the binding sights of SHBG proteins. One limitation of this study is that we could not determine precisely the proportions of SHBG protein coupled and uncoupled with sex steroids when excess amounts of sex hormones were added. However, considering that very small amounts of sex steroids were present as contaminants of Abcam's SHBG protein and that the addition of excess amounts of sex steroids did not affect the actions of SHBG, it is reasonable to regard SHBG as exerting anti-inflammatory and lipolytic actions regardless of whether or not it is coupled with sex hormones.

It is possible that the actions of SHBG observed herein might be modified by residual steroids in cells, which had

proliferated in serum-containing media before the experiments. Sex hormones exert effects on certain cell types via SHBG and the putative SHBG receptor complex, as previously reported [56, 57].

In rodents, the Shbg gene is not expressed in the liver postnatally. The role of SHBG in rodents might be limited during the fetal period. However, our findings suggest that human SHBG protein exerts activity on adipocytes and macrophages derived from mice. These findings are concordant with the report that human SHBG-Tg mice with the db/db background are resistant to the development of obesity [11]. Effects of human SHBG protein on human adipocytes or macrophages warrant further examinations.

SHBG concentration changes have previously been considered to result from metabolic abnormalities including inflammation [58] and hepatic lipogenesis [59]. Thus, SHBG may be regarded as a good biomarker for metabolic syndrome. However, our results also raise the possibility that SHBG suppresses chronic inflammation, in good agreement with several previous studies employing SHBG transgenic mice, and also exerts direct effects on numerous cell types, as mentioned in the Introduction.

5. Conclusions

In conclusion, at a physiological concentration, SHBG suppresses inflammation and lipid accumulation in macrophages and adipocytes, which may be among the mechanisms underlying the protective effect of SHBG which acts to suppress the development of metabolic syndrome.

Conflicts of Interest

The authors have no conflicts of interest to declare regarding the publication of this paper.

Acknowledgments

The authors thank Natsuko Horikawa-Shirai, Kiyoko Shimazaki, Masako Fujita, and Yuriko Muta-Fukuda for their technical assistance. This study was supported by the Institute for Adult Diseases Asahi Life Foundation grant for medical research and JSPS KAKENHI (Grant no. JP17K16158).

References

[1] G. V. Avvakumov, A. Cherkasov, Y. A. Muller, and G. L. Hammond, "Structural analyses of sex hormone-binding globulin reveal novel ligands and function," *Molecular and Cellular Endocrinology*, vol. 316, no. 1, pp. 13–23, 2010.

[2] Z. Sumer-Bayraktar, T. Nguyen-Khuong, R. Jayo et al., "Micro- and macroheterogeneity of *N*-glycosylation yields size and charge isoforms of human sex hormone binding globulin circulating in serum," *Proteomics*, vol. 12, no. 22, pp. 3315–3327, 2012.

[3] G. L. Hammond, "Diverse roles for sex hormone-binding globulin in reproduction," *Biology of Reproduction*, vol. 85, no. 3, pp. 431–441, 2011.

[4] C. H. Liao, H. Y. Li, H. J. Yu et al., "Low serum sex hormone-binding globulin: marker of inflammation?," *Clinica Chimica Acta*, vol. 413, no. 7-8, pp. 803–807, 2012.

[5] M. Maggio, G. P. Ceda, F. Lauretani et al., "SHBG, sex hormones, and inflammatory markers in older women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 4, pp. 1053–1059, 2011.

[6] Q. Wang, A. J. Kangas, P. Soininen et al., "Sex hormone-binding globulin associations with circulating lipids and metabolites and the risk for type 2 diabetes: observational and causal effect estimates," *International Journal of Epidemiology*, vol. 44, no. 2, pp. 623–637, 2015.

[7] C. Wang, G. Jackson, T. H. Jones et al., "Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes," *Diabetes Care*, vol. 34, no. 7, pp. 1669–1675, 2011.

[8] J. Zhang, X. Huang, M. Liao et al., "Both total testosterone and sex hormone-binding globulin are independent risk factors for metabolic syndrome: results from Fangchenggang Area Male Health and Examination Survey in China," *Diabetes/Metabolism Research and Reviews*, vol. 29, no. 5, pp. 391–397, 2013.

[9] P. Skalba, M. Wojtowicz, and J. Sikora, "Androgen and SHBG serum concentrations in late post-menopause women," *Medical Science Monitor*, vol. 9, no. 3, pp. Cr152–Cr156, 2003.

[10] E. L. Ding, Y. Song, V. S. Malik, and S. Liu, "Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-analysis," *JAMA*, vol. 295, no. 11, pp. 1288–1299, 2006.

[11] C. Saéz-López, M. Rivera-Giménez, C. Hernández, R. Simó, and D. M. Selva, "SHBG-C57BL/ksJ-db/db: a new mouse model to study SHBG expression and regulation during obesity development," *Endocrinology*, vol. 156, no. 12, pp. 4571–4581, 2015.

[12] C. Saez-Lopez, A. Barbosa-Desongles, C. Hernandez et al., "Sex hormone-binding globulin reduction in metabolic disorders may play a role in NAFLD development," *Endocrinology*, vol. 158, no. 3, pp. 545–559, 2017.

[13] E. L. Ding, Y. Song, J. E. Manson et al., "Sex hormone-binding globulin and risk of type 2 diabetes in women and men," *The New England Journal of Medicine*, vol. 361, no. 12, pp. 1152–1163, 2009.

[14] A. M. Nakhla, M. S. Khan, N. P. Romas, and W. Rosner, "Estradiol causes the rapid accumulation of cAMP in human prostate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 12, pp. 5402–5405, 1994.

[15] H. Li, T. Pham, B. C. McWhinney et al., "Sex hormone binding globulin modifies testosterone action and metabolism in prostate cancer cells," *International Journal of Endocrinology*, vol. 2016, Article ID 6437585, 10 pages, 2016.

[16] C. S. Porto, M. F. M. Lazari, L. C. Abreu, C. W. Bardin, and G. L. Gunsalus, "Receptors for androgen-binding proteins: internalization and intracellular signalling," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 53, no. 1–6, pp. 561–565, 1995.

[17] G. Queipo, M. Deas, C. Arranz, C. Carino, R. Gonzalez, and F. Larrea, "Sex hormone-binding globulin stimulates chorionic gonadotrophin secretion from human cytotrophoblasts in culture," *Human Reproduction*, vol. 13, no. 5, pp. 1368–1373, 1998.

[18] A. Hammes, T. K. Andreassen, R. Spoelgen et al., "Role of endocytosis in cellular uptake of sex steroids," *Cell*, vol. 122, no. 5, pp. 751–762, 2005.

[19] E.-J. Hong, B. Sahu, O. A. Jänne, and G. L. Hammond, "Cytoplasmic accumulation of incompletely glycosylated SHBG enhances androgen action in proximal tubule epithelial cells," *Molecular Endocrinology*, vol. 25, no. 2, pp. 269–281, 2011.

[20] M. Iwashita, H. Sakoda, A. Kushiyama et al., "Valsartan, independently of AT1 receptor or PPARγ, suppresses LPS-induced macrophage activation and improves insulin resistance in cocultured adipocytes," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 302, no. 3, pp. E286–E296, 2012.

[21] Y. Nakatsu, H. Sakoda, A. Kushiyama et al., "Peptidyl-prolyl cis/trans isomerase NIMA-interacting 1 associates with insulin receptor substrate-1 and enhances insulin actions and adipogenesis," *The Journal of Biological Chemistry*, vol. 286, no. 23, pp. 20812–20822, 2011.

[22] J. M. Ntambi and K. Young-Cheul, "Adipocyte differentiation and gene expression," *The Journal of Nutrition*, vol. 130, no. 12, pp. 3122S–3126S, 2000.

[23] Y. Berthois, J. A. Katzenellenbogen, and B. S. Katzenellenbogen, "Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 8, pp. 2496–2500, 1986.

[24] J. F. Dunn, B. C. Nisula, and D. Rodbard, "Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma," *The Journal of Clinical Endocrinology & Metabolism*, vol. 53, no. 1, pp. 58–68, 1981.

[25] N. Ouchi, A. Higuchi, K. Ohashi et al., "Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity," *Science*, vol. 329, no. 5990, pp. 454–457, 2010.

[26] A. Haschemi, P. Kosma, L. Gille et al., "The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism," *Cell Metabolism*, vol. 15, no. 6, pp. 813–826, 2012.

[27] J. Liang, J. Wang, Y. Saad, L. Warble, E. Becerra, and P. E. Kolattukudy, "Participation of MCP-induced protein 1 in lipopolysaccharide preconditioning-induced ischemic stroke tolerance by regulating the expression of proinflammatory cytokines," *Journal of Neuroinflammation*, vol. 8, no. 1, p. 182, 2011.

[28] H. Sha, Y. He, H. Chen et al., "The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis," *Cell Metabolism*, vol. 9, no. 6, pp. 556–564, 2009.

[29] M. Schupp, F. Chen, E. R. Briggs et al., "Metabolite and transcriptome analysis during fasting suggest a role for the p53-Ddit4 axis in major metabolic tissues," *BMC Genomics*, vol. 14, no. 1, p. 758, 2013.

[30] T. Wada, H. Kenmochi, Y. Miyashita et al., "Spironolactone improves glucose and lipid metabolism by ameliorating hepatic steatosis and inflammation and suppressing enhanced gluconeogenesis induced by high-fat and high-fructose diet," *Endocrinology*, vol. 151, no. 5, pp. 2040–2049, 2010.

[31] C. M. Taniguchi, K. Ueki, and C. R. Kahn, "Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism," *Journal of Clinnical Investigation*, vol. 115, no. 3, pp. 718–727, 2005.

[32] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante Jr, "Obesity is associated with macrophage accumulation in adipose tissue," *J Clin Invest*, vol. 112, no. 12, pp. 1796–1808, 2003.

[33] M. Taher, M. Z. A. Mohamed Amiroudine, T. M. F. S. Tengku Zakaria et al., "α-Mangostin improves glucose uptake and inhibits adipocytes differentiation in 3T3-L1 cells via PPARγ, GLUT4, and leptin expressions," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 740238, 9 pages, 2015.

[34] P. Chakrabarti and K. V. Kandror, "FoxO1 controls insulin-dependent adipose triglyceride lipase (ATGL) expression and lipolysis in adipocytes," *The Journal of Biological Chemistry*, vol. 284, no. 20, pp. 13296–13300, 2009.

[35] A. Coppola, Z. W. Liu, Z. B. Andrews et al., "A central thermogenic-like mechanism in feeding regulation: an interplay between arcuate nucleus T3 and UCP2," *Cell Metabolism*, vol. 5, no. 1, pp. 21–33, 2007.

[36] S. Y. Cheng, Y. H. Chou, F. L. Liao et al., "Losartan reduces ensuing chronic kidney disease and mortality after acute kidney injury," *Scientific Reports*, vol. 6, no. 1, article 34265, 2016.

[37] D. Brina, A. Miluzio, S. Ricciardi et al., "eIF6 coordinates insulin sensitivity and lipid metabolism by coupling translation to transcription," *Nature Communications*, vol. 6, no. 1, p. 8261, 2015.

[38] M. E. Shaul, G. Bennett, K. J. Strissel, A. S. Greenberg, and M. S. Obin, "Dynamic, M2-like remodeling phenotypes of CD11c + adipose tissue macrophages during high-fat diet–induced obesity in mice," *Diabetes*, vol. 59, no. 5, pp. 1171–1181, 2010.

[39] X. Zhange, R. Goncalves, and D. M. Mosser, "Unit 14.1 the isolation and characterization of murine macrophages," *Current Protocols in Immunology*, vol. 14, p. 11, 2008.

[40] Y. Nakatsu, Y. Otani, H. Sakoda et al., "Role of Pin1 protein in the pathogenesis of nonalcoholic steatohepatitis in a rodent mode," *Journal of Biological Chemistry*, vol. 287, no. 53, pp. 44526–44535, 2012.

[41] P. Greenspan, E. P. Mayer, and S. D. Fowler, "Nile red: a selective fluorescent stain for intracellular lipid droplets," *Journal of Cell Biology*, vol. 100, no. 3, pp. 965–973, 1985.

[42] H. Kanda, S. Tateya, Y. Tamori et al., "MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity," *Journal of Clinical Investigation*, vol. 116, no. 6, pp. 1494–1505, 2006.

[43] P. D. Cani, J. Amar, M. A. Iglesias et al., "Metabolic endotoxemia initiates obesity and insulin resistance," *Diabetes*, vol. 56, no. 7, pp. 1761–1772, 2007.

[44] S. J. Creely, P. G. McTernan, C. M. Kusminski et al., "Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 292, no. 3, pp. E740–E747, 2007.

[45] P. Brun, I. Castagliuolo, V. D. Leo et al., "Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 292, no. 2, pp. G518–G525, 2007.

[46] A. Chawla, K. D. Nguyen, and Y. P. S. Goh, "Macrophage-mediated inflammation in metabolic disease," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 738–749, 2011.

[47] M. W. Elmlinger, W. Kühnel, H. Wormstall, and P. C. Döller, "Reference intervals for testosterone, androstenedione and SHBG levels in healthy females and males from birth until old age," *Clinical Laboratory*, vol. 51, no. 11-12, pp. 625–632, 2005.

[48] M. Maggio, F. Lauretani, S. Basaria et al., "Sex hormone binding globulin levels across the adult lifespan in women–the role of body mass index and fasting insulin," *Journal of Endocrinological Investigation*, vol. 31, no. 7, pp. 597–601, 2008.

[49] L. A. J. O'Neill and A. G. Bowie, "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 7, no. 5, pp. 353–364, 2007.

[50] T. Kawai and S. Akira, "TLR signaling," *Cell Death Differ*, vol. 13, no. 5, pp. 816–825, 2006.

[51] S. L. Deshmane, S. Kremlev, S. Amini, S. Amini, and B. E. Sawaya, "Monocyte chemoattractant protein-1 (MCP-1): an overview," *Journal of Interferon & Cytokine Research*, vol. 29, no. 6, pp. 313–326, 2009.

[52] L. de Toni, D. Guidolin, V. de Filippis et al., "Osteocalcin and sex hormone binding globulin compete on a specific binding site of GPRC6A," *Endocrinology*, vol. 157, no. 11, pp. 4473–4486, 2016.

[53] S. N. Pope and I. R. Lee, "Yeast two-hybrid identification of prostatic proteins interacting with human sex hormone-binding globulin," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 94, no. 1-3, pp. 203–208, 2005.

[54] K.-M. Ng, M. G. Catalano, T. Pinós et al., "Evidence that fibulin family members contribute to the steroid-dependent extravascular sequestration of sex hormone-binding globulin," *The Journal of Biological Chemistry*, vol. 281, no. 23, pp. 15853–15861, 2006.

[55] M. M. Pugeat, J. F. Dunn, and B. C. Nisula, "Transport of steroid hormones: interaction of 70 drugs with testosterone-binding globulin and corticosteroid-binding globulin in human plasma," *The Journal of Clinical Endocrinology & Metabolism*, vol. 53, no. 1, pp. 69–75, 1981.

[56] D. J. Hryb, M. S. Khan, N. A. Romas, and W. Rosner, "The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones," *The Journal of Biological Chemistry*, vol. 265, no. 11, pp. 6048–6054, 1990.

[57] S. M. Kahn, D. J. Hryb, A. M. Nakhla, N. A. Romas, and W. Rosner, "Sex hormone-binding globulin is synthesized in target cells," *Journal of Endocrinology*, vol. 175, no. 1, pp. 113–120, 2002.

[58] R. Simó, A. Barbosa-Desongles, C. Sáez-Lopez, A. Lecube, C. Hernandez, and D. M. Selva, "Molecular mechanism of TNFα-induced down-regulation of SHBG expression," *Molecular Endocrinology*, vol. 26, no. 3, pp. 438–446, 2012.

[59] D. M. Selva, K. N. Hogeveen, S. M. Innis, and G. L. Hammond, "Monosaccharide-induced lipogenesis regulates the human hepatic sex hormone-binding globulin gene," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3979–3987, 2007.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3601454, 14 pages
https://doi.org/10.1155/2018/3601454

Research Article

GLP-1 Analogue Liraglutide Enhances SP-A Expression in LPS-Induced Acute Lung Injury through the TTF-1 Signaling Pathway

Tao Zhu,[1,2] Changyi Li,[2] Xue Zhang,[3] Chunyan Ye,[4] Shuo Tang,[5] Wei Zhang,[6] Jiayang Sun,[7] Niwen Huang,[7] Fuqiang Wen,[1] Daoxin Wang,[2] Huojin Deng,[8] Jing He,[2] Di Qi,[2] Wang Deng,[2] and Tao Yang[9]

[1]*Division of Pulmonary Diseases, State Key Laboratory of Biotherapy of China, and Department of Respiratory Medicine, West China Hospital of Sichuan University, Chengdu 610041, China*
[2]*Respiratory Medicine, Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China*
[3]*Luoyang Orthopedic Hospital of Henan Province, Luoyang 471000, China*
[4]*School Hospital of Southern Medical University, Guangzhou 510280, China*
[5]*Pain Medicine, Shenzhen Nanshan Hospital, Shenzhen 518052, China*
[6]*Respiratory Medicine, First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan 610500, China*
[7]*Respiratory Medicine, Affiliated Hospital of Guiyang Medical University, Guiyang 550004, China*
[8]*Respiratory Medicine, Zhujiang Hospital of Southern Medical University, Guangzhou 510280, China*
[9]*Thoracic Surgery, First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China*

Correspondence should be addressed to Fuqiang Wen; wenfuqiang.scu@gmail.com
and Daoxin Wang; wangdaoxin2011@sina.com

Tao Zhu and Changyi Li contributed equally to this work.

Received 16 December 2017; Revised 1 March 2018; Accepted 28 March 2018; Published 22 May 2018

Academic Editor: Joilson O. Martins

The reduction of pulmonary surfactant (PS) is essential for decreased pulmonary compliance and edema in acute lung injury (ALI). Thyroid transcription factor-1 (TTF-1) plays a major role in the regulation of surfactant protein-A (SP-A), the most abundant protein component of PS. Simultaneously, the glucagon-like peptide-1 (GLP-1) analogue can enhance SP-A expression in the lung. However, the underlying mechanism is still unknown. The purpose of this study was to explore whether liraglutide, a GLP-1 analogue, upregulates SP-A expression through the TTF-1 signaling pathway in ALI. In vivo, a murine model of ALI was induced by lipopolysaccharide (LPS). Pulmonary inflammation, edema, insulin level, ultrastructural changes in type II alveolar epithelial (ATII) cells, and SP-A and TTF-1 expression were analyzed. In vitro, rat ATII cells were obtained. SP-A and TTF-1 expression in cells was measured. ShRNA-TTF-1 transfection was performed to knock down TTF-1 expression. Our data showed that LPS-induced lung injury and increase in insulin level, and LPS-induced reduction of SP-A and TTF-1 expression in both the lung and cells, were significantly compromised by liraglutide. Furthermore, we also found that these effects of liraglutide were markedly blunted by shRNA-TTF-1. Taken together, our findings suggest that liraglutide enhances SP-A expression in ATII cells and attenuates pulmonary inflammation in LPS-induced ALI, most likely through the TTF-1 signaling pathway.

1. Introduction

Pulmonary surfactant (PS), a complex of various lipids and proteins lining the alveolar surface, is synthesized by type II alveolar epithelial (ATII) cells [1, 2]. Its principle function is to maintain pulmonary compliance and pulmonary fluid balance, to prevent the lung from collapsing at the end of expiration, and to regulate the size of alveoli [1, 2].

Meanwhile, PS also plays a role in pulmonary immune defenses [3].

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is one of most common fatal diseases with an extremely high morbidity rate in critically ill patients [4]. Alveolar epithelial and endothelial cells are the major injury targets of ALI/ARDS [5, 6]. It is well known that reduction of PS is essential for decreased pulmonary compliance and pulmonary edema in ALI/ARDS [7, 8]. Surfactant protein-A (SP-A), a hydrophilic collectin, is the most abundant protein component of PS [9]. The main function of SP-A is to facilitate tubular myelin formation; however, it also plays an active role in defense against pathogens and immunological regulation in the lung [3, 9–11]. Simultaneously, several studies have shown that thyroid transcription factor-1 (TTF-1), also known as Nkx2.1, contributed substantially to the regulation of SP-A expression in ATII cells [12–14]. Chen et al. found that hypoxia-induced reduction of SP-A expression was attenuated by leptin through promoting TTF-1 translation in rat ATII cells [13].

Glucagon like peptide-1 (GLP-1), a peptide hormone synthesized and secreted by the L-cells in guts, is essential for glycometabolism and lipid metabolism. Recently, the GLP-1 receptor (GLP-1R), a transmembrane G-protein coupled receptor, has been found in a variety of tissues outside of the pancreas, such as the lung, heart, blood vessels, and kidney [15–17]. Viby et al. showed that GLP-1R was wildly expressed in the airway and alveolar epithelium, in both mice and humans [16]. Romaní-Pérez et al. reported that liraglutide, a GLP-1 analogue, upregulated SP-A expression in the fetal lung and promoted lung development in newborn rats [17]. However, the underlying mechanism remains unclear. In addition, some studies also confirmed that GLP-1 possessed a potent anti-inflammatory property in different inflammatory conditions [15, 18–20]. Our recent study also showed that ovalbumin- (OVA-) induced airway inflammation and mucus hypersecretion were significantly suppressed by liraglutide in a murine model of asthma [15].

Therefore, the purpose of the current study was to explore whether GLP-1 analogue liraglutide upregulates SP-A expression in ATII cells and attenuates inflammation in LPS-induced ALI and to elucidate its underlying mechanism.

2. Methods and Materials

2.1. Animals. All procedures involving animals were approved by the Animal Experimental Ethics Committee of West China Medical School of Sichuan University. The current study was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals. All surgeries were performed using sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Six–eight-week-old male BALB/c mice (18–22 g) and male Sprague-Dawley (SD) rats (200–220 g) were maintained under specific pathogen-free conditions in the animal center facilities of our University. The mice and rats were kept in a temperature-controlled room (12 h dark/light cycles) and offered ad libitum access to food and water. Animals underwent an acclimatization period of at least 1 week before study.

2.2. Knockdown of TTF-1 by shRNA in Mice. To silence the expression of TTF-1 in the lung, a recombinant lentiviral vector for TTF-1 (shRNA-TTF-1, sc-36757-V, Santa Cruz Biotechnology, CA, USA) was used. Lentivirus-expressing nontargeting sequences (sc-108080; Santa Cruz Biotechnology, CA, USA) were used as the negative control (shRNA-scramble). In brief, thirty male BALB/c mice were divided into 3 groups—control, shRNA-scramble, and shRNA-TTF-1 groups—with 10 mice in each group. After anesthesia, shRNA-TTF-1 lentiviral vector (40 μL) for the shRNA-TTF-1 group or negative control lentivirus (40 μL) for the shRNA-scramble group was given by intratracheal injection. The mice in the control group were then administered sterile saline. Three days after transfection, the left lower lung was resected. Histology changes were observed by HE staining. The efficiency of shRNA transfection was measured by qPCR and Western blot analysis.

2.3. Murine Model of LPS-Induced ALI. Fifty male BALB/c mice were randomly and evenly divided into 5 groups: control, liraglutide (Lira), LPS, LPS+Lira, and LPS+Lira +shRNA-TTF-1 groups, with 10 mice in each group. ALI was induced by intratracheal injection of LPS (*Escherichia coli*; serotype 0111:B4; Sigma-Aldrich, St. Louis, MO, USA) [6, 21]. In brief, mice were anesthetized with 30 mg/kg of pentobarbital sodium, followed by intratracheal injection of 10 μg of LPS in 50 μL sterile saline with a 3-gauge needle. ShRNA-TTF-1 (40 μL) was also given by intratracheal injection 3 days before LPS stimulation. The mice in the control group were administrated sterile saline instead. According to our previous studies, liraglutide (2 mg/kg in 200 μL sterile saline, Novo Nordisk A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark) was given by intraperitoneal injection, 20 min after LPS injection [15, 22]. In 3 days, liraglutide was given every 12 h (7 times in total).

2.4. Bronchoalveolar Lavage Fluid (BALF) and Cell Counting. Seventy-two hours later, mice were sacrificed under anesthesia by pentobarbital (50 mg/kg i.p.). BALF was collected by cannulating the upper part of the trachea, by lavage 3 times with 1.0 mL phosphate-buffered saline (pH 7.2) [23]. The fluid recovery rate was about 90%. The sediment cells were stained with Diff-Quik (International Reagents Corp., Japan) for cytospin preparations. The number of total cells, neutrophils, macrophages, and lymphocytes was then counted double-blindly with a hemocytometer.

2.5. TNF-α, IL-6, and IL-1β in BALF. As described before, the BALF supernatant was collected and stored at −80°C before performing the cytokine assay [6, 21]. TNF-α, IL-6, and IL-1β expression levels in BALF were measured by ELISA (R&D Systems, USA).

2.6. Myeloperoxidase (MPO) Activity Assay. Seventy-two hours after LPS injection, mice were sacrificed and their lungs were collected. According to our previous studies,

MPO activity was detected. Results are expressed as units of MPO activity per gram of lung tissue [6, 21].

2.7. Lung Wet/Dry Weight Ratio. Seventy-two hours after LPS injection, mice were sacrificed and their lungs were collected. The severity of pulmonary edema was assessed by the wet-to-dry ratio (W/D) [6, 21]. The left lower lung was weighed and then dehydrated at 60°C for 72 h in an oven.

2.8. H&E Staining and Lung Injury Score. Seventy-two hours after LPS injection, mice were sacrificed and their lungs were collected. The right lower lung of each mouse was fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with H&E to observe the pathological alterations in the lung tissues. According to our previous studies, the lung injury score was measured by a blinded pathologist with a 0- to 4-point scale according to combined assessments of inflammatory cell infiltration in the airspace or vessel wall, alveolar congestion, hemorrhage, alveolar wall thickness, and hyaline membrane formation [6, 21]. Five microscope fields from each histological section were taken and scored. The final score of each sample was the average of 5 scores. Briefly, a score of 0 represented no damage, 1 represented mild damage, 2 represented moderate damage, 3 represented severe damage, and 4 represented very severe histological changes.

2.9. Immunohistochemistry. Seventy-two hours after LPS injection, mice were sacrificed and their lungs were collected. SP-A expression in the lung was determined by immunohistochemistry. Briefly, tissue sections (right lower lung) were deparaffinated and rehydrated. Samples were treated with Target Retrieval (Dako, Glostrup, Denmark) at 95°C, blocked at room temperature with Protein Block Serum-Free (Dako, Glostrup, Denmark), and incubated with anti-SP-A antibody (1 : 400; sc-7699, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, sections were incubated with biotin-conjugated anti-rabbit immunoglobulin G (IgG) for 30 min at room temperature. The biotinylated reagents were detected with ABC complex HRP (Dako, Glostrup, Denmark).

2.10. Transmission Electron Microscopy (TEM). Seventy-two hours after LPS injection, mice were sacrificed and their lungs were collected. TEM was performed to observe the ultrastructural changes in pulmonary epithelial cells and to identify rat ATII cells [21, 24]. Lamellar bodies, the principal storage site of pulmonary surfactant, are the specific organelle and the feature of ATII cells. In brief, the fresh left upper lung tissues and isolated cells were obtained for observation. Images were taken with an electron microscope (H-600IV, Hitachi, Tokyo, Japan).

2.11. Fasting Serum Insulin. Blood was taken from the tail vein without anesthesia, 72 h after LPS injection and 6 h after the last feeding, before sacrifice. The procedure was done in the afternoon. According to the instructions of the manufacturer, the fasting serum insulin level was analyzed using an ELISA kit (ALPCO Diagnostics).

2.12. Rat ATII Cell Isolation and Characterization. Rat ATII cells were isolated from male pathogen-free SD rats (200–220 g) as described previously [25]. In brief, the lungs were perfused via the pulmonary artery to remove the blood. The lung was digested with intratracheally instilled 3 U/mL elastase (Sangon Biotech, Shanghai, China) three times at 37°C for 40 minutes. Due to Fcγ receptors (FcγR), the receptor of IgG, only expressed on non-ATII cells in the lung, rat IgG can be used to remove non-ATII cells in the lung. In our study, ATII cells were purified by differential adhesion to IgG-pretreated dishes (Boster Biological Technology, Wuhan, China). More than 95% of the cells obtained were viable, which was assessed by trypan blue exclusion assay. Cells were resuspended in DMEM supplemented with FBS (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were then used in experiments after a 24 h culture period. TEM was used to identify the ATII cells, as mentioned above.

2.13. Cell Transfection with shRNA. According to our previous study, ATII cells were transfected at 70% confluence with shRNA-TTF-1 (336312, Qiagen, Valencia, CA) or shRNA-scrambled (sc-108060, Santa Cruz Biotechnology, CA, USA) [26]. Twenty-four hours after transfection, ATII cells were used for further experiments. Meanwhile, total and nuclear proteins and mRNA were extracted from cells and kept at −80°C for qPCR and Western blot. Then, knockdown of TTF-1 expression was analyzed by qPCR and Western blot. β-Actin was used as an internal control.

2.14. Cell Intervention. ShRNA-TTF-1-transfected and non-transfected ATII cells were pretreated with liraglutide (100 nM) for 4 h and then stimulated with LPS (100 ng/mL) for 4 h [21, 26, 27]. Then, total and nuclear proteins and mRNA were extracted from cells and kept at −80°C for qPCR and Western blot. MTT assay (Promega, Madison, WI) was used to assess cell viability at 0 h and 4 h after interventions.

2.15. Quantitative PCR. The mRNA expression of SP-A and TTF-1 was detected by qPCR [15, 22]. β-Actin was used as an internal reference. Briefly, mice were sacrificed and their lungs were collected. The right upper lung tissues were kept at −80°C. Then, total RNA of lung tissues and rat ATII cells were isolated by TRIzol reagent. PrimeScript® RT reagent kit with gDNA eraser (Takara Bio Inc., Otsu, Japan) was used for reverse transcription. PCR was then performed with iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA) and a SYBR Green PCR kit (Takara Bio Inc., Otsu, Japan) in a final volume of 20 μL, containing 1.6 μL cDNA template, forward and backward primers (0.8 μL each), 10 μL SYBR® Premix Ex Taq™ II, and 6.8 μL dH_2O. The primers and TaqMan probes were designed using Primer Premier (PREMIER Biosoft International, Canada). The premier sequences were as follows. mTTF-1: (forward) 5′-AACAGC GGCCATGCAG CAGC AC-3′ and (reverse) 5′-CCATG TTCTTGC TCACGTCC-3′; mSP-A: (forward) 5′-TCGGA GGCAGACA TCCACA-3′ and (reverse) 5′-GCCAGCA ACAACAGTC AAGAAG AG-3′; mβ-actin: (forward) 5′-GATTA CTGCTCTGG CT CCTAGC-3′ and (reverse) 5′-ACTCAT CGTACTCC TGC

TTGCT-3′; rTTF-1: (forward) 5′-AAATT TGGGGGT CT TTCTGG-3′ and (reverse) 5′-AGAGT GCATCCA CAGG GAAG-3′; rSP-A: (forward) 5′-AGCCTG CAGGTCTG TATGTGGA-3′ and (reverse) 5′-TTGCAC TTGATACCA GCGACAAC-3′; and rβ-actin: (forward) 5′-ATCATGTT TGAGACCT TCAACA-3′ and (reverse) 5′-CATCTC TT GCTCGA AGTCCA-3′. Changes in the expression of target genes were calculated using the $2^{-\triangle\triangle Ct}$ method, $\triangle\triangle Ct = (Ct_{target} - Ct_{\beta-actin})_{sample} - (Ct_{target} - Ct_{\beta-actin})_{control}$.

2.16. Western Blot. Western blot was performed to evaluate the protein expression [21, 28]. Briefly, 72 hours after LPS injection, mice were sacrificed and their lungs were collected. The lung tissues were kept at −80°C. Protein lysates from the left upper lung tissues and rat ATII cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Antibodies against mSP-A, mTTF-1, rSP-A, rTTF-1, mβ-actin, and rβ-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The relative protein levels of mSP-A, rSP-A, mTTF-1, and rTTF-1 were normalized to that of β-actin.

2.17. Statistical Analysis. Statistical analyses were performed with SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). All data were presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) test was performed. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. TTF-1 Expression Was Inhibited after Transfection of the Lung with shRNA-TTF-1. After 3 days of transfection, no pathological alterations were observed in the control, shRNA-scrambled, shRNA-TTF-1 groups (Figure 1(a)). However, TTF-1 expression was largely inhibited by shRNA-TTF-1 in the lung (Figures 1(b) and 1(c)).

3.2. Liraglutide Attenuated Pulmonary Inflammation and Pulmonary Edema in LPS-Induced ALI. After 72 h of LPS injection, severe and typical pulmonary pathological alterations were observed, including severe and extensive inflammatory cell infiltration, interstitial and intra-alveolar edema and patchy hemorrhage, interalveolar septal thickening, and hyaline membrane formation with some alveoli collapsed (Figure 2(a)). As shown in Figure 2(c), the W/D ratio was significantly increased after 72 h of LPS injection. Figure 2(d) demonstrates that MPO activity was significantly enhanced by LPS injection. However, LPS-induced lung injury, W/D ratio, and MPO activity were all notably suppressed by liraglutide (Figure 2). Furthermore, these effects of liraglutide were substantially blunted by shRNA-TTF-1. No pathological changes were observed in the control and Lira groups (Figure 2(a)).

3.3. Liraglutide Reduced Inflammatory Cell Counts and Inflammatory Mediators in BALF. As shown in Figure 3, the number of total cells, neutrophils, and macrophages and the levels of TNF-α, IL-6, and IL-1β in BALF were notably increased after 72 h of LPS injection. Meanwhile, our data also demonstrated that the increase in the number of total cells, neutrophils, and macrophages and increased levels of TNF-α, IL-6, and IL-1β in BALF, induced by LPS, were remarkably reduced by liraglutide. These effects of liraglutide were substantially blocked by shRNA-TTF-1. No difference in the number of lymphocytes in BALF was found among all groups (Figure 3(a)).

3.4. Liraglutide Alleviated Pathological Alterations in ATII Cell Ultrastructure in LPS-Induced ALI. After 72 h of LPS injection, significant ultrastructural pathological alterations were observed in ATII cells. These features included cell swelling with a cytoplasm of low electronic density, unclear cell structure, mitochondrial edema with dilated mitochondrial cristae, chromatin margination, and reduced and indistinct cell surface microvilli, along with decreased and vacuolated lamellar bodies (Figure 4). Meanwhile, as shown in Figure 4, these ultrastructural alterations were alleviated by liraglutide. However, shRNA-TTF-1 notably abolished this effect of liraglutide. No ultrastructural pathological change was observed in the control and Lira groups.

3.5. Liraglutide Enhanced SP-A Expression by Increasing TTF-1 in LPS-Induced ALI. As shown in Figures 5(b) and 5(c), the expression of SP-A was substantially decreased after 72 h of LPS injection, and liraglutide significantly enhanced SP-A expression in the lung after LPS injection. In addition, SP-A expression in the ATII cells was reduced after LPS administration (Figure 5(a)). Subsequently, as shown in Figure 6, LPS-induced suppression of TTF-1 expression was markedly alleviated by liraglutide. Furthermore, our data also demonstrated that these effects of liraglutide were largely abrogated by shRNA-TTF-1.

3.6. Liraglutide Reduced Serum Insulin Level in LPS-Induced ALI. Fasting serum insulin level (72 h after LPS injection, 6 h after last feeding) was measured. Fasting serum insulin level was markedly increased after 72 h of LPS injection. Meanwhile, as shown in Figure 7, the increased level of fasting serum insulin induced by LPS was significantly reduced by liraglutide. This effect of liraglutide was substantially blocked by shRNA-TTF-1. No difference in the level of serum insulin was found among the control, Lira, and shRNA-TTF-1 groups.

3.7. Identification of Rat ATII Cells and Evaluation of Cell Viability. Isolation primary rat ATII cells were confirmed by TEM. Lamellar bodies, the characteristic organelles of ATII cells, were observed in our isolated cells (Figure 8(a)). To evaluate the cell viability of rat ATII cells, MTT assay was performed. As shown in Figure 8(b), no significant difference in cell viability was found between different groups after 4 h of intervention.

3.8. The Expression of TTF-1 Was Inhibited after shRNA-TTF-1 Transfection in Rat ATII Cells. QPCR and Western blot were performed to analyze the mRNA and protein

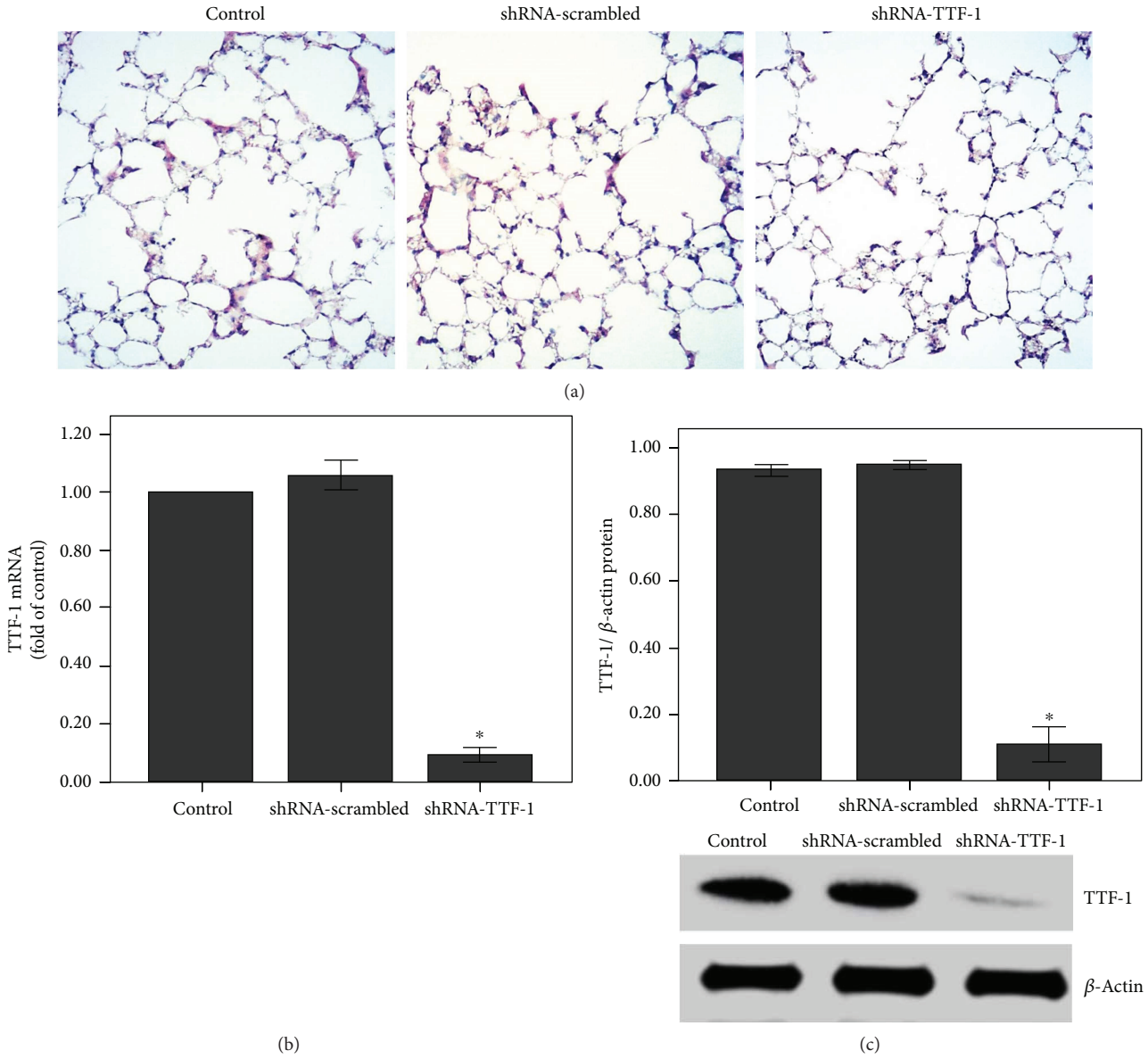


Figure 1: The expression of TTF-1 was inhibited after shRNA-TTF-1 transfection in the lung. Mice were transfected with shRNA-TTF-1 or shRNA-scrambled by intratracheal injection. Seventy-two hours after transfection, the expression of TTF-1 was measured. (a) After 72 h of transfection, mice were sacrificed and their right lower lungs were fixed. The tissue sections were then stained with H&E. The figure demonstrates a representative view (×200) from each group. (b) QPCR was used to analyze the mRNA expression of TTF-1. (c) Western blot was performed to evaluate the protein expression of TTF-1. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control.

expression of TTF-1 in rat ATII cells. After 24 h of transfection, TTF-1 expression was markedly inhibited by shRNA-TTF-1 in rat ATII cells (Figure 9).

3.9. Liraglutide Promoted SP-A Expression by Increasing TTF-1 in Rat ATII Cells. As shown in Figures 10(a) and 10(b), the expression of SP-A was notably reduced after 4 h of LPS stimulation. Liraglutide remarkably enhanced SP-A expression in ATII cells after LPS stimulation. Subsequently, LPS-induced suppression of TTF-1 expression was also notably abolished by liraglutide in ATII cells (Figures 10(c) and 10(d)). Furthermore, our data also demonstrated that these effects of liraglutide were substantially abrogated by shRNA-TTF-1.

4. Discussion

In the current study, our results showed that LPS-induced pulmonary inflammation, pulmonary edema, increase in insulin level and alveolar cell injuries, and LPS-induced reduction of SP-A expression were markedly compromised by GLP-1 analogue liraglutide both in vivo and in vitro. Furthermore, our data also indicated that this SP-A-enhancing property of liraglutide was most likely mediated via the TTF-1 signaling pathway.

ALI/ARDS, characterized by damage to the alveolar-capillary barrier, is induced by self-amplified and uncontrolled lung inflammation [4]. Although substantial progress has

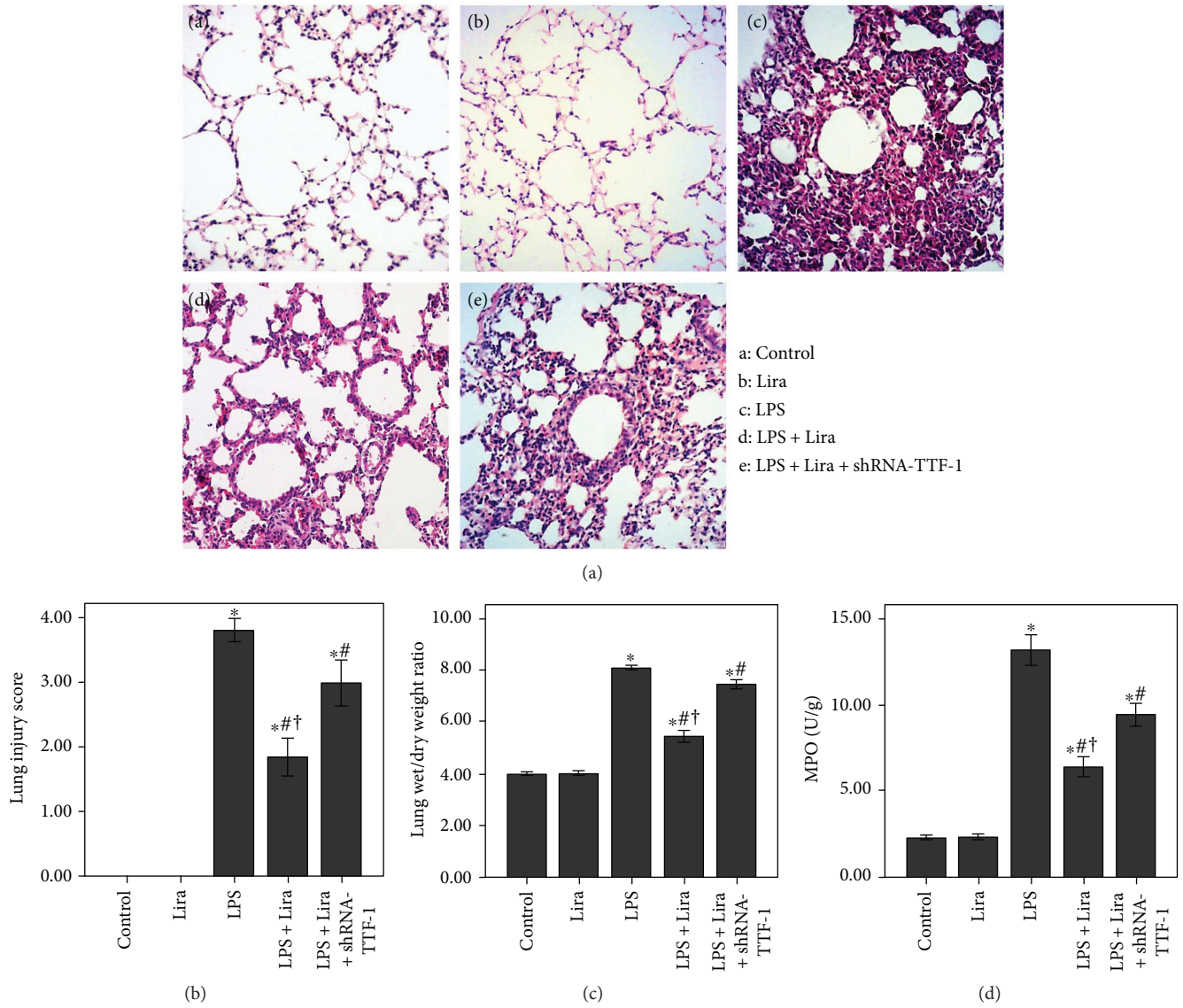
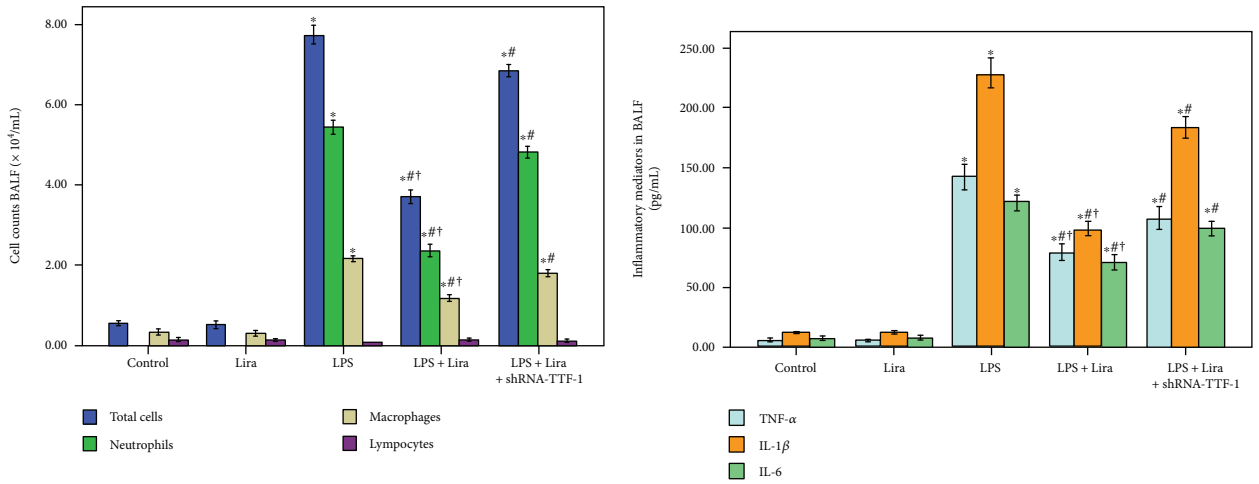


Figure 2: Liraglutide attenuated pulmonary inflammation and pulmonary edema in LPS-induced ALI. (a) After 72 h of intervention, mice were sacrificed and their right lower lungs were fixed. The tissue sections were then stained with H&E. The figure demonstrates a representative view (×200) from each group. (b) Severity of lung injury was measured by the lung injury scoring system. (c) The left lower lungs were obtained to evaluate the W/D ratio of the lung tissues. (d) MPO activity was measured to evaluate the accumulation and activation of neutrophils in the lung tissues. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+Lira+shRNA-TTF-1.

been made in the understanding of ALI/ARDS, effective treatments are still limited in clinical practice.

GLP-1 is mainly synthesized and secreted by intestinal L-cells [18]. GLP-1 analogues, including liraglutide and exenatide, have been successfully used in type 2 diabetes mellitus treatment. Several recent studies have confirmed that GLP-1R is also expressed in many extrapancreatic tissues, including endothelial cells, airway and alveolar epithelial cells, macrophages, gastrointestinal tract, myocardium, and kidney [15–17]. A previous study revealed the protective role of liraglutide in a murine model of obstructive lung disease [16]. Moreover, our previous investigation also showed that OVA-induced airway inflammation and mucus hypersecretion were markedly inhibited by liraglutide in mice [15]. Meanwhile, our other study demonstrated that bleomycin-induced pulmonary inflammation and fibrosis were notably attenuated by liraglutide in mice [22]. After 72 h of LPS injection, severe and typical pathological changes in the lungs were observed (Figure 2(a)). Our results showed that these typical pathological alterations and enhanced lung injury scores were both significantly improved by liraglutide (Figures 2(a) and 2(b)). Meanwhile, according to previous studies, MPO activity is a marker of accumulation and activation of neutrophils in inflammatory processes [6, 21]. Our findings demonstrated that LPS-induced increase in MPO activity in the lung was largely inhibited by liraglutide



(a) (b)

FIGURE 3: Liraglutide reduced inflammatory cell counts and inflammatory mediators in BALF. (a) Cells in BALF were collected, and cytospin preparations were made. The number of total cells, neutrophils, macrophages, and lymphocytes in BALF were assessed. (b) TNF-α, IL-6, and IL-1β levels in BALF were detected by ELISA. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+Lira+shRNA-TTF-1.

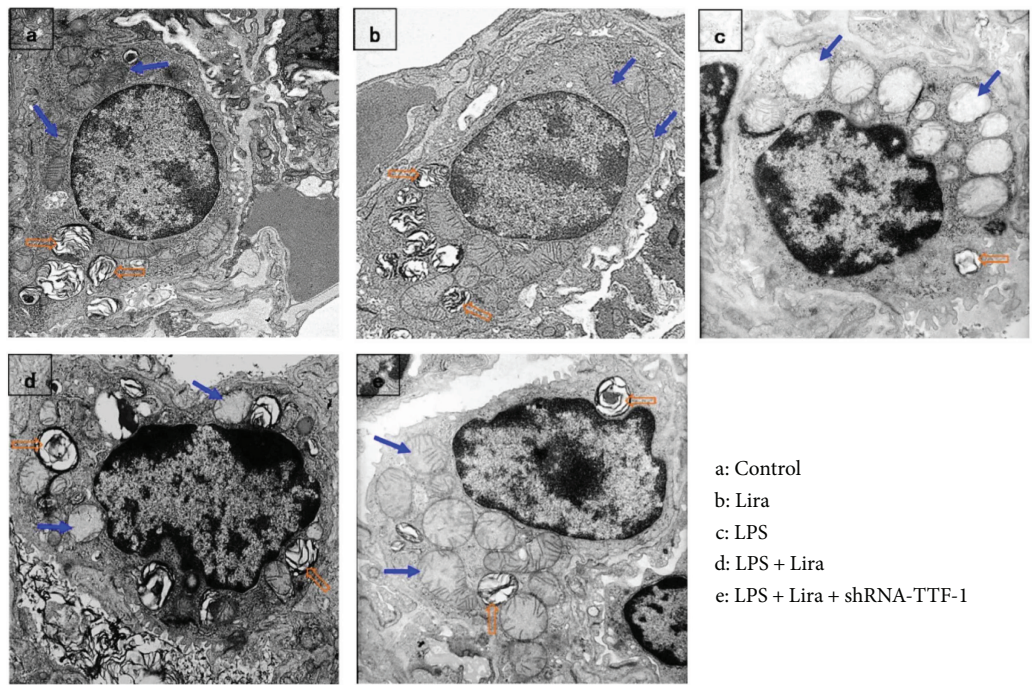


FIGURE 4: Liraglutide alleviated pathological alterations in ATII cell ultrastructure in LPS-induced ALI. After mice were sacrificed, the left upper lung tissues were taken and observed by TEM. The figure demonstrates a representative view (×6000) from each group. Mitochondrion, blue arrow; lamellar body, orange open arrow.

(Figure 2(d)). At the initial stage of infection-induced acute inflammation, pathogen-associated molecular patterns (PAMPs), including LPS and CpG DNA, are recognized and interacted by inflammatory cells with pattern recognition receptors (PPRs), such as mannose receptor (MR) and toll-like receptors (TLRs) [29, 30]. Following this, a wide range of inflammatory mediators, including TNF-α, IL-6, and IL-1β, are released [29, 30]. In our study, we found that LPS-induced increase in the number of total cells, neutrophils, and macrophages and LPS-induced increase in TNF-α, IL-6, and IL-1β levels in BALF were remarkably compromised by liraglutide (Figure 3). Additionally, noncardiogenic pulmonary edema is another major pathological feature of ARDS, a major cause of the failure of oxygenation [31]. Our data showed that LPS-induced increase in the W/D ratio was markedly reduced by liraglutide (Figure 2(c)).

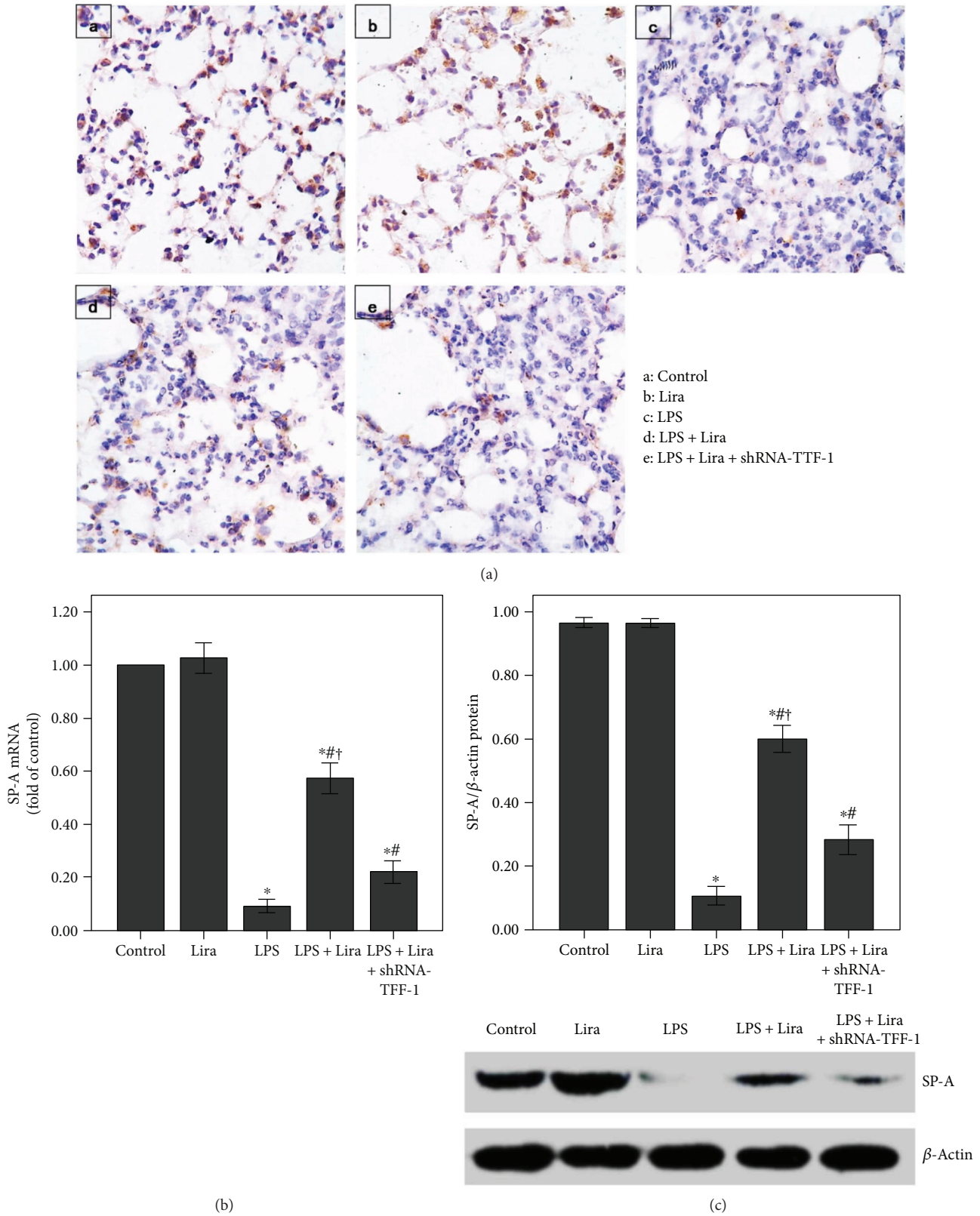


Figure 5: Liraglutide enhanced SP-A expression in LPS-induced ALI. (a) After 72 h of interventions, mice were sacrificed and their right lower lungs were fixed. Immunohistochemistry was then performed to observe SP-A expression in the lung. (b) QPCR was used to analyze the mRNA expression of SP-A. (c) Western blot was performed to evaluate the protein expression of SP-A. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+ Lira+ shRNA-TTF-1.

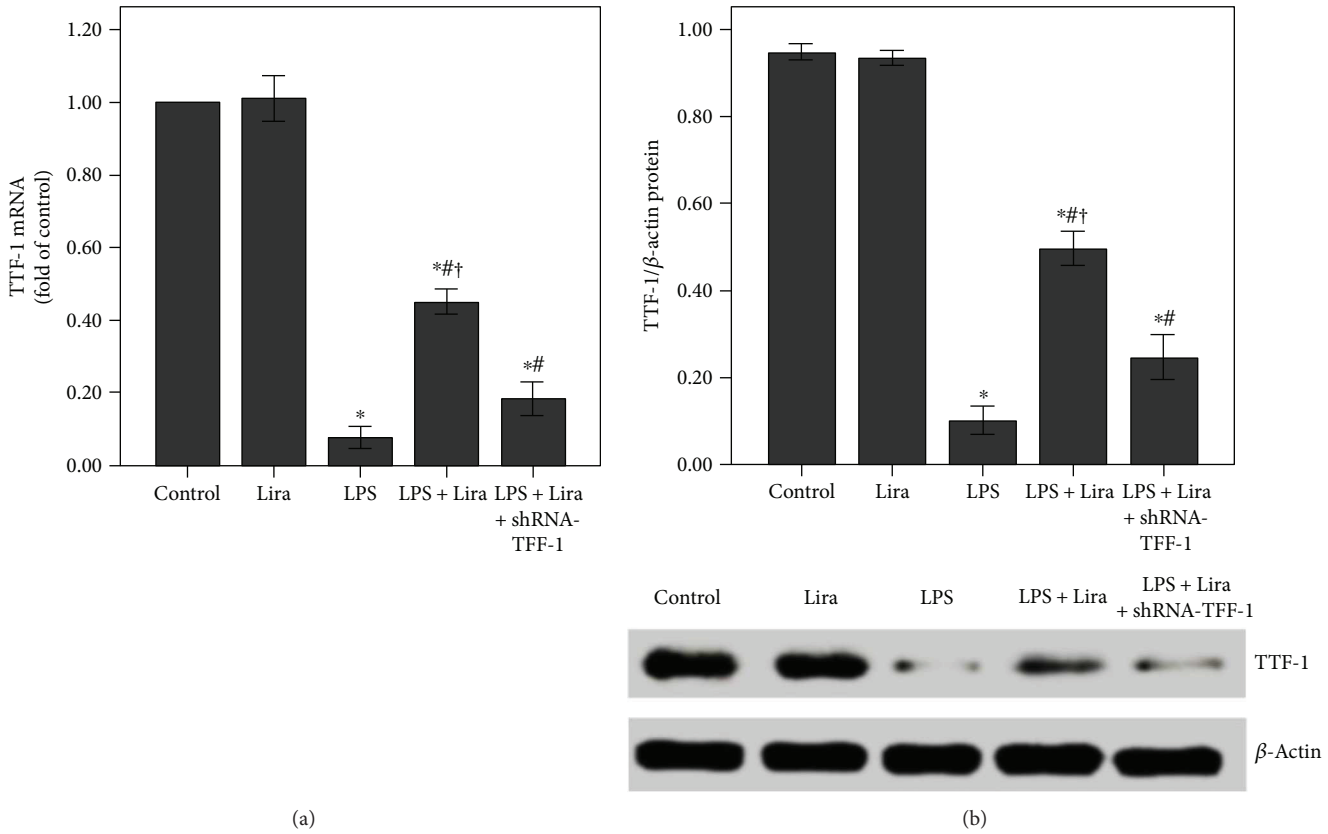


Figure 6: Liraglutide increased the expression of TTF-1 in LPS-induced ALI. (a) QPCR was used to analyze the mRNA expression of TTF-1. (b) Western blot was performed to evaluate the protein expression of TTF-1. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+Lira+shRNA-TTF-1.

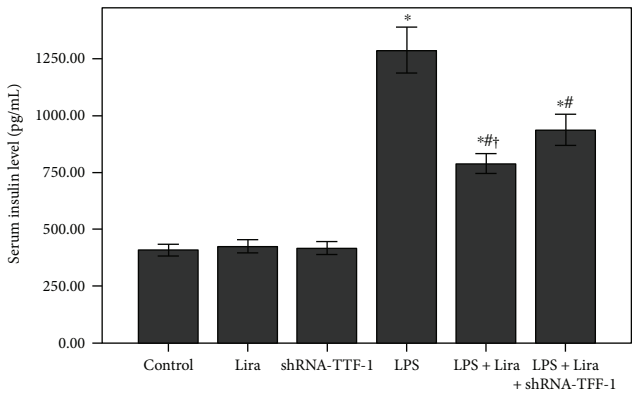
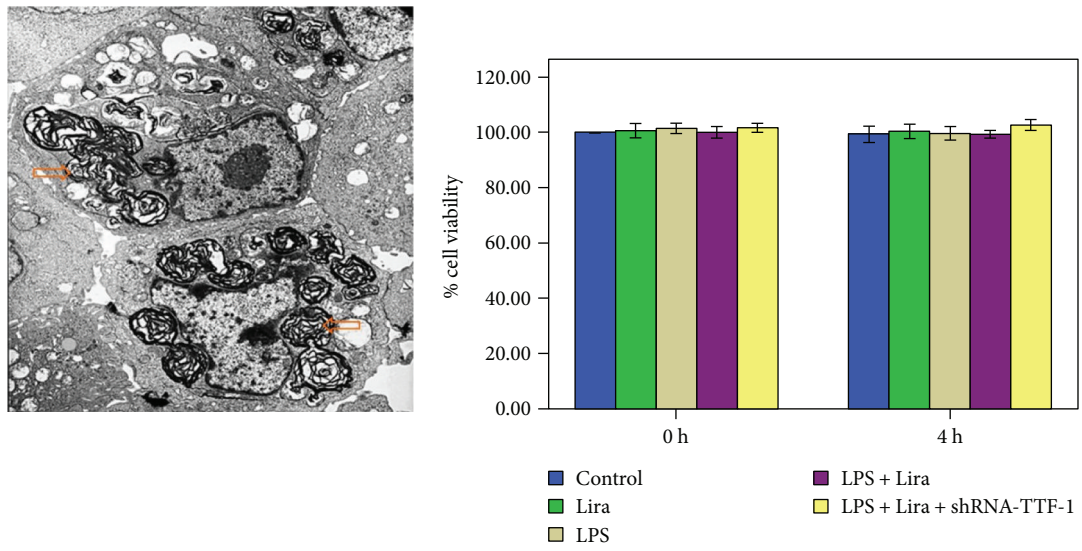


Figure 7: Liraglutide reduced the serum insulin level in LPS-induced ALI. Fasting serum insulin level (72 h after LPS injection and 6 h after last feeding) was detected by ELISA. Each bar represents the mean ± SEM of 10 mice. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+Lira+shRNA-TTF-1.

Therefore, these findings indicated that LPS-induced pulmonary inflammation, injury, and edema were substantially attenuated by liraglutide in mice.

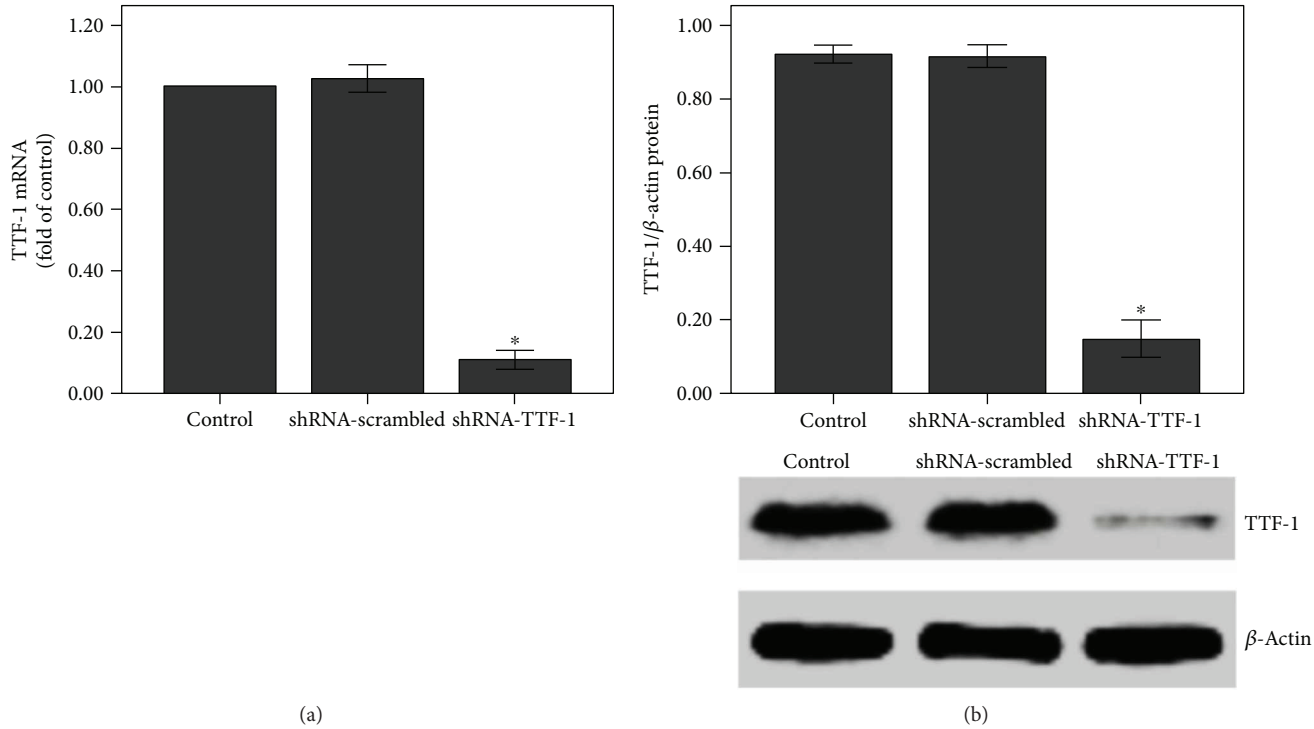
GLP-1 is a key regulator of insulin secretion. It has been found that the effect of GLP-1 in promoting pancreatic β cell insulin secretion was dependent on blood glucose concentration [32]. Fransson et al. showed that liraglutide did not influence serum insulin level in nondisease mice; however, corticosterone-induced hyperinsulinemia was alleviated by liraglutide [33]. It is well known that insulin tolerance is impaired in severe conditions, such as ARDS, severe sepsis, and severe burn. Inflammation plays a critical role in insulin intolerance in these conditions. Landgraf et al. found that LPS-induced insulin tolerance impairment was alleviated by leptin, resulting from its anti-inflammatory effect, in mice [34]. In the current study, our data showed that LPS-increased fasting serum insulin was suppressed by liraglutide (Figure 7). Our data also demonstrated that fasting serum insulin level was not influenced by liraglutide in nondisease mice. This result indicates that LPS-increased insulin level was remarkably suppressed by liraglutide in mice, possibly stemming from its anti-inflammatory effect.

PS is a complex of various lipids (90%) and proteins (10%), mainly synthesized by ATII cells [35, 36]. PS is responsible for increasing pulmonary compliance, preventing the lung from collapsing at the end of expiration, maintaining fluid balance in the lung, regulating the size of alveoli, and modulating the lung's innate immune system [3, 35, 36]. Lamellar bodies, the main sites for the synthesis and metabolism of surfactants, are the specialized organelles of ATII cells [1, 36]. Simultaneously, it is well known that mitochondria, the powerhouse of the cell, is the most sensitive and vulnerable organelle, responding to injury and



(a) (b)

Figure 8: Identification of rat ATII cells and evaluation of cell viability. (a) Isolated and cultivated rat ATII cells were identified by TEM. The figure demonstrates a representative view (×6000). Lamellar body, orange open arrow. (b) MTT assay was performed to evaluate rat ATII cell viabilities. Quantitative data were presented as mean ± SEM ($n = 5$). $^*P < 0.05$ compared with control.



(a) (b)

Figure 9: The expression of TTF-1 was inhibited after shRNA-TTF-1 transfection in rat ATII cells. Rat ATII cells were transfected with shRNA-TTF-1 or shRNA-scrambled. Twenty-four hours after transfection, the expression of TTF-1 was measured. (a) QPCR was used to analyze the mRNA expression of TTF-1. (b) Western blot was performed to analyze the protein expression of TTF-1. Quantitative data were presented as mean ± SEM ($n = 5$). $^*P < 0.05$ compared with control.

hypoxia. Even slight injury can cause mitochondrial dysfunction and structure changes which usually is the first and most common finding after cell damage. ATII cell is the major injury target of ARDS [21]. Some investigations have shown that the degree of functional impairment and structural abnormality of lamellar bodies and mitochondria were correlated with the severity of ALI [21, 37, 38]. Our data also showed that LPS-induced ultrastructural alterations, particularly lamellar bodies and mitochondria, in ATII cells were notably attenuated by liraglutide in mice (Figure 4). Thus,

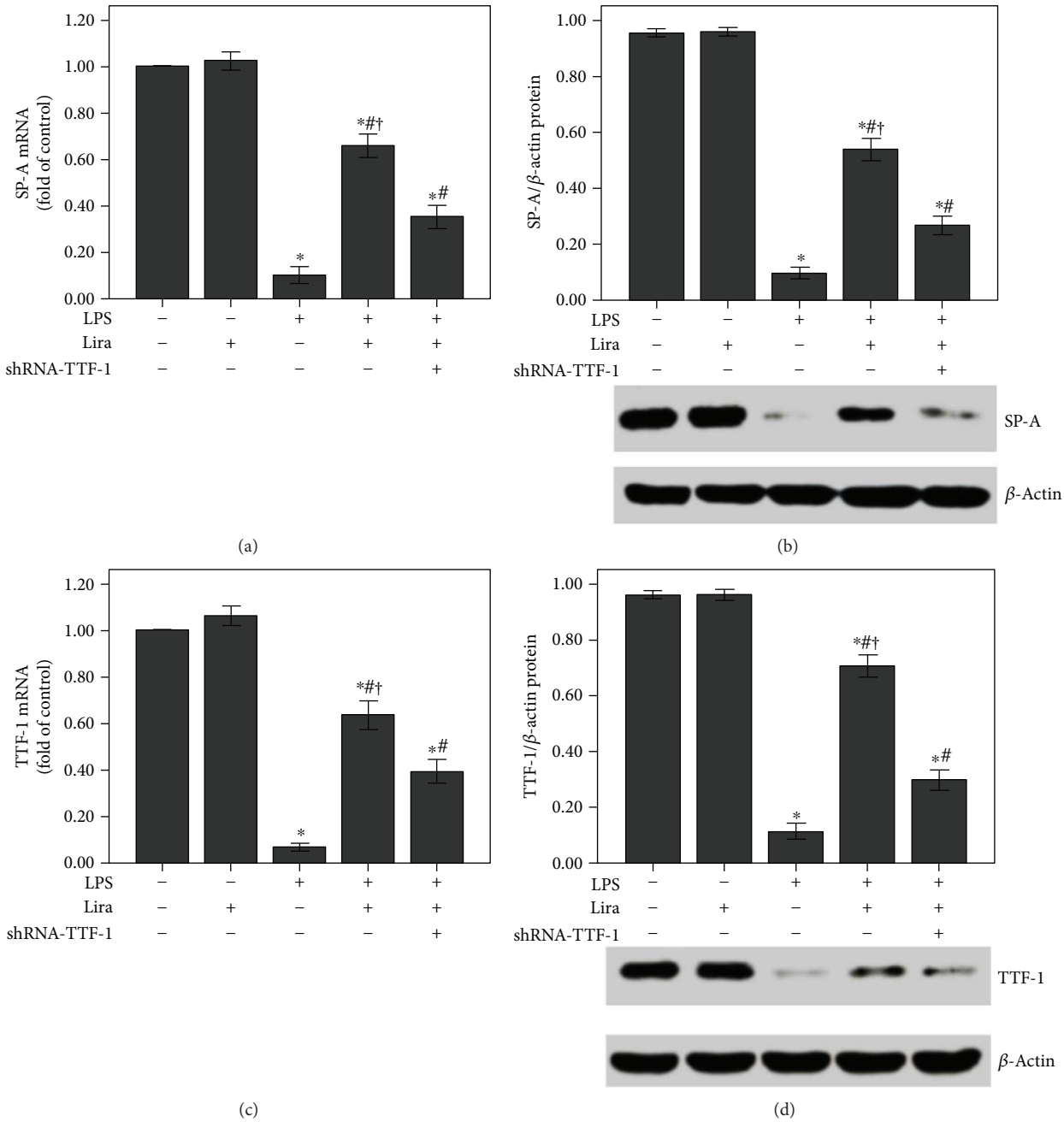


Figure 10: Liraglutide increased the expression of SP-A and TTF-1 in rat ATII cells. ShRNA-TTF-1-transfected and nontransfected ATII cells were pretreated with liraglutide (100 nM) for 4 h and then stimulated with LPS (100 ng/mL) for 4 h. (a and c) QPCR was used to analyze the mRNA expression of SP-A and TTF-1. (b and d) Western blot was performed to analyze the protein expression of SP-A and TTF-1. Quantitative data were presented as mean ± SEM ($n = 5$). $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with LPS. $^{\dagger}P < 0.05$ compared with LPS+Lira+shRNA-TTF-1.

this result suggested the protective role of liraglutide in LPS-induced ATII cell injury.

SP-A is the most abundant protein component of PS [1, 39]. SP-A plays an indispensable role in tubular myelin formation and recycling of PS [35]. Tubular myelin, the precursor of a monomolecular surfactant form, is an ultrathin film lining the surface of the alveolus [35]. Moreover, a number of studies have reported that SP-A is essential for innate immunity in the lung [3, 10, 39]. Many previous studies have shown that SP-A expression is significantly reduced in ALI/ARDS [40, 41]. It is well known that reduction of PS is essential for decreased pulmonary compliance and edema in ALI/ARDS. There is broad evidence that the expressions of SP-A and other PS components were reduced after ATII injury in ALI [21, 42–45]. Then, in the current study, our results also indicate that the reduction of SP-A expression

in the lung was correlated with the severity of lamellar body damage and ATII cell injury in ALI (Figures 4 and 5). Therefore, we presumed that upregulation of SP-A might benefit in treating ARDS. Simultaneously, GLP-1 played a critical role in regulation of SP-A and lung development. Previously, it was found that SP-A expression was upregulated and the lung size was increased by liraglutide in fetal and neonatal rats [17]. Furthermore, another study also showed that streptozotocin-induced reduction of SP-A expression was prevented by liraglutide through the TTF-1 signaling pathway in a rat model of diabetes [46]. In the current study, our data demonstrated that LPS-induced downregulation of SP-A expression could be abolished by liraglutide (Figure 5).

TTF-1 is considered to be a central regulator of SP-A transcription in ATII cells [12, 13]. Meanwhile, it is reported that TTF-1 also played a role in LPS and other inflammatory stimulator-induced ATII cell injuries [42, 45]. Das et al. figured out that TNF-α could directly inhibit TTF-1 expression by binding the TTF-1 proximal promoter in H441 and primary alveolar type II cells [42]. TNF-α, one of the most critical inflammatory mediators, has been implicated in the pathogenesis of ARDS and inhibits surfactant protein levels [21, 45]. The TTF-1-binding element (TBE) has been identified in the promoter region of *SP-A* [12]. Chen et al. revealed that SP-A expression in rat ATII cells could be upregulated by leptin via the TTF-1 signaling pathway under hypoxic conditions [13]. In the current study, our data indicated that liraglutide upregulated SP-A expression through the TTF-1 signaling pathway in LPS-induced ALI (Figures 6 and 10).

SP-A is essential for modulating lung inflammation [3, 39]. Some studies have determined the anti-inflammatory role of SP-A in different conditions [10, 11]. Du et al. found that pneumonia severity and intestinal injury were notably attenuated by SP-A in a murine model of *Staphylococcus aureus* pneumonia [47]. Minutti et al. demonstrated that SP-A attenuated IFN-γ/LPS-induced alveolar macrophage activation [48]. Another study also reported that TTF-1 played a role in the regulation of pulmonary inflammation [49]. In the current study, our data revealed that the effects of liraglutide on LPS-induced pulmonary inflammation, pulmonary edema, increase in insulin level, and ATII cell injury were significantly blunted by shRNA-TTF-1. Thus, this result suggested that the effects of liraglutide were induced by increasing TTF-1 expression. Nevertheless, the underlying mechanism of how liraglutide regulates TTF-1 expression in ATII cells in ALI is still unclear. However, several studies, including our previous study, found that the cAMP/PKA signal pathway was essential for the bioactivity of GLP-1 in different conditions [15, 18]. Steven et al. demonstrated that GLP-1 receptor activation in platelets by linagliptin and liraglutide attenuated LPS-induced microvascular thrombosis, systemic inflammation, vascular dysfunction, and end organ damage by a cAMP/PKA-dependent mechanism [18]. Therefore, we highly supposed that the cAMP/PKA signal pathway would play a critical role in liraglutide-induced TTF-1 expression in ATII cells in ALI. Meanwhile, we also supposed that one or more intermediate molecules probably were involved in this process. Thus, we presume that liraglutide-induced TTF-1 expression in ALI is indirect. And this is an important area that requires further research.

Additionally, it is reported that haploinsufficient mutations in *TTF-1* are associated with pulmonary disease in infants and with variable inhibitory effects on the expression of SPs in human [50, 51]. However, in our current study, shRNA-TTF-1 was given 3 days before LPS injection. The time period was too short to cause significant pulmonary histological changes. As shown in Figure 1, after 3 days of transfection, no pathological alterations were observed in the control, shRNA-scrambled, and shRNA-TTF-1 groups of mice. Meanwhile, in the current study, TTF-1 knockdown via shRNA is not sufficient to completely abrogate TTF-1 expression in ATII cells. TTF-1 conditional knockout mice should be used to further confirm this mechanism. Interestingly, we also noticed that liraglutide alone could not promote SP-A expression both in vivo and in vitro (Figures 5 and 10). This result can probably be attributed to the complex SP-A metabolic balance. The molecular mechanisms responsible for SP-A metabolic balance in ATII cells should be investigated.

For the safety and tolerance of liraglutide, a comparable high dosage of liraglutide (2 mg/kg) was given to mice in the current study [15, 22]. It is reported that body weight and food intake reduction are the major side effects of GLP-1 analogues in both human and animals [52, 53]. Then, we also observed that the food intake of mice with liraglutide administration was suppressed in our study. And the role of energy and food consumption in the pathogenesis of ALI needs to be elucidated in the future.

Taken together, our results indicated that liraglutide upregulated SP-A expression in ATII cells and attenuated pulmonary inflammation, pulmonary edema, and increase in insulin level in LPS-induced ALI, most likely through the TTF-1 signaling pathway, suggesting that liraglutide may be considered an effective drug for the potential treatment of ARDS in the future.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (81230001, 81470236, and 81670038), China Postdoctoral Science Foundation (2014M552369), Natural Science Foundation of Guangdong Province (201707010282), Scientific Research Project of Guangzhou (2017A030310286), Medical Scientific Research Foundation of Sichuan Province (150123), Medical Science Foundation for Yong Innovative Project of Sichuan Province (Q15002), and Science and Technology Planning Project of Guangdong Province (2014A020212627 and 2016A020215099).

References

[1] E. Lopez-Rodriguez and J. Perez-Gil, "Structure-function relationships in pulmonary surfactant membranes: from

biophysics to therapy," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1838, no. 6, pp. 1568–1585, 2014.

[2] J. Perez-Gil and T. E. Weaver, "Pulmonary surfactant pathophysiology: current models and open questions," *Physiology*, vol. 25, no. 3, pp. 132–141, 2010.

[3] A. Nayak, E. Dodagatta-Marri, A. G. Tsolaki, and U. Kishore, "An insight into the diverse roles of surfactant proteins, SP-A and SP-D in innate and adaptive immunity," *Frontiers in Immunology*, vol. 3, p. 131, 2012.

[4] The ARDS Definition Task Force, "Acute respiratory distress syndrome: the Berlin Definition," *JAMA*, vol. 307, no. 23, pp. 2526–2533, 2012.

[5] M. A. Matthay and R. L. Zemans, "The acute respiratory distress syndrome: pathogenesis and treatment," *Annual Review of Pathology*, vol. 6, no. 1, pp. 147–163, 2011.

[6] M. Xiao, T. Zhu, W. Zhang et al., "Emodin ameliorates LPS-induced acute lung injury, involving the inactivation of NF-κB in mice," *International Journal of Molecular Sciences*, vol. 15, no. 11, pp. 19355–19368, 2014.

[7] Y. Sun, R. Yang, J. G. Zhong et al., "Aerosolised surfactant generated by a novel noninvasive apparatus reduced acute lung injury in rats," *Critical Care*, vol. 13, no. 2, article R31, 2009.

[8] K. Raghavendran, D. Willson, and R. H. Notter, "Surfactant therapy for acute lung injury and acute respiratory distress syndrome," *Critical Care Clinics*, vol. 27, no. 3, pp. 525–559, 2011.

[9] U. Kishore, T. J. Greenhough, P. Waters et al., "Surfactant proteins SP-A and SP-D: structure, function and receptors," *Molecular Immunology*, vol. 43, no. 9, pp. 1293–1315, 2006.

[10] N. T. Veith, T. Tschernig, B. Gutbier et al., "Surfactant protein A mediates pulmonary clearance of *Staphylococcus aureus*," *Respiratory Research*, vol. 15, no. 1, p. 85, 2014.

[11] S. P. Keese, K. Brandenburg, M. Roessle, and A. B. Schromm, "Pulmonary surfactant protein A-induced changes in the molecular conformation of bacterial deep-rough LPS lead to reduced activity on human macrophages," *Innate Immunity*, vol. 20, no. 8, pp. 787–798, 2014.

[12] D. Liu, M. Yi, M. Smith, and C. R. Mendelson, "TTF-1 response element is critical for temporal and spatial regulation and necessary for hormonal regulation of human *surfactant protein-A2* promoter activity," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 295, no. 2, pp. L264–L271, 2008.

[13] H. Chen, J. P. Zhang, H. Huang, Z. H. Wang, R. Cheng, and W. B. Cai, "Leptin promotes fetal lung maturity and upregulates SP-A expression in pulmonary alveoli type-II epithelial cells involving TTF-1 activation," *PLoS One*, vol. 8, no. 7, article e69297, 2013.

[14] W. Guo, H. Benlhabib, and C. R. Mendelson, "The microRNA 29 family promotes type II cell differentiation in developing lung," *Molecular and Cellular Biology*, vol. 36, no. 16, p. 2141, 2016.

[15] T. Zhu, X. L. Wu, W. Zhang, and M. Xiao, "Glucagon like peptide-1 (GLP-1) modulates OVA-induced airway inflammation and mucus secretion involving a protein kinase A (PKA)-dependent nuclear factor-κB (NF-κB) signaling pathway in mice," *International Journal of Molecular Sciences*, vol. 16, no. 9, pp. 20195–20211, 2015.

[16] N. E. Viby, M. S. Isidor, K. B. Buggeskov, S. S. Poulsen, J. B. Hansen, and H. Kissow, "Glucagon-like peptide-1 (GLP-1) reduces mortality and improves lung function in a model of experimental obstructive lung disease in female mice," *Endocrinology*, vol. 154, no. 12, pp. 4503–4511, 2013.

[17] M. Romaní-Pérez, V. Outeiriño-Iglesias, M. Gil-Lozano, L. C. González-Matías, F. Mallo, and E. Vigo, "Pulmonary GLP-1 receptor increases at birth and exogenous GLP-1 receptor agonists augmented surfactant-protein levels in litters from normal and nitrofen-treated pregnant rats," *Endocrinology*, vol. 154, no. 3, pp. 1144–1155, 2013.

[18] S. Steven, K. Jurk, M. Kopp et al., "Glucagon-like peptide-1 receptor signalling reduces microvascular thrombosis, nitro-oxidative stress and platelet activation in endotoxaemic mice," *British Journal of Pharmacology*, vol. 174, no. 12, pp. 1620–1632, 2017.

[19] Y. Bao, L. Jiang, H. Chen, J. Zou, Z. Liu, and Y. Shi, "The neuroprotective effect of liraglutide is mediated by glucagon-like peptide 1 receptor-mediated activation of cAMP/PKA/CREB pathway," *Cellular Physiology and Biochemistry*, vol. 36, no. 6, pp. 2366–2378, 2015.

[20] S. Ben-Shlomo, I. Zvibel, M. Shnell et al., "Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase," *Journal of Hepatology*, vol. 54, no. 6, pp. 1214–1223, 2011.

[21] T. Zhu, D. X. Wang, W. Zhang et al., "Andrographolide protects against LPS-induced acute lung injury by inactivation of NF-κB," *PLoS One*, vol. 8, no. 2, article e56407, 2013.

[22] S. Gou, T. Zhu, W. Wang, M. Xiao, X. C. Wang, and Z. H. Chen, "Glucagon like peptide-1 attenuates bleomycin-induced pulmonary fibrosis, involving the inactivation of NF-κB in mice," *International Immunopharmacology*, vol. 22, no. 2, pp. 498–504, 2014.

[23] T. Zhu, W. Zhang, D. X. Wang et al., "Rosuvastatin attenuates mucus secretion in a murine model of chronic asthma by inhibiting the gamma-aminobutyric acid type A receptor," *Chinese Medical Journal*, vol. 125, no. 8, pp. 1457–1464, 2012.

[24] X. G. Guo, T. X. Ji, Y. Xia, and Y. Y. Ma, "Autophagy protects type II alveolar epithelial cells from *Mycobacterium tuberculosis* infection," *Biochemical and Biophysical Research Communications*, vol. 432, no. 2, pp. 308–313, 2013.

[25] M. Bhaskaran, N. Kolliputi, Y. Wang, D. Gou, N. R. Chintagari, and L. Liu, "Trans-differentiation of alveolar epithelial type II cells to type I cells involves autocrine signaling by transforming growth factor β1 through the Smad pathway," *Journal of Biological Chemistry*, vol. 282, no. 6, pp. 3968–3976, 2007.

[26] T. Zhu, W. Zhang, S. Feng, and H.-P. Yu, "Emodin suppresses LPS-induced inflammation in RAW264.7 cells through a PPARγ-dependent pathway," *International Immunopharmacology*, vol. 34, pp. 16–24, 2016.

[27] B. Schisano, A. L. Harte, K. Lois et al., "GLP-1 analogue, liraglutide protects human umbilical vein endothelial cells against high glucose induced endoplasmic reticulum stress," *Regulatory Peptides*, vol. 174, no. 1–3, pp. 46–52, 2012.

[28] M. Xiao, T. Zhu, T. Wang, and F. Q. Wen, "Hydrogen-rich saline reduces airway remodeling via inactivation of NF-κB in a murine model of asthma," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 8, pp. 1033–1043, 2013.

[29] H. Herwald and A. Egesten, "On PAMPs and DAMPs," *Journal of Innate Immunity*, vol. 8, no. 5, pp. 427-428, 2016.

[30] T. Ito, "PAMPs and DAMPs as triggers for DIC," *Journal of Intensive Care*, vol. 2, no. 1, p. 67, 2014.

[31] T. Zhu, W. Zhang, and D. X. Wang, "Insulin up-regulates epithelial sodium channel in LPS-induced acute lung injury model in rats by SGK1 activation," *Injury*, vol. 43, no. 8, pp. 1277–1283, 2012.

[32] D. A. Sandoval and D. A. D'Alessio, "Physiology of proglucagon peptides: role of glucagon and GLP-1 in health and disease," *Physiological Reviews*, vol. 95, no. 2, pp. 513–548, 2015.

[33] L. Fransson, C. dos Santos, P. Wolbert, Å. Sjöholm, A. Rafacho, and H. Ortsäter, "Liraglutide counteracts obesity and glucose intolerance in a mouse model of glucocorticoid-induced metabolic syndrome," *Diabetology & Metabolic Syndrome*, vol. 6, no. 1, p. 3, 2014.

[34] M. A. Landgraf, R. C. Silva, M. Corrêa-Costa et al., "Leptin downregulates LPS-induced lung injury: role of corticosterone and insulin," *Cellular Physiology and Biochemistry*, vol. 33, no. 3, pp. 835–846, 2014.

[35] J. A. Whitsett, S. E. Wert, and T. E. Weaver, "Diseases of pulmonary surfactant homeostasis," *Annual Review of Pathology*, vol. 10, no. 1, pp. 371–393, 2015.

[36] S. Han and R. K. Mallampalli, "The role of surfactant in lung disease and host defense against pulmonary infections," *Annals of the American Thoracic Society*, vol. 12, no. 5, pp. 765–774, 2015.

[37] R. Haque, T. M. Umstead, P. Ponnuru et al., "Role of surfactant protein-A (SP-A) in lung injury in response to acute ozone exposure of SP-A deficient mice," *Toxicology and Applied Pharmacology*, vol. 220, no. 1, pp. 72–82, 2007.

[38] Q. Du, C. Wang, N. Zhang et al., "In vivo study of the effects of exogenous hydrogen sulfide on lung mitochondria in acute lung injury in rats," *BMC Anesthesiology*, vol. 14, no. 1, p. 117, 2014.

[39] L. E. Carreto-Binaghi, E. M. Aliouat, and M. L. Taylor, "Surfactant proteins, SP-A and SP-D, in respiratory fungal infections: their role in the inflammatory response," *Respiratory Research*, vol. 17, no. 1, p. 66, 2016.

[40] N. Mittal and S. N. Sanyal, "Cycloxygenase inhibition enhances the effects of surfactant therapy in endotoxin-induced rat model of ARDS," *Inflammation*, vol. 34, no. 2, pp. 92–98, 2011.

[41] D. Xinmin, D. Yunyou, P. Chaosheng et al., "Dexamethasone treatment attenuates early seawater instillation-induced acute lung injury in rabbits," *Pharmacological Research*, vol. 53, no. 4, pp. 372–379, 2006.

[42] A. Das, S. Acharya, K. R. Gottipati et al., "Thyroid transcription factor-1 (TTF-1) gene: identification of ZBP-89, Sp1, and TTF-1 sites in the promoter and regulation by TNF-α in lung epithelial cells," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 301, no. 4, pp. L427–L440, 2011.

[43] L. Cui, D. Zheng, Y. H. Lee et al., "Metabolomics investigation reveals metabolite mediators associated with acute lung injury and repair in a murine model of influenza pneumonia," *Scientific Reports*, vol. 6, no. 1, article 26076, 2016.

[44] L. Wang, J. Cang, and Z. Xue, "Protective effects of thoracic epidural anesthesia on hypoxia-induced acute lung injury in rabbits," *Experimental and Therapeutic Medicine*, vol. 11, no. 5, pp. 2021–2027, 2016.

[45] B. Xiao, J. Xu, G. Wang et al., "Troglitazone-activated PPARγ inhibits LPS-induced lung alveolar type II epithelial cells injuries via TNF-α," *Molecular Biology Reports*, vol. 38, no. 8, pp. 5009–5015, 2011.

[46] M. Romaní-Pérez, V. Outeiriño-Iglesias, C. M. Moya et al., "Activation of the GLP-1 receptor by liraglutide increases ACE2 expression, reversing right ventricle hypertrophy, and improving the production of SP-A and SP-B in the lungs of type 1 diabetes rats," *Endocrinology*, vol. 156, no. 10, pp. 3559–3569, 2015.

[47] X. Du, Q. Meng, A. Sharif et al., "Surfactant proteins SP-A and SP-D ameliorate pneumonia severity and intestinal injury in a murine model of Staphylococcus aureus pneumonia," *Shock*, vol. 46, no. 2, pp. 164–172, 2016.

[48] C. M. Minutti, B. García-Fojeda, A. Sáenz et al., "Surfactant protein A prevents IFN-γ/IFN-γ receptor interaction and attenuates classical activation of human alveolar macrophages," *The Journal of Immunology*, vol. 197, no. 2, pp. 590–598, 2016.

[49] Y. Maeda, G. Chen, Y. Xu et al., "Airway epithelial transcription factor NK2 homeobox 1 inhibits mucous cell metaplasia and Th2 inflammation," *American Journal of Respiratory and Critical Care Medicine*, vol. 184, no. 4, pp. 421–429, 2011.

[50] A. Hamvas, R. R. Deterding, S. E. Wert et al., "Heterogeneous pulmonary phenotypes associated with mutations in the thyroid transcription factor gene *NKX2-1*," *Chest*, vol. 144, no. 3, pp. 794–804, 2013.

[51] C. Galambos, H. Levy, C. L. Cannon et al., "Pulmonary pathology in thyroid transcription factor-1 deficiency syndrome," *American Journal of Respiratory and Critical Care Medicine*, vol. 182, no. 4, pp. 549–554, 2010.

[52] K. Raun, P. von Voss, C. F. Gotfredsen, V. Golozoubova, B. Rolin, and L. B. Knudsen, "Liraglutide, a long-acting glucagon-like peptide-1 analog, reduces body weight and food intake in obese candy-fed rats, whereas a dipeptidyl peptidase-IV inhibitor, vildagliptin, does not," *Diabetes*, vol. 56, no. 1, pp. 8–15, 2007.

[53] L. van Bloemendaal, R. G. IJzerman, J. S. ten Kulve et al., "GLP-1 receptor activation modulates appetite- and reward-related brain areas in humans," *Diabetes*, vol. 63, no. 12, pp. 4186–4196, 2014.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 2456579, 11 pages
https://doi.org/10.1155/2018/2456579

Review Article

Diabetes Mellitus and Liver Surgery: The Effect of Diabetes on Oxidative Stress and Inflammation

Mariana Mendes-Braz and Joilson O. Martins

Laboratory of Immunoendocrinology, Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences of University Sao Paulo (FCF/USP), São Paulo, SP, Brazil

Correspondence should be addressed to Mariana Mendes-Braz; marianamb@usp.br and Joilson O. Martins; martinsj@usp.br

Received 22 October 2017; Revised 2 April 2018; Accepted 11 April 2018; Published 8 May 2018

Academic Editor: Fumio Tsuji

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia and high morbidity worldwide. The detrimental effects of hyperglycaemia include an increase in the oxidative stress (OS) response and an enhanced inflammatory response. DM compromises the ability of the liver to regenerate and is particularly associated with poor prognosis after ischaemia-reperfusion (I/R) injury. Considering the growing need for knowledge of the impact of DM on the liver following a surgical procedure, this review aims to present recent publications addressing the effects of DM (hyperglycaemia) on OS and the inflammatory process, which play an essential role in I/R injury and impaired hepatic regeneration after liver surgery.

1. Introduction

To extirpate a macroscopic lesion or accomplish a transplant, the blood flow to the liver must be interrupted to avoid the haemorrhagic process. Despite the safety of surgical procedures that involve the interruption of blood flow to the liver (ischaemia), this interruption contributes to tissue damage, which is potentiated by the restoration of blood flow (reperfusion). This phenomenon, known as ischaemia-reperfusion (I/R) injury [1, 2], is associated with inflammation and oxidative stress (OS) [3].

Diabetes mellitus (DM) is a metabolic disorder resulting from deficient insulin secretion and/or insulin action, leading to hyperglycaemia (high blood glucose) [4], which causes oxidative damage and activates inflammatory signalling cascades [5], in addition to acting as a damaging agent exacerbating the pathological conditions of DM [6, 7]. Considering the growing need for knowledge about the impact of DM on livers undergoing a surgical procedure, the present review aims to present recent data concerning the effects of DM (hyperglycaemia) on OS and the inflammatory process.

2. Oxidative Stress

Under normal conditions, the hepatic production of prooxidants, such as reactive oxygen species (ROS), is counterbalanced by antioxidants. An imbalance in favour of prooxidants corresponds to OS, and the direct action of ROS on cell viability and function is directly related to the occurrence of several pathological processes in the liver [8]. OS plays an essential role in liver surgery [9], and diabetes is generally followed by increased free radical production [10–13] or reduced antioxidant protection [14, 15]. To better understand the effect of DM (hyperglycaemia) on OS, this section will describe research findings that help clarify the association of DM with liver surgery.

2.1. Diabetes Mellitus and Ischaemia-Reperfusion Injury. Hydrogen peroxide (H_2O_2), a mild and relatively stable oxidant that is formed in tissues exposed to I/R, has been considered a representative ROS for evaluating the response of cells to OS [16]. Although H_2O_2 is not a free radical, its accumulation may promote the formation of more toxic species, such as hydroxyl radicals (•OH), through

the Fenton reaction [17]. H_2O_2 can cause permanent growth arrest [18, 19] and apoptosis [20–22] in a number of cell types. Nuclear (8-hydroxy-2′-deoxyguanosine) 8-OHdG formation indicates the presence of OS in nuclei [23]. The liver is a major organ affected by ROS [24] and is susceptible to the effects of OS induced by hyperglycaemia, causing liver injury [25–27]. Zhang et al. [28] found that serum H_2O_2 and nuclear 8-OHdG levels were higher in streptozotocin-(STZ-) induced diabetic rats subjected to I/R compared with the diabetic control group. ROS induce lipid peroxidation, which causes membrane injury, in addition to changes in ion permeability, enzyme activity, and, ultimately, cell death. Malondialdehyde (MDA), an indicator of oxidative injury produced via lipid peroxidation [29], is significantly enhanced in STZ-induced diabetic rats compared with normal rats and increases after I/R [28, 30] (Figure 1).

Apoptosis and necrosis can occur after I/R. An intense injury leads to initial necrotic killing, whereas late apoptosis may follow moderate injury [31]. STZ-induced diabetic rats exposed to an ischaemic period present significantly increased hepatocyte degeneration, sinusoidal dilatation, nuclear pyknosis, and cellular necrosis compared with the diabetes sham group [30]. In spite of this experimental difference, Behrends et al. [32] reported that necrosis is the preferential form of cell death in the liver of hyperglycemic rats (due to intraperitoneal injection of 25% glucose) subjected to I/R. The authors [32] noted that this increased injury may be associated with the inhibition of heat shock proteins (HSPs), which is only possible through the association of hyperglycaemia and I/R. The hyperglycaemia alone was not enough for HSP32 and HSP70 downregulation. HSPs are considered to be an indispensable protective agent against I/R injury because they are able to protect the liver from OS [33] (Figure 1).

Cell adaptation to OS is a consequence of the upregulation of distinct cytoprotective genes responsible for buffering the antioxidant capacity of the cell [34]. Under physiological conditions, an antioxidant defence system protects the body against the harmful effects of free radicals [35]. Diabetic livers are vulnerable to attack by oxygen free radicals because they present overall antioxidant depression [14]. Release of ROS and the concurrent consumption of endogenous antioxidants and cell death (apoptosis or necrosis) occur during hepatic I/R [36]. After I/R, nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a transcription factor that mediates the expression of many endogenous antioxidants plays an important role in opposing hepatic injury [37]. Zhang et al. [28] reported that, after I/R injury, hepatocytes pretreated with high glucose (25 mM) exhibited a reduction in the antioxidative ability of the Nrf2 pathway and a substantial increase in nuclear factor kappa B (NF-κB) translocation; however, NF-κB activation was already enhanced in these hepatocytes before I/R injury. Interestingly, NF-κB, a transcription factor that reacts to redox signals, may directly repress Nrf2 signalling at the transcriptional level [38, 39]. Zhang et al. [28] postulated that high glucose-induced ROS overproduction could initiate the inhibitory interaction between NF-κB and Nrf2 (Figure 1). However, the precise mechanisms involved in the NF-κB and Nrf2 interaction under hyperglycaemic conditions require further elucidation.

Under normal conditions, the body presents a potent antioxidant system that is responsible for protecting it from the harmful effects of ROS [40]. Endogenous antioxidant enzymes attenuate I/R injury in the liver [36]. In both type 1 and type 2 DM, antioxidant defence enzymes are deficient, and there is an increase in oxidative damage [41]. High levels of ROS such as superoxide (O_2^-) are found in diabetes and especially during I/R injury [42]. Cem Sezen et al. [30] showed that there is an increase in glutathione s-transferase (GST) in STZ-induced diabetic mice post-I/R with respect to diabetic rats. Between these two groups, there was no difference in the level of superoxide dismutase (SOD); however, compared with the sham group (nondiabetic), there was a marked decrease in SOD levels. The orchestrated actions of several antioxidants in mammalian cells are essential for efficiently detoxifying free radicals. Therefore, any impairment in this pathway will influence the activities of other enzymes [43, 44]. Reduction in the activity of SOD will result in an increased level of O_2^- [45]. GST is known to be an early and sensitive marker of liver injury and has been shown to increase after liver ischaemia/reperfusion [46]. This increased activity of GST could be explained as a compensatory mechanism to protect the organism against injury [47]. These findings are not only in accord with the diverse signalling pathways related to postoperative liver injury associated with DM (Figure 1) but also indicate the importance of the determination of increased ROS production and its characteristic consequences in post-ischaemic tissues, permitting the identification of interventions that stimulates ROS detoxification, and consequently protect against reperfusion injury [16], mainly in a diabetic context (Figure 1).

2.2. Diabetes Mellitus and Liver Regeneration. An increase in lipid peroxidation was found to be important for a normal proliferative process to occur in the liver remnant after partial hepatectomy (PH) [48, 49]. Francés et al. [50] reported that OS is increased by hyperglycaemia and is juxtaposed with the effect of PH in STZ-induced diabetic rats. Postoperative recovery depends on the regenerative capacity of the residual liver. The liver presents altered intracellular signalling pathways in type 1 DM specimens [51–53] and a consequent deficient regenerative response [54]. STZ-induced diabetic rats were found to present an increase in •OH production, which could result in DNA damage [55, 56] (Figure 1). Hyperglycaemia in STZ-induced diabetic rats leads to an increase in hepatic ROS production and is further enhanced after PH. STZ-induced diabetic rats subjected to PH present a decrease in the level of proliferating cell nuclear antigen (PCNA) and a significant decrease in cyclin D1 levels, suggesting that few hepatocytes are capable of entering the cell cycle [50].

Hyperglycaemia enhances •OH radical levels and consequent Bax protein induction. After PH, STZ-induced diabetic rats were found to present an increase in proapoptotic events (Bax/Bcl-xL ratio, caspase-3 activity, and cytosolic cytochrome c) compared with the diabetic group

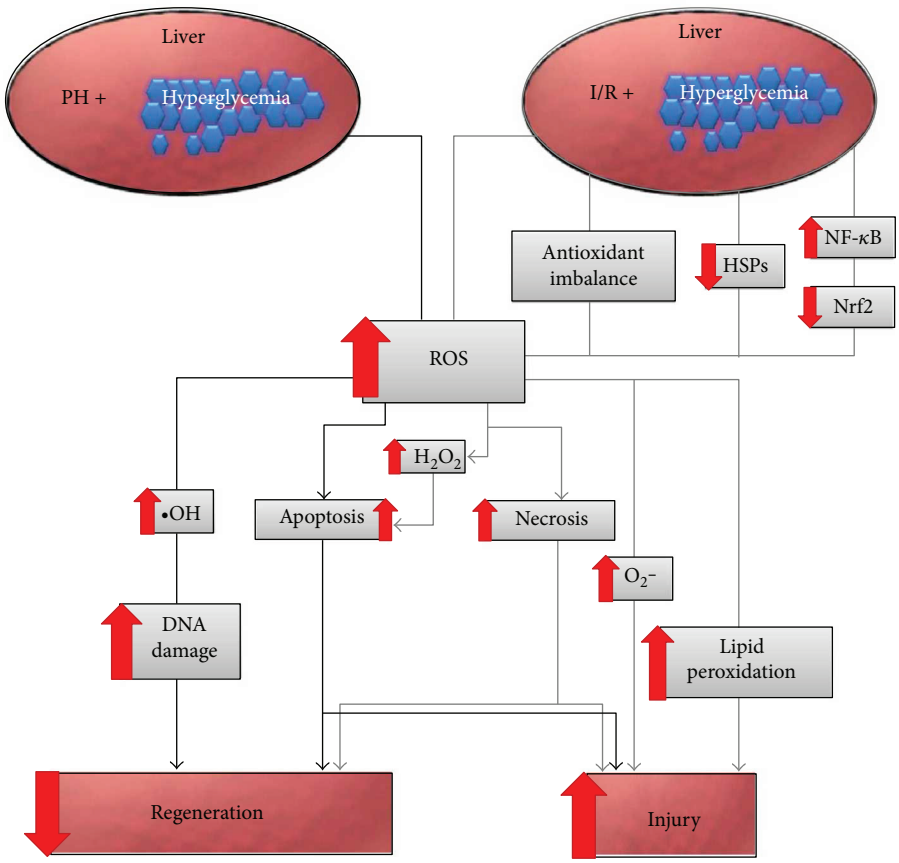


Figure 1: Mechanisms of OS in the promotion of liver damage and impaired regeneration after liver surgery in association with DM. The illustration shows the molecular events subsequent to the surgical procedure performed on the diabetic liver, which leads to a significant increase of ROS, inducing liver injury and regeneration. PH, partial hepatectomy; I/R, ischaemia-reperfusion; O_2^-, superoxide anion; HSP, heat shock protein; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor (erythroid-derived 2)-like-2 factor; H_2O_2, hydrogen peroxide; •OH, hydroxyl radical.

[50] (Figure 1). The diversity of the results of different studies [30, 32, 50, 55] shows that the association of hyperglycaemia with different surgical modalities leads to differences in the type of cell death. It is imperative to identify the effects of diabetes on cell death after more complex surgical procedures leading to pronounced liver injury, such as liver transplantation and PH under I/R.

3. Inflammation

Hepatic inflammation is a complex process that is initiated in response to stressful conditions to protect hepatocytes from injury. However, overly intense inflammatory responses are followed by massive hepatocyte loss, causing irreversible parenchymal damage [57]. Liver damage is a serious complication in DM [58]. Surgical procedures induce acute inflammation, which is characterized by the production and release of various chemical mediators, including cytokines [59]. In the next section, the effects of DM (hyperglycaemia) on the hepatic inflammatory process after a surgical procedure will be discussed.

3.1. Diabetes Mellitus and Ischaemia-Reperfusion Injury. The pathophysiology of hepatic I/R injury is not only related to the direct cell impairment caused by ischaemic insult but also results from the restoration of blood flow, which triggers the proinflammatory environment. Diabetic patients present a variety of deficient immune cell functions [60, 61], and diabetic animals exhibit abnormalities in the course of the inflammatory response, with a consequent decrease in the number of leukocytes in inflammatory injuries [62, 63], the airway inflammatory response to antigen challenge [64, 65], mast cell degranulation [66, 67], superoxide generation, and tumour necrosis factor- (TNF-) α release by leukocytes upon exposure to lipopolysaccharides [68]. The difficulty in arriving at any consistent conclusion is due to the conflicting views regarding the impact of hyperglycaemia on inflammatory responses between different reports. Since clinical observations have revealed that the association between hyperglycaemia and immune alterations could increase the risk for rejection in transplantation, the substantial inflammatory response associated with I/R injury appears to be mediated by an exaggerated adhesion of leukocytes to the endothelium [69, 70].

The hyperinflammatory phenotype associated with DM may induce a liver immune response against I/R, which could favour an increase in parenchymal damage [71]. In the initial phase of liver injury, different events trigger a complex

inflammatory pathway that leads to hepatic accumulation of neutrophils [72]. Through the release of oxidants and proteases, hepatocytes are directly damaged by recruited neutrophils, which are involved in by the later phase of liver injury induced by I/R [73]. In the livers of hyperglycaemic rats subjected to I/R, Behrends et al. [32] observed an increase in neutrophil infiltration (Figure 2). Interestingly, in association with microvascular dysfunction in response to I/R, neutrophil infiltration is exacerbated in DM, suggesting that DM predisposes tissues to the detrimental consequences of I/R, which is a deleterious process that is broadly mediated by neutrophils [69].

The immune system responds to liver injury and/or stress through the activation of resident Kupffer cells (KCs), which release proinflammatory cytokines and other factors [74]. A prominent feature of liver injury is an increase in the hepatic macrophage population [75]. Considering cellular and molecular mechanisms, Yue et al. [71] showed that I/R stimulates the release of advanced glycation end products (AGE) into the blood of STZ-induced diabetic mice and that KCs express higher levels of the receptor for AGE (RAGE). The authors [71] proposed that RAGE may exhibit different functions in a cell type-specific manner. In normal mice, RAGE regulates hepatocyte proliferation during the restoration phase of I/R, whereas in diabetic mice, RAGE activates the hepatic immune system. These findings support the hypothesis that DM may be a factor involved in the course and evolution of I/R injury after liver surgery.

Activated KCs respond with a classic inflammatory reaction and consequent production of proinflammatory cytokines [76–80]. At 6 hours after reperfusion, TNF-α and interleukin- (IL-) 6 levels were found to be increased, while the IL-10 level was decreased on STZ-induced diabetic mice [71, 81] (Figure 2), whereas in control mice, KCs not only presented increases in TNF-α and IL-6 but also an increase in IL-10 [81]. The activation of IL-10 during a proinflammatory response may represent an important agent in the regulation of intensive inflammation in a stressful situation. These findings not only illustrate the defensive role of KCs during liver I/R injury in opposing the hyperinflammatory response through IL-10 expression but also show that hyperglycemic mice subjected to I/R present a significant decrease in IL-10 secretion, by KCs, which is related to uncontrolled inflammation and robust hepatic I/R injury [81].

Several studies suggest that endoplasmic reticulum stress and CHOP signalling could be upregulated by RAGE signalling [82–85]. After 6 hours of reperfusion, C/EBP homologous protein (CHOP) levels in KCs were found to be stimulated by I/R and were further increased in STZ-induced hyperglycemic mice. In hyperglycemic KCs, overactivation of CHOP is related to the inhibition of STAT3 and STAT6 activation. The signal transducers and activators of transcription (STATs) regulate the polarization of macrophages [86], and diabetic mice present M2 KC phenotype inhibition, which results in increased inflammation under hepatic I/R when the rodents exhibit interruption of IL-10-secreting M2 differentiation [81]. Additionally, mice that are only subjected to ischaemia show development of M2-type macrophages, which protect livers from I/R via an IL-10-dependent mechanism [87] (Figure 2).

In the pathogenesis of DM, activated innate immunity and inflammation are important factors. Type 2 DM involves inflammatory elements [88, 89], and type 1 DM is regarded as an inflammatory process [90]. NF-κB is a transcription factor that is activated in the diabetic liver [91–93] and is involved in events that lead to inflammation [94]. NF-κB regulates the expression of many inflammatory cytokines, including monocyte chemotactic protein- (MCP-) 1, IL-6, and TNF-α [95, 96], which are proinflammatory cytokines that may activate neutrophils and KCs [97]. Zhang et al. [28] showed that after 6 hours of reperfusion, the levels of these hepatic cytokines were significantly higher in STZ-induced diabetic rats and further increased after the ischaemic period. These results suggested that NF-κB might also be involved in hepatic I/R in diabetic rats (Figure 2). The investigation of NF-κB activation in diabetic livers subjected to surgical procedures should be extended to cell death. Between NF-κB and TNF-α, there is an autocrine-reinforcing loop [98, 99]. The hepatic increase of TNF-α in STZ-induced diabetic rats leads to pronounced upregulation of the NF-κB pathway [100], and NF-κB activation induced by hyperglycaemia mediates cell apoptosis [101, 102].

Several inflammatory cytokines (e.g., TNF-α) and arachidonic acid metabolites (prostaglandins and thromboxanes) are involved in liver injury induced by I/R. Cyclooxygenase (COX) regulates the production of prostanoids [103], and inhibition of COX-2 protects against hepatic I/R injury [104, 105], which suggests that COX-2 is associated with organ injury and contributes to hepatic microvascular and hepatocellular injuries through TNF-α production [103]. Hepatocyte apoptosis stimulated by TNF is associated with c-Jun N-terminal kinase (JNK) activation [106]. Conversely, Francés et al. [107] showed that STZ-induced diabetic COX-2 transgenic mice presented a substantial decrease in apoptosis and that COX-2 overexpression could prevent the increase in JNK activity stimulated by high glucose. The authors [107] also showed that the increased expression of COX-2 in diabetic COX-2 transgenic mice induces an increase of phosphoinositide 3-kinase (PI3K) activity compared with diabetic wild-type mice, in addition to favouring the activation of Akt and producing an antiapoptotic signal [107]. These studies call attention not only to the contradictory roles of diabetes in orchestrating hepatocyte activity but also to the necessity of clearly understanding the consequences of diabetes for cell death after liver surgery (Figure 2).

3.2. Diabetes Mellitus and Liver Regeneration. In a model of type 2 DM (ob/ob murine), liver regeneration was found to be impaired after 70% PH, which resulted in 90% mortality [108]. The regenerative ability of the liver is compromised in type 1 diabetic rats subjected to PH [51, 52, 109]. In patients subjected to a major hepatectomy, DM tends to induce postoperative liver failure [110]. Considering the mechanisms of regeneration failure, diabetic and obese KK-Ay mice exhibit abnormal responses after PH [111] and present excessive induction of hepatic TNF-α

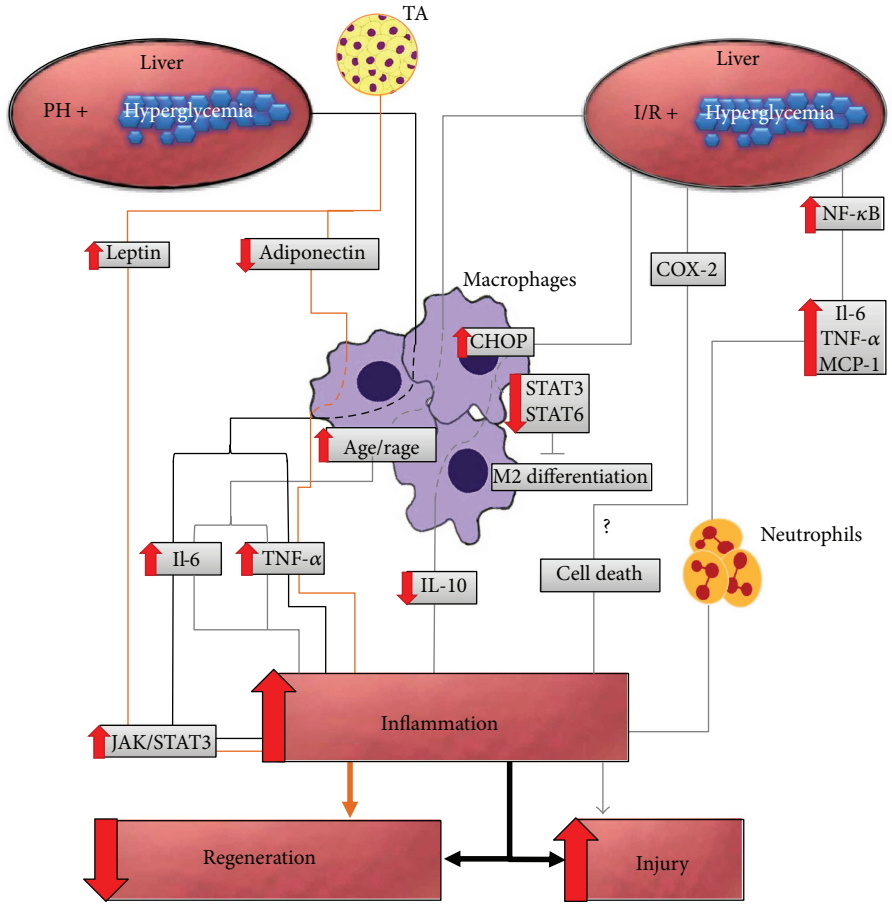


Figure 2: Inflammatory mechanisms underlying the promotion of liver damage and impaired regeneration after liver surgery in association with DM. The illustration shows the molecular events subsequent to the surgical procedure performed on the diabetic liver, inducing the participation of inflammatory cells and consequent cytokine production, leading to liver injury and regeneration. TA, adipose tissue; AGE, advanced glycation end products; RAGE, receptor for AGE; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumour necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; CHOP, C/EBP homologous protein; NF-κB, nuclear factor kappa B; COX-2, cyclooxygenase-2; PH, partial hepatectomy; I/R, ischaemia-reperfusion.

expression. Although TNF-α is important for the initiation of normal hepatic regeneration [112, 113], excess induction of TNF-α in KCs might interfere with the regenerative process [111] (Figure 2).

Adipose tissue is involved in a number of biological functions, including inflammation, and acts as an endocrine organ through the secretion of several biologically active substances known as "adipokines" [114]. During liver regeneration, systemic adipose stores are required as a source of various adipokines, such as adiponectin, which is an essential signal for liver regeneration [115]. Aoyama et al. [111] showed that the serum adiponectin level was significantly reduced in KK-Ay mice before PH and tended to decrease gradually after PH. Adiponectin has been found to inhibit the lipopolysaccharide-dependent activation of macrophages [116, 117]. The significant hypoadiponectinemia presented by KK-Ay mice could be related to the fact that the KCs of these animals are more susceptible to certain stimuli; moreover, the hypoadiponectinemia caused by this susceptibility could be further associated with the increased production of TNF-α by KCs, which may interfere with regenerative responses [111] (Figure 2). Adiponectin mediates anti-inflammatory effects. However, since this role for adiponectin was found to depend on surgical conditions, the function of adiponectin in the inflammatory process is a controversial issue [118]. While injurious effects of adiponectin on steatotic livers subjected to warm ischaemia (60 minutes) were identified by Massip-Salcedo et al. [119], the beneficial (anti-inflammatory) effects of adiponectin on small fatty grafts subjected to cold ischaemia (40 minutes) were observed by Man et al. [120]. Although these findings were obtained in steatotic livers, these results suggest opportunities for investigation of the effect of adiponectin on diabetic livers subjected to different surgical procedures.

IL-6 is a protein synthesized by fibroblasts, monocytes, macrophages, T cells, and endothelial cells [121] that plays an important role in hepatic regeneration [122, 123]. Adipokines exhibit proinflammatory or anti-inflammatory activities [124], and leptin presents proinflammatory properties [125, 126]. IL-6 and leptin function in the Janus kinase-(JAK-) STAT3 signalling pathway [111]. KK-Ay mice present a substantial increase in the levels of IL-6 and leptin

following PH [111]. Despite the important role of the JAK-STAT pathway in hepatic protection against different hepatic injuries [127, 128] and the evidence that IL-6, leptin, and the JAK-STAT signalling pathway are essential to liver regeneration [129–132], Aoyama et al. [111] showed that the role of the JAK-STAT pathway in hepatic regeneration seems to be complex and dependent on the intensity of the stimulus, showing that hyperphosphorylation of STAT3 favours poor hepatic regeneration as a result of direct downregulation of cyclin D1 expression (Figure 2).

4. Diabetes Mellitus in Clinical Situations

There is an absence of clinical studies elucidating signalling pathways related to liver damage and impaired regeneration in diabetic patients undergoing surgery. Nevertheless, it is indispensable to discuss and generate hypotheses about this issue, which is quite controversial because some studies have shown that DM patients present a poorer prognosis after hepatic surgery in comparison with non-DM patients, whereas others show no difference [133].

Focusing on the issues addressed in this review (OS and inflammation), Li et al. [133] and Shields et al. [134] described the typical change in microcirculation that occurs in diabetic patients after liver surgery. The ischaemic period and liver perfusion recovery are important factors related to hepatocellular damage because microcirculatory collapse is followed by a pronounced reduction of tissue oxygenation [135], which might result in degeneration and necrosis of hepatocytes and consequent liver dysfunction [136]. Experimental models of I/R injury have offered evidence that insufficient hepatic microcirculatory perfusion, inflammatory cell activation, and consequent generation of ROS, cytokines, and chemokines can be considered essential in I/R syndrome [137]. Although the authors [133, 134] did not report the relationship between diabetic liver failure after liver surgery and microcirculation collapse, we take this opportunity to raise this question for the development of future studies.

The alterations of hepatic haemodynamics are also related to hepatic steatosis, and a decrease in portal vein haemodynamics is observed in patients with a fatty liver disease [138, 139]. Moreover, experimental animals with steatosis present decreased parenchymal microcirculation [140]. Hepatic steatosis has long been reported in type 1 [141] and type 2 DM [142]. Steatosis is common in diabetic patients (36% incidence) [143], and increased steatosis raises the sensibility of the liver parenchyma to I/R injury [144]. In steatotic livers, the parenchymal regeneration ability is impaired, particularly after a surgical procedure [115], which may partially explain the incapacity of some diabetic patients to resist liver surgery. The high mortality observed in diabetic patients is absent in nondiabetic patients with steatosis [143]. In hepatocytes, increased accumulation of fatty acids induces OS arising from mitochondria, peroxisomes, or microsomes. ROS and lipid peroxidation products can influence KCs and stimulate NF-κB activation, which in turn stimulates the production of TNF-α and several proinflammatory cytokines, such as IL-6 [143], which are presented in this review as factors involved in decreased regeneration and increased liver damage.

5. Conclusion

The purpose of this review was to discuss the literature addressing the damaging effect of DM on liver recovery after a surgical procedure and, especially, to highlight the need to expand knowledge of this issue to benefit patients with DM subjected to surgical procedures, which are increasing in clinical practice. Extensive work is still necessary to assess the differences between the diabetic and nondiabetic liver after a surgical procedure. Exploring this subject will enable the development of new treatments that will improve the success of diabetic liver recovery after surgery.

Conflicts of Interest

The authors declare that they have no conflicts of interest. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Acknowledgments

The authors apologize to the many researchers whose work they have not been able to discuss in this limited review. The authors are supported by Grants 2016/24992-0 and 2017/11540-7 from the São Paulo Research Foundation (FAPESP), Grant 301617/2016-3 from the National Counsel of Technological and Scientific Development (CNPq, PQ-1D), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

[1] D. J. Powner, "Factors during donor care that may affect liver transplantation outcome," *Progress in Transplantation*, vol. 14, no. 3, pp. 241–249, 2004.

[2] J. M. Henderson, "Liver transplantation and rejection: an overview," *Hepato-Gastroenterology*, vol. 46, Supplement 2, pp. 1482–1484, 1999.

[3] F. Serracino-Inglott, N. A. Habib, and R. T. Mathie, "Hepatic ischemia-reperfusion injury," *The American Journal of Surgery*, vol. 181, no. 2, pp. 160–166, 2001.

[4] S. Celik, S. Erdogan, and M. Tuzcu, "Caffeic acid phenethyl ester (CAPE) exhibits significant potential as an antidiabetic and liver-protective agent in streptozotocin-induced diabetic rats," *Pharmacological Research*, vol. 60, no. 4, pp. 270–276, 2009.

[5] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[6] A. Guven, O. Yavuz, M. Cam et al., "Effects of melatonin on streptozotocin-induced diabetic liver injury in rats," *Acta Histochemica*, vol. 108, no. 2, pp. 85–93, 2006.

[7] M. Romagnoli, M. C. Gomez-Cabrera, M. G. Perrelli et al., "Xanthine oxidase-induced oxidative stress causes activation of NF-κB and inflammation in the liver of type I diabetic rats," *Free Radical Biology & Medicine*, vol. 49, no. 2, pp. 171–177, 2010.

[8] L. A. Videla, R. Rodrigo, M. Orellana et al., "Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients," *Clinical Science*, vol. 106, no. 3, pp. 261–268, 2004.

[9] T. Fukuda, K. Ikejima, M. Hirose, Y. Takei, S. Watanabe, and N. Sato, "Taurine preserves gap junctional intercellular communication in rat hepatocytes under oxidative stress," *Journal of Gastroenterology*, vol. 35, no. 5, pp. 361–368, 2000.

[10] J. W. Baynes and S. R. Thorpe, "Role of oxidative stress in diabetic complications: a new perspective on an old paradigm," *Diabetes*, vol. 48, no. 1, pp. 1–9, 1999.

[11] J. W. Baynes, "Role of oxidative stress in development of complications in diabetes," *Diabetes*, vol. 40, no. 4, pp. 405–412, 1991.

[12] K. C. Chang, S. Y. Chung, W. S. Chong et al., "Possible superoxide radical-induced alteration of vascular reactivity in aortas from streptozotocin-treated rats," *The Journal of Pharmacology and Experimental Therapeutic*, vol. 266, no. 2, pp. 992–1000, 1993.

[13] I. S. Young, S. Tate, J. H. Lightbody, D. McMaster, and E. R. Trimble, "The effects of desferrioxamine and ascorbate on oxidative stress in the streptozotocin diabetic rat," *Free Radical Biology & Medicine*, vol. 18, no. 5, pp. 833–840, 1995.

[14] A. K. Saxena, P. Srivastava, R. K. Kale, and N. Z. Baquer, "Impaired antioxidant status in diabetic rat liver. Effect of vanadate," *Biochemical Pharmacology*, vol. 45, no. 3, pp. 539–542, 1993.

[15] S. V. McLennan, S. Heffernan, L. Wright et al., "Changes in hepatic glutathione metabolism in diabetes," *Diabetes*, vol. 40, no. 3, pp. 344–348, 1991.

[16] D. N. Granger and P. R. Kvietys, "Reperfusion injury and reactive oxygen species: the evolution of a concept," *Redox Biology*, vol. 6, pp. 524–551, 2015.

[17] W. Jassem, S. V. Fuggle, M. Rela, D. D. H. Koo, and N. D. Heaton, "The role of mitochondria in ischemia/reperfusion injury," *Transplantation*, vol. 73, no. 4, pp. 493–499, 2002.

[18] Q. Chen and B. N. Ames, "Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 10, pp. 4130–4134, 1994.

[19] D. A. Clopton and P. Saltman, "Low-level oxidative stress causes cell-cycle specific arrest in cultured cells," *Biochemical and Biophysical Research Communications*, vol. 210, no. 1, pp. 189–196, 1995.

[20] G. B. Corcoran, L. Fix, D. P. Jones et al., "Apoptosis: molecular control point in toxicity," *Toxicology and Applied Pharmacology*, vol. 128, no. 2, pp. 169–181, 1994.

[21] D. P. de Bono and W. D. Yang, "Exposure to low concentrations of hydrogen peroxide causes delayed endothelial cell death and inhibits proliferation of surviving cells," *Atherosclerosis*, vol. 114, no. 2, pp. 235–245, 1995.

[22] E. R. Whittemore, D. T. Loo, and C. W. Cotman, "Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons," *Neuroreport*, vol. 5, no. 12, pp. 1485–1488, 1994.

[23] V. N. Anisimov, "Ageing and the mechanisms of carcinogenesis: some practical implications," *Journal of Experimental & Clinical Cancer Research*, vol. 17, no. 3, pp. 263–268, 1998.

[24] V. Sanchez-Valle, N. C. Chavez-Tapia, M. Uribe, and N. Mendez-Sanchez, "Role of oxidative stress and molecular changes in liver fibrosis: a review," *Current Medicinal Chemistry*, vol. 19, no. 28, pp. 4850–4860, 2012.

[25] E. Bugianesi, A. J. McCullough, and G. Marchesini, "Insulin resistance: a metabolic pathway to chronic liver disease," *Hepatology*, vol. 42, no. 5, pp. 987–1000, 2005.

[26] P. Manna, J. Das, J. Ghosh, and P. C. Sil, "Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IκBα/NF-κB, MAPKs, and mitochondria-dependent pathways: prophylactic role of arjunolic acid," *Free Radical Biology & Medicine*, vol. 48, no. 11, pp. 1465–1484, 2010.

[27] P. Palsamy, S. Sivakumar, and S. Subramanian, "Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin-nicotinamide-induced experimental diabetic rats," *Chemico-Biological Interactions*, vol. 186, no. 2, pp. 200–210, 2010.

[28] Y. Zhang, D. Yuan, W. Yao et al., "Hyperglycemia aggravates hepatic ischemia reperfusion injury by inducing chronic oxidative stress and inflammation," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 3919627, 16 pages, 2016.

[29] T. Şahin, Z. Begeç, H. İ. Toprak et al., "The effects of dexmedetomidine on liver ischemia-reperfusion injury in rats," *Journal of Surgical Research*, vol. 183, no. 1, pp. 385–390, 2013.

[30] S. Cem Sezen, B. Işık, M. Bilge et al., "Effect of dexmedetomidine on ischemia-reperfusion injury of liver and kidney tissues in experimental diabetes and hepatic ischemia-reperfusion injury induced rats," *Anaesthesia, Pain & Intensive Care*, vol. 20, no. 2, pp. 143–149, 2016.

[31] H. Malhi, G. J. Gores, and J. J. Lemasters, "Apoptosis and necrosis in the liver: a tale of two deaths?," *Hepatology*, vol. 43, Supplement 1, pp. S31–S44, 2006.

[32] M. Behrends, G. Martinez-Palli, C. U. Niemann, S. Cohen, R. Ramachandran, and R. Hirose, "Acute hyperglycemia worsens hepatic ischemia/reperfusion injury in rats," *Journal of Gastrointestinal Surgery*, vol. 14, no. 3, pp. 528–535, 2010.

[33] H. Yamamoto, Y. Yamamoto, K. Yamagami et al., "Heat-shock preconditioning reduces oxidative protein denaturation and ameliorates liver injury by carbon tetrachloride in rats," *Research in Experimental Medicine*, vol. 199, no. 6, pp. 309–318, 2000.

[34] M. E. Rinaldi Tosi, V. Bocanegra, W. Manucha, A. Gil Lorenzo, and P. G. Vallés, "The Nrf2–Keap1 cellular defense pathway and heat shock protein 70 (Hsp70) response. Role in protection against oxidative stress in early neonatal unilateral ureteral obstruction (UUO)," *Cell Stress and Chaperones*, vol. 16, no. 1, pp. 57–68, 2011.

[35] B. Halliwell and J. M. Gutteridge, "Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy," *The Lancet*, vol. 1, no. 8391, pp. 1396-1397, 1984.

[36] G. K. Glantzounis, H. J. Salacinski, W. Yang, B. R. Davidson, and A. M. Seifalian, "The contemporary role of antioxidant therapy in attenuating liver ischemia-reperfusion injury: a review," *Liver Transplantation*, vol. 11, no. 9, pp. 1031–1047, 2005.

[37] K. Kudoh, H. Uchinami, M. Yoshioka, E. Seki, and Y. Yamamoto, "Nrf2 activation protects the liver from ischemia/reperfusion injury in mice," *Annals of Surgery*, vol. 260, no. 1, pp. 118–127, 2014.

[38] M. Buelna-Chontal and C. Zazueta, "Redox activation of Nrf2 & NF-κB: a double end sword?," *Cellular Signalling*, vol. 25, no. 12, pp. 2548–2557, 2013.

[39] G. H. Liu, J. Qu, and X. Shen, "NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1783, no. 5, pp. 713–727, 2008.

[40] D. E. Francés, P. I. Ingaramo, M. T. Ronco, and C. E. Carnovale, "Diabetes, an inflammatory process: oxidative stress and TNF-alpha involved in hepatic complication," *Journal of Biomedical Science and Engineering*, vol. 6, no. 6, pp. 645–653, 2013.

[41] I. C. West, "Radicals and oxidative stress in diabetes," *Diabetic Medicine*, vol. 17, no. 3, pp. 171–180, 2000.

[42] J. W. Elrod, M. R. Duranski, W. Langston et al., "eNOS gene therapy exacerbates hepatic ischemia-reperfusion injury in diabetes: a role for eNOS uncoupling," *Circulation Research*, vol. 99, no. 1, pp. 78–85, 2006.

[43] P.-M. Sinet and P. Garber, "Inactivation of the human CuZn superoxide dismutase during exposure to O_2^- and H_2O_2," *Archives of Biochemistry and Biophysics*, vol. 212, no. 2, pp. 411–416, 1981.

[44] Y. Kono and I. Fridovich, "Superoxide radical inhibits catalase," *Journal of Biological Chemistry*, vol. 257, no. 10, pp. 5751–5754, 1982.

[45] A. T. H. Mossa, A. A. Refaie, A. Ramadan, and J. Bouajila, "Amelioration of prallethrin-induced oxidative stress and hepatotoxicity in rat by the administration of *Origanum majorana* essential oil," *BioMed Research International*, vol. 2013, Article ID 859085, 11 pages, 2013.

[46] A. Choukér, A. Martignoni, R. J. Schauer et al., "α-Gluthathione S-transferase as an early marker of hepatic ischemia/reperfusion injury after liver resection," *World Journal of Surgery*, vol. 29, no. 4, pp. 528–534, 2005.

[47] C. E. Carnovale, J. A. Monti, C. Favre, C. Scapini, and M. C. Carrillo, "Is intestinal cytosolic glutathione S-transferase an alternative detoxification pathway in two-thirds hepatectomized rats?," *Life Sciences*, vol. 57, no. 9, pp. 903–910, 1995.

[48] M. T. Ronco, M. L. de Alvarez, J. Monti et al., "Modulation of balance between apoptosis and proliferation by lipid peroxidation (LPO) during rat liver regeneration," *Molecular Medicine*, vol. 8, no. 12, pp. 808–817, 2002.

[49] I. Aguilar-Delfín, F. López-Barrera, and R. Hernández-Muñoz, "Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CC14 administration," *Hepatology*, vol. 24, no. 3, pp. 657–662, 1996.

[50] D. E. Francés, M. T. Ronco, P. I. Ingaramo et al., "Role of reactive oxygen species in the early stages of liver regeneration in streptozotocin-induced diabetic rats," *Free Radical Research*, vol. 45, no. 10, pp. 1143–1153, 2011.

[51] S. Chin, S. Ramirez, L. E. Greenbaum, A. Naji, and R. Taub, "Blunting of the immediate-early gene and mitogenic response in hepatectomized type 1 diabetic animals," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 269, no. 4, pp. E691–E700, 1995.

[52] A. A. Aznar, C. T. Sanchez, M. L. B. Remacha, A. A. Lopez, and P. L. Diaz, "Effect of alloxan-induced diabetes on hepatic regeneration," *Revista Española de Enfermedades Digestivas*, vol. 79, pp. 313–319, 1991.

[53] P. W. Mola, B. Sudha, and C. S. Paulose, "Effect of insulin on DNA synthesis and kinetic parameters of thymidine kinase during liver regeneration," *Biochemistry and Molecular Biology International*, vol. 40, no. 5, pp. 1067–1075, 1996.

[54] S. S. Devi and H. M. Mehendale, "Disrupted G1 to S phase clearance via cyclin signaling impairs liver tissue repair in thioacetamide-treated type 1 diabetic rats," *Toxicology and Applied Pharmacology*, vol. 207, no. 2, pp. 89–102, 2005.

[55] D. E. Francés, M. T. Ronco, J. A. Monti et al., "Hyperglycemia induces apoptosis in rat liver through the increase of hydroxyl radical: new insights into the insulin effect," *Journal of Endocrinology*, vol. 205, no. 2, pp. 187–200, 2010.

[56] G. Andican and G. Burçak, "Oxidative damage to nuclear DNA in streptozotocin-diabetic rat liver," *Clinical and Experimental Pharmacology and Physiology*, vol. 32, no. 8, pp. 663–666, 2005.

[57] C. Brenner, L. Galluzzi, O. Kepp, and G. Kroemer, "Decoding cell death signals in liver inflammation," *Journal of Hepatology*, vol. 59, no. 3, pp. 583–594, 2013.

[58] J. Mohamed, A. H. Nazratun Nfizah, A. H. Zariyantey, and S. B. Budin, "Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation," *Sultan Qaboos University Medical Journal*, vol. 16, no. 2, pp. e132–e141, 2016.

[59] E. Faist, C. Schinkel, and S. Zimmer, "Update on the mechanisms of immune suppression of injury and immune modulation," *World Journal of Surgery*, vol. 20, no. 4, pp. 454–459, 1996.

[60] K. K. S. Sunahara, P. Sannomiya, and J. O. Martins, "Briefs on insulin and innate immune response," *Cellular Physiology and Biochemistry*, vol. 29, no. 1-2, pp. 1–8, 2012.

[61] S. C. Trevelin, D. Carlos, M. Beretta, J. S. da Silva, and F. Q. Cunha, "Diabetes mellitus and sepsis: a challenging association," *Shock*, vol. 47, no. 3, pp. 276–287, 2017.

[62] I. Gavrylenko and M. Khomenko, "Morphological and functional state of immune organs in rats with experimental type 1 diabetes mellitus (DM-1)," *Journal of Medical and Dental Science Research*, vol. 4, no. 1, pp. 6–10, 2017.

[63] F. Spiller, D. Carlos, F. O. Souto et al., "α1-Acid glycoprotein decreases neutrophil migration and increases susceptibility to sepsis in diabetic mice," *Diabetes*, vol. 61, no. 6, pp. 1584–1591, 2012.

[64] D. M. André, M. C. Calixto, C. Sollon et al., "High-fat diet-induced obesity impairs insulin signaling in lungs of allergen-challenged mice: improvement by resveratrol," *Scientific Reports*, vol. 7, no. 1, p. 17296, 2017.

[65] D. M. André, M. C. Calixto, C. Sollon et al., "Therapy with resveratrol attenuates obesity-associated allergic airway inflammation in mice," *International Immunopharmacology*, vol. 38, pp. 298–305, 2016.

[66] B. L. Diaz, M. F. Serra, A. C. Alves et al., "Alloxan diabetes reduces pleural mast cell numbers and the subsequent eosinophil influx induced by allergen in sensitized rats," *International Archives of Allergy and Immunology*, vol. 111, no. 1, pp. 36–43, 1996.

[67] S. C. Cavalher-Machado, W. T. de Lima, A. S. Damazo et al., "Down-regulation of mast cell activation and airway reactivity in diabetic rats: role of insulin," *The European Respiratory Journal*, vol. 24, no. 4, pp. 552–558, 2004.

[68] E. Boichot, P. Sannomiya, N. Escofier, N. Germain, Z. B. Fortes, and V. Lagente, "Endotoxin-induced acute lung injury

in rats. Role of insulin," *Pulmonary Pharmacology and Therapeutics*, vol. 12, no. 5, pp. 285–290, 1999.

[69] J. Panés, I. Kurose, M. D. Rodriguez-Vaca et al., "Diabetes exacerbates inflammatory responses to ischemia-reperfusion," *Circulation*, vol. 93, no. 1, pp. 161–167, 1996.

[70] J. Parekh, C. U. Niemann, K. Dang, and R. Hirose, "Intraoperative hyperglycemia augments ischemia reperfusion injury in renal transplantation: a prospective study," *Journal of Transplantation*, vol. 2011, 7 pages, 2011.

[71] S. Yue, H. M. Zhou, J. J. Zhu et al., "Hyperglycemia and liver ischemia reperfusion injury: a role for the advanced glycation endproduct and its receptor pathway," *American Journal of Transplantation*, vol. 15, no. 11, pp. 2877–2887, 2015.

[72] H. Jaeschke, A. Farhood, and C. W. Smith, "Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo," *FASEB Journal*, vol. 4, no. 15, pp. 3355–3359, 1990.

[73] A. B. Lentsch, A. Kato, H. Yoshidome, K. M. McMasters, and M. J. Edwards, "Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury," *Hepatology*, vol. 32, no. 2, pp. 169–173, 2000.

[74] M. Shuh, H. Bohorquez, Loss GE Jr, and A. J. Cohen, "Tumor necrosis factor-α: life and death of hepatocytes during liver ischemia/reperfusion injury," *The Ochsner Journal*, vol. 13, no. 1, pp. 119–130, 2013.

[75] K. R. Karlmark, R. Weiskirchen, H. W. Zimmermann et al., "Hepatic recruitment of the inflammatory $Gr1^+$ monocyte subset upon liver injury promotes hepatic fibrosis," *Hepatology*, vol. 50, no. 1, pp. 261–274, 2009.

[76] N. Hisama, Y. Yamaguchi, N. Miyanari et al., "Ischemia-reperfusion injury: the role of Kupffer cells in the production of cytokine induced neutrophil chemoattractant, a member of the interleukin-8 family," *Transplant Proceeding*, vol. 27, no. 2, pp. 1604–1606, 1995.

[77] N. Hisama, Y. Yamaguchi, T. Ishiko et al., "Kupffer cell production of cytokine-induced neutrophil chemoattractant following ischemia/reperfusion injury in rats," *Hepatology*, vol. 24, no. 5, pp. 1193–1198, 1996.

[78] G. A. Wanner, P. E. Müller, W. Ertel, M. Bauer, M. D. Menger, and K. Messmer, "Differential effect of anti-TNF-alpha antibody on proinflammatory cytokine release by Kupffer cells following liver ischemia and reperfusion," *Shock*, vol. 11, no. 6, pp. 391–395, 1999.

[79] A. Nakamitsu, E. Hiyama, Y. Imamura, Y. Matsuura, and T. Yokoyama, "Kupffer cell function in ischemic and nonischemic livers after hepatic partial ischemia/reperfusion," *Surgery Today*, vol. 31, no. 2, pp. 140–148, 2001.

[80] B. Mosher, R. Dean, J. Harkema, D. Remick, J. Palma, and E. Crockett, "Inhibition of Kupffer cells reduced CXC chemokine production and liver injury," *Journal of Surgical Research*, vol. 99, no. 2, pp. 201–210, 2001.

[81] Z. Rao, J. Sun, X. Pan et al., "Hyperglycemia aggravates hepatic ischemia and reperfusion injury by inhibiting liver-resident macrophage M2 polarization via C/EBP homologous protein-mediated endoplasmic reticulum stress," *Frontiers in Immunology*, vol. 8, pp. 1–10, 2017.

[82] Y. T. Zhao, Y. W. Qi, C. Y. Hu, S. H. Chen, and Y. Liu, "Advanced glycation end products inhibit testosterone secretion by rat Leydig cells by inducing oxidative stress and endoplasmic reticulum stress," *International Journal of Molecular Medicine*, vol. 38, no. 2, pp. 659–665, 2016.

[83] C. Adamopoulos, E. Farmaki, E. Spilioti, H. Kiaris, C. Piperi, and A. G. Papavassiliou, "Advanced glycation end-products induce endoplasmic reticulum stress in human aortic endothelial cells," *Clinical Chemistry and Laboratory Medicine*, vol. 52, no. 1, pp. 151–160, 2014.

[84] J. Xu, M. Xiong, B. Huang, and H. Chen, "Advanced glycation end products upregulate the endoplasmic reticulum stress in human periodontal ligament cells," *Journal of Periodontology*, vol. 86, no. 3, pp. 440–447, 2015.

[85] S. Yamabe, J. Hirose, Y. Uehara et al., "Intracellular accumulation of advanced glycation end products induces apoptosis via endoplasmic reticulum stress in chondrocytes," *FEBS Journal*, vol. 280, no. 7, pp. 1617–1629, 2013.

[86] T. Lawrence and G. Natoli, "Transcriptional regulation of macrophage polarization: enabling diversity with identity," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 750–761, 2011.

[87] S. Yue, J. Rao, J. Zhu et al., "Myeloid PTEN deficiency protects livers from ischemia reperfusion injury by facilitating M2 macrophage differentiation," *The Journal of Immunology*, vol. 192, no. 11, pp. 5343–5353, 2014.

[88] S. Müller, S. Martin, W. Koenig et al., "Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-alpha or its receptors," *Diabetologia*, vol. 45, no. 6, pp. 805–812, 2002.

[89] M. Crook, "Type 2 diabetes mellitus: a disease of the innate immune system? An update," *Diabetic Medicine*, vol. 21, no. 3, pp. 203–207, 2004.

[90] K. I. Alexandraki, C. Piperi, P. D. Ziakas et al., "Cytokine secretion in long-standing diabetes mellitus type 1 and 2: associations with low-grade systemic inflammation," *Journal of Clinical Immunology*, vol. 28, no. 4, pp. 314–321, 2008.

[91] G. Boden, P. She, M. Mozzoli et al., "Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-κB pathway in rat liver," *Diabetes*, vol. 54, no. 12, pp. 3458–3465, 2005.

[92] Y. Iwasaki, M. Kambayashi, M. Asai, M. Yoshida, T. Nigawara, and K. Hashimoto, "High glucose alone, as well as in combination with proinflammatory cytokines, stimulates nuclear factor kappa-B-mediated transcription in hepatocytes in vitro," *Journal of Diabetes and its Complications*, vol. 21, no. 1, pp. 56–62, 2007.

[93] Y. Bi, W. P. Sun, X. Chen et al., "Effect of early insulin therapy on nuclear factor κB and cytokine gene expressions in the liver and skeletal muscle of high-fat diet, streptozotocin-treated diabetic rats," *Acta Diabetologica*, vol. 45, no. 3, pp. 167–178, 2008.

[94] U. Siebenlist, G. Franzoso, and K. Brown, "Structure, regulation and function of NF-κB," *Annual Review of Cell Biology*, vol. 10, no. 1, pp. 405–455, 1994.

[95] M. J. Morgan and Z. G. Liu, "Crosstalk of reactive oxygen species and NF-κB signaling," *Cell Research*, vol. 21, no. 1, pp. 103–115, 2011.

[96] M. Niwa, A. Hara, Y. Kanamori et al., "Nuclear factor-κB activates dual inhibition sites in the regulation of tumor necrosis factor-α-induced neutrophil apoptosis," *European Journal of Pharmacology*, vol. 407, no. 3, pp. 211–219, 2000.

[97] R. F. Saidi and S. K. H. Kenari, "Liver ischemia/reperfusion injury: an overview," *Journal of Investigative Surgery*, vol. 27, no. 6, pp. 366–379, 2014.

[98] A. R. Brasier and J. Li, "Mechanisms for inducible control of angiotensinogen gene transcription," *Hypertension*, vol. 27, no. 3, pp. 465–475, 1996.

[99] J. Li and A. R. Brasier, "Angiotensinogen gene activation by angiotensin II is mediated by the rel A (nuclear factor-kappaB p65) transcription factor: one mechanism for the renin angiotensin system positive feedback loop in hepatocytes," *Molecular Endocrinology*, vol. 10, no. 3, pp. 252–264, 1996.

[100] P. I. Ingaramo, M. T. Ronco, D. E. A. Francés et al., "Tumor necrosis factor alpha pathways develops liver apoptosis in type 1 diabetes mellitus," *Molecular Immunology*, vol. 48, no. 12-13, pp. 1397–1407, 2011.

[101] P. Dandona, A. Chaudhuri, H. Ghanim, and P. Mohanty, "Proinflammatory effects of glucose and antiinflammatory effect of insulin: relevance to cardiovascular disease," *American Journal of Cardiology*, vol. 99, no. 4A, pp. 15B–26B, 2007.

[102] F. M. Ho, W. W. Lin, B. C. Chen et al., "High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF-κB and c-Jun NH2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway," *Cellular Signalling*, vol. 18, no. 3, pp. 391–399, 2006.

[103] Y. Ito, H. Katagiri, K. Ishii, A. Kakita, I. Hayashi, and M. Majima, "Effects of selective cyclooxygenase inhibitors on ischemia/reperfusion-induced hepatic microcirculatory dysfunction in mice," *European Surgical Research*, vol. 35, no. 5, pp. 408–416, 2003.

[104] Y. Sunose, I. Takeyoshi, S. Ohwada et al., "The effect of cyclooxygenase-2 inhibitor FK3311 on ischemia-reperfusion injury in a canine total hepatic vascular exclusion model," *Journal of the American College of Surgeons*, vol. 192, no. 1, pp. 54–62, 2001.

[105] I. Takeyoshi, Y. Sunose, S. Iwazaki et al., "The effect of a selective cyclooxygenase-2 inhibitor in extended liver resection with ischemia in dogs," *Journal of Surgical Research*, vol. 100, no. 1, pp. 25–31, 2001.

[106] A. Wullaert, K. Heyninck, and R. Beyaert, "Mechanisms of crosstalk between TNF-induced NF-κB and JNK activation in hepatocytes," *Biochemical Pharmacology*, vol. 72, no. 9, pp. 1090–1101, 2006.

[107] D. E. A. Francés, P. I. Ingaramo, R. Mayoral et al., "Cyclooxygenase-2 over-expression inhibits liver apoptosis induced by hyperglycemia," *Journal of Cellular Biochemistry*, vol. 114, no. 3, pp. 669–680, 2013.

[108] M. Torbenson, S. Q. Yang, H. Z. Liu, J. Huang, W. Gage, and A. M. Diehl, "STAT-3 overexpression and p21 up-regulation accompany impaired regeneration of fatty livers," *American Journal of Pathology*, vol. 161, no. 1, pp. 155–161, 2002.

[109] T. Matsumoto, M. Yamaguchi, M. Kuzume et al., "Insulin gene transfer improves posthepatectomized status of diabetic rats," *Transplantation Proceedings*, vol. 32, no. 7, pp. 2378-2379, 2000.

[110] K. Shirabe, M. Shimada, T. Gion et al., "Postoperative liver failure after major hepatic resection for hepatocellular carcinoma in the modern era with special reference to remnant liver volume," *Journal of American College of Surgeons*, vol. 188, no. 3, pp. 304–309, 1999.

[111] T. Aoyama, K. Ikejima, K. Kon, K. Okumura, K. Arai, and S. Watanabe, "Pioglitazone promotes survival and prevents hepatic regeneration failure after partial hepatectomy in obese and diabetic KK-Ay mice," *Hepatology*, vol. 49, no. 5, pp. 1636–1644, 2009.

[112] P. Akerman, P. Cote, S. Q. Yang et al., "Antibodies to tumor necrosis factor-alpha inhibit liver regeneration after partial hepatectomy," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 263, no. 4, pp. G579–G585, 1992.

[113] A. M. Diehl, S. Q. Yang, M. Yin, H. Z. Lin, S. Nelson, and G. Bagby, "Tumor necrosis factor-alpha modulates CCAA T/enhancer binding proteins-DNA binding activities and promotes hepatocyte-specific gene expression during liver regeneration," *Hepatology*, vol. 22, no. 1, pp. 252–261, 1995.

[114] S. Nepal and P. H. Park, "Modulation of cell death and survival by adipokines in the liver," *Biological and Pharmaceutical Bulletin*, vol. 38, no. 7, pp. 961–965, 2015.

[115] M. Mendes-Braz, M. Elias-Miró, B. Kleuser et al., "The effects of glucose and lipids in steatotic and non-steatotic livers in conditions of partial hepatectomy under ischaemia-reperfusion," *Liver International*, vol. 34, no. 7, pp. e271–e289, 2014.

[116] T. Masaki, S. Chiba, H. Tatsukawa et al., "Adiponectin protects LPS-induced liver injury through modulation of TNF-α in KK-Ay obese mice," *Hepatology*, vol. 40, no. 1, pp. 177–184, 2004.

[117] H. Matsumoto, S. Tamura, Y. Kamada et al., "Adiponectin deficiency exacerbates lipopolysaccharide/D-galactosamine induced liver injury in mice," *World Journal of Gastroenterology*, vol. 12, no. 21, pp. 3352–3358, 2006.

[118] M. B. Jiménez-Castro, A. Casillas-Ramírez, M. Mendes-Braz et al., "Adiponectin and resistin protect steatotic livers undergoing transplantation," *Journal of Hepatology*, vol. 59, no. 6, pp. 1208–1214, 2013.

[119] M. Massip-Salcedo, M. A. Zaouali, S. Padrissa-Altés et al., "Activation of peroxisome proliferator-activated receptor-α inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion," *Hepatology*, vol. 47, no. 2, pp. 461–472, 2008.

[120] K. Man, Y. Zhao, A. Xu et al., "Fat-derived hormone adiponectin combined with FTY720 significantly improves small-for-size fatty liver graft survival," *American Journal of Transplantation*, vol. 6, no. 3, pp. 467–476, 2006.

[121] D. Schmidt-Arras and S. Rose-John, "IL-6 pathway in the liver: from physiopathology to therapy," *Journal of Hepatology*, vol. 64, no. 6, pp. 1403–1415, 2016.

[122] R. Taub, "Liver regeneration: from myth to mechanism," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 10, pp. 836–847, 2004.

[123] N. Fausto, J. S. Campbell, and K. J. Riehle, "Liver regeneration," *Hepatology*, vol. 43, Supplement 1, pp. S45–S53, 2006.

[124] N. Ouchi, J. L. Parker, J. J. Lugus, and K. Walsh, "Adipokines in inflammation and metabolic disease," *Nature Reviews Immunology*, vol. 11, no. 2, pp. 85–97, 2011.

[125] S. Loffreda, S. Q. Yang, H. Z. Lin et al., "Leptin regulates proinflammatory immune responses," *FASEB Journal*, vol. 12, no. 1, pp. 57–65, 1998.

[126] G. Fantuzzi and R. Faggioni, "Leptin in the regulation of immunity, inflammation, and hematopoiesis," *Journal of Leukocyte Biology*, vol. 68, no. 4, pp. 437–446, 2000.

[127] K. L. Streetz, F. Tacke, L. Leifeld et al., "Interleukin 6/gp130-dependent pathways are protective during chronic liver diseases," *Hepatology*, vol. 38, no. 1, pp. 218–229, 2003.

[128] C. Klein, T. Wüstefeld, U. Assmus et al., "The IL-6-gp130-STAT3 pathway in hepatocytes triggers liver protection in T cell-mediated liver injury," *The Journal of Clinical Investigation*, vol. 115, no. 4, pp. 860–869, 2005.

[129] D. E. Cressman, L. E. Greenbaum, R. A. DeAngelis et al., "Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice," *Science*, vol. 274, no. 5291, pp. 1379–1383, 1996.

[130] K. Streetz, T. Luedde, M. Manns, and C. Trautwein, "Interleukin 6 and liver regeneration," *Gut*, vol. 47, no. 2, pp. 309–312, 2000.

[131] T. Wüstefeld, T. Rakemann, S. Kubicka, M. P. Manns, and C. Trautwein, "Hyperstimulation with interleukin 6 inhibits cell cycle progression after hepatectomy in mice," *Hepatology*, vol. 32, no. 3, pp. 514–522, 2000.

[132] I. A. Leclercq, J. Field, and G. C. Farrell, "Leptin-specific mechanisms for impaired liver regeneration in ob/ob mice after toxic injury," *Gastroenterology*, vol. 124, no. 5, pp. 1451–1464, 2003.

[133] Q. Li, Y. Wang, T. Ma, Y. Lv, and R. Wu, "Clinical outcomes of patients with and without diabetes mellitus after hepatectomy: a systematic review and meta-analysis," *PLoS One*, vol. 12, no. 2, article e0171129, 2017.

[134] P. L. Shields, H. Tang, J. M. Neuberger, B. K. Gunson, P. McMaster, and J. Pirenne, "Poor outcome in patients with diabetes mellitus undergoing liver transplantation," *Transplantation*, vol. 68, no. 4, pp. 530–535, 1999.

[135] B. Vollmar, S. Richter, and M. D. Menger, "Liver ischemia/reperfusion induces an increase of microvascular leukocyte flux, but not heterogeneity of leukocyte trafficking," *Liver*, vol. 17, no. 2, pp. 93–98, 1997.

[136] Y. Wan, J. Garner, N. Wu et al., "Role of stem cells during diabetic liver injury," *Journal of Cellular and Molecular Medicine*, vol. 20, no. 2, pp. 195–203, 2016.

[137] H. Jaeschke, "Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning," *American Journal of Physiology, Gastrointestinal and Liver Physiology*, vol. 284, no. 1, pp. G15–G26, 2003.

[138] A. Balci, S. Karazincir, H. Sumbas, Y. Oter, E. Egilmez, and T. Inandi, "Effects of diffuse fatty infiltration of the liver on portal vein flow hemodynamics," *Journal of Clinical Ultrasound*, vol. 36, no. 3, pp. 134–140, 2008.

[139] B. Erdogmus, A. Tamer, R. Buyukkaya et al., "Portal vein hemodynamics in patients with non-alcoholic fatty liver disease," *Tohoku Journal of Experimental Medicine*, vol. 215, no. 1, pp. 89–93, 2008.

[140] A. M. Seifalian, C. Piasecki, A. Agarwal, and B. R. Davidson, "The effect of graded steatosis on flow in the hepatic parenchymal microcirculation," *Transplantation*, vol. 68, no. 6, pp. 780–784, 1999.

[141] S. E. Regnell and Å. Lernmark, "Hepatic steatosis in type 1 diabetes," *The Review of Diabetic Studies*, vol. 8, no. 4, pp. 454–467, 2011.

[142] K. G. Tolman, V. Fonseca, A. Dalpiaz, and M. H. Tan, "Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease," *Diabetes Care*, vol. 30, no. 3, pp. 734–743, 2007.

[143] S. A. Little, W. R. Jarnagin, R. P. DeMatteo, L. H. Blumgart, and Y. Fong, "Diabetes is associated with increased perioperative mortality but equivalent long-term outcome after hepatic resection for colorectal cancer," *Journal of Gastrointestinal Surgery*, vol. 6, no. 1, pp. 88–94, 2002.

[144] M. Selzner, H. Rüdiger, D. Sindram, J. Madden, and P. Clavien, "Mechanisms of ischemic injury are different in the steatotic and normal rat liver," *Hepatology*, vol. 32, no. 6, pp. 1280–1288, 2000.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 2503950, 17 pages
https://doi.org/10.1155/2018/2503950

Review Article

Wound Healing and Omega-6 Fatty Acids: From Inflammation to Repair

Jéssica R. Silva, Beatriz Burger, Carolina M. C. Kühl, Thamiris Candreva, Mariah B. P. dos Anjos, and Hosana G. Rodrigues

Laboratory of Nutrients and Tissue Repair, School of Applied Sciences, University of Campinas, Limeira, SP, Brazil

Correspondence should be addressed to Hosana G. Rodrigues; hosana.rodrigues@fca.unicamp.br

Received 4 December 2017; Accepted 8 March 2018; Published 12 April 2018

Academic Editor: Naïma Moustaïd-Moussa

Wound healing is an evolutionarily conserved process that is essential for species survival. Wound healing involves a series of biochemical and cellular events that are tightly controlled, divided into 3 concomitant and overlapping phases: inflammation, proliferation, and remodelling. Poor wound healing or a chronic wound represents a silent epidemic that affects billions of people worldwide. Considering the involvement of immune cells in its resolution, recent studies are focused on investigating the roles of immune nutrients such as amino acids, minerals, and fatty acids on wound healing. Among the fatty acids, much attention has been given to omega-6 (ω-6) fatty acids since they can modulate cell migration and proliferation, phagocytic capacity, and production of inflammatory mediators. The present review summarizes current knowledge about the role of ω-6 fatty acids in the wound healing context.

1. Wound Healing: A Vital Process

Wound healing occurs after a chemical, physical, or biological insult results in epithelial barrier disruption. This process involves activation of platelets, neutrophils, macrophages, endothelial cells, keratinocytes, and fibroblasts; moreover, the production and release of protein mediators (growth factors and cytokines) released by these cell types and lipid mediators (prostaglandins, leukotrienes, thromboxanes, and lipoxins) are needed to coordinate the tissue repair and to reestablish tissue homeostasis [1, 2].

The process is divided into 3 concomitant and overlapping phases: inflammation, proliferation, and remodelling (Figure 1).

After a tissue lesion, the disruption in vasculature blocks the oxygen and nutrient supply to the injured area, leading to a hypoxic condition that induces the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [3–5], initiating a coagulation cascade. Blood elements such as platelets, erythrocytes, and fibrin form a framework for the recruitment of immune cells [6, 7]. Platelets produce platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF) that induce migration and activation of immune cells [8].

The extracellular matrix (ECM), which is composed of fibronectin, fibrinogen, fibrin, thrombospondin, and vitronectin, fills the tissue defect and enables migration of different cell types required for the healing process [9].

Inflammation is the body's natural and essential defence mechanism responsible for combating antigens, restoring homeostasis, and repairing tissue damage [10, 11]. The inflammatory response consists of a variety of events triggered by immune cells, which involves influx of leukocytes to the injured area and production of pro- and anti-inflammatory mediators [12].

Neutrophils are the predominant cells in the first hours after the tissue injury, and they respond to proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) at the lesion site, phagocytizing microorganisms and cellular debris [10, 13]. For microorganism destruction, the degranulation process occurs, releasing granule enzymes such as defensins,

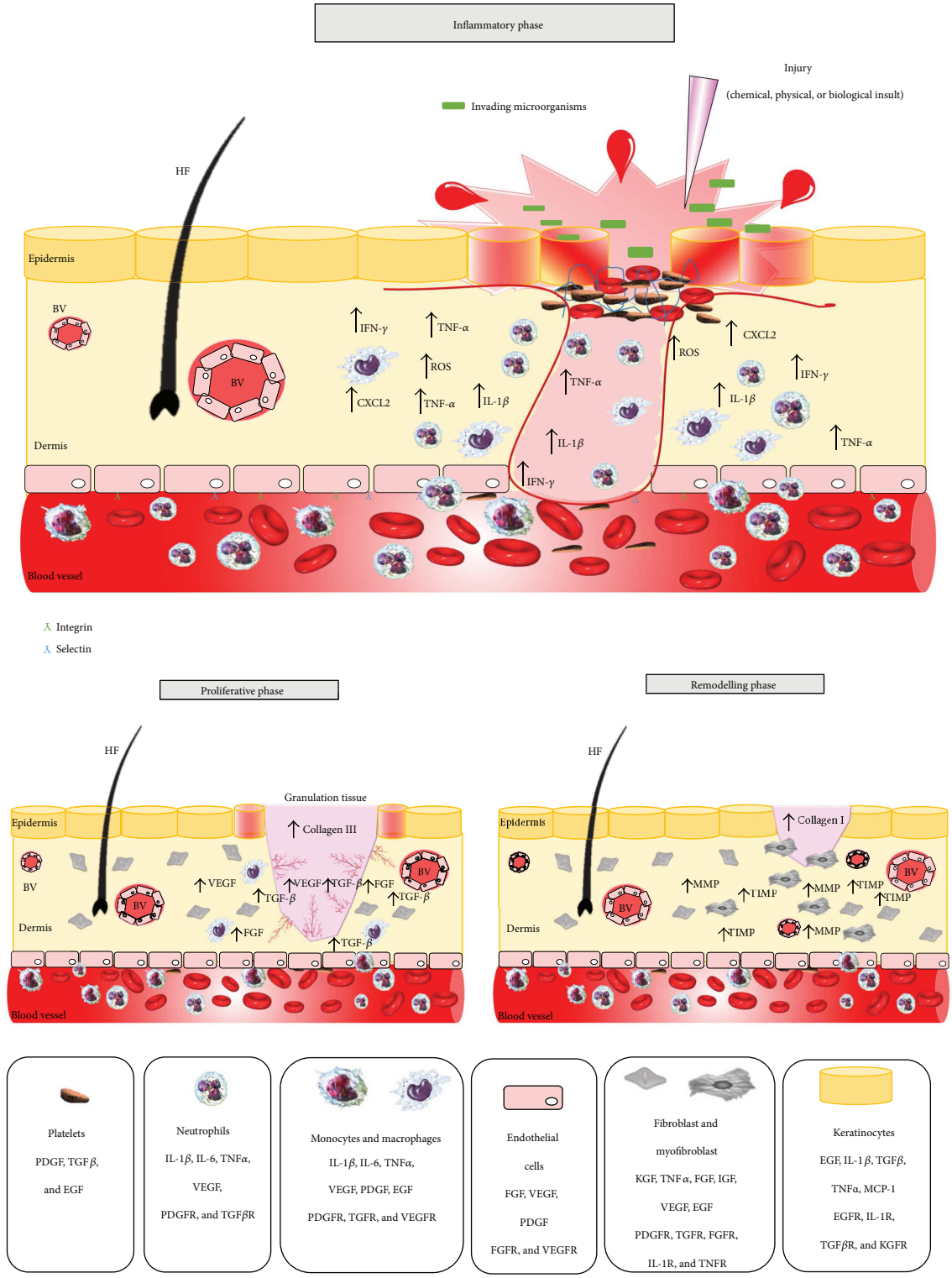


Figure 1: Wound healing process. The illustration shows the inflammatory, proliferative, and remodelling phases of wound healing. Early stages of wound healing include coagulation and activation of inflammatory cells. The proliferative stage involves proliferation of fibroblasts and angiogenesis. The remodelling phase includes restoration of the barrier and contraction of the wound by myofibroblasts. The process is orchestrated by immune cells and growth factors and cytokines and chemokines (listed below) [8]. HF = hair follicle; BV = blood vessels; TNF = tumor necrosis factor; IL-1beta = interleucina 1beta; IL-6 = interleucina 6; ROS = reactive oxygen species; CXCL2 = chemokine (C-X-C motif) ligand 2; IFN-gamma = interferon-gamma; VEGF = vascular endothelial growth factor; TGF-beta = transforming growth factor beta; FGF = fibroblast growth factor; KGF = keratinocyte growth factor; MCP1 = monocyte chemoattractant protein-1; IGF = insulin growth factor; TIMPs = tissue inhibitors of metalloproteinases; MMPs = matrix matalloproteinases; PDGF = platelet-derived growth factor; EGF = epithelial growth factor.

cathelicidins, elastase, myeloperoxidase (MPO), lactoferrins, and cathepsins inside the phagosome. In addition to their microbicidal activities, these molecules also act in the chemoattraction of macrophages to the lesion site. They also amplify the production of cytokines and chemokines, such as chemokine C-X-C motif ligand-2 (CXCL2) that will attract macrophages to the wound area. Neutrophils produce IL-1β, TNF-α, and vascular endothelial growth factor (VEGF) and express PDGF and TGF-β receptors [8]. These cytokines also induce the expression of adhesion molecules on the endothelial cell surface that will interact with selectins and integrins expressed in macrophages, facilitating the rolling, attachment, and transmigration of these cells to injured areas [13–15].

Macrophages are phagocytes that have PDGF, TGF-β, and VEGF receptors, and thus, they migrate in response to mediators produced by platelets and neutrophils in the injured tissue [8].

After 72 hours, macrophages are the predominant cells at the wound site, and they release growth factors (VEGF, PDGF, and EGF) and cytokines (IL-1β, IL-6, and TNF-α) that promote the migration of other cells such as fibroblasts and endothelial cells [10, 16]. They also produce prostaglandins that activate endothelial cells and act as potent vasodilators, affecting the permeability of microblood vessels [17].

The lack of control in the amplitude and time to resolve inflammation is one of the factors that most influence the genesis of chronic inflammatory diseases, such as cardiovascular diseases, diabetes, cancer, asthma, dementia, and Alzheimer's disease [11, 12, 18], as well as chronic wounds.

The proliferative stage begins within the first 48 hours and can unfold up to the 14th day after a tissue injury [17]. This phase is characterized by angiogenesis and fibroplasia, restoring the blood vessels and forming the granulation tissue [10].

Angiogenesis is the formation of new blood vessels from preexisting vessels and it is initiated by growth factors such as VEGF, PDGF, and fibroblast growth factor (FGF) [10, 19]. Fibroplasia is the formation of granulation tissue, and its main characteristic is proliferation of fibroblasts in response to PDGF, TGF-β, FGF, IL-1, and TNFα. At this time, the production of collagen occurs, and there is a release of growth factors such as keratinocyte growth factor (KGF), TNF-α, FGF, insulin growth factor (IGF), VEGF, and EGF [8, 16]. Then, the provisional matrix initially formed is replaced by granulation tissue composed of fibroblasts, granulocytes, macrophages, and blood vessels in complex with collagen bundles that form the basis for cell adhesion and migration, growth, and differentiation [10, 19].

The remodelling phase occurs approximately from the 21st day after injury and can last for years. During this period, there is intense production and digestion of collagen as well as the substitution of collagen III for collagen I. These events are aimed at maintaining the fibres in the same direction as in unwounded tissue to reestablish its functions and mechanical forces [20].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade ECM components (collagens), and their gene expression is regulated at the transcriptional level by cytokines and growth factors as well as by their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [21].

In all of the processes cited above, it is important to emphasize that exogenous and endogenous factors can modulate such events and influence the healing process. More specifically, systemic disorders, such as diabetes, immunosuppression, and venous stasis as well as those resulting from external agents, such as the use of corticotherapy and smoking, can hinder the early closure of the wound.

Chronic wounds are defined as wounds with a full thickness in depth and a slow healing tendency. The time an open wound needs to remain to define chronicity is still not well established, ranging from 4 weeks to more than 3 months [22]. Including diabetic foot ulcers, venous leg ulcers, and pressure ulcers, they represent a silent epidemic that affects a large fraction of the world population, becoming a public health problem [23, 24].

Complications of chronic wounds include infection that can lead to lower extremity amputations and impacts on health and life quality of patients because they cause pain and suffering, loss of function, loss of productivity, depression, distress and anxiety, social isolation, prolonged hospital stays, and chronic morbidity or even death [25].

Moreover, the treatment of chronic wounds causes economic expenditures by the individual and the healthcare system, and therefore, they are a matter of political interest [26]. In the USA, for example, chronic wounds affect 6.5 million patients, with nearly US$ 50 billion spent on treatment of chronic wounds and complications related to them per year [22, 25].

In developed countries, 1 to 2% of the population will experience a chronic wound throughout their lives [22] due to population ageing and increases in noncommunicable diseases such as obesity, diabetes, and cancer [27, 28].

Animal and human studies have shown that in the elderly population (age over 60 years), there is an increase in the number of cases of poor wound healing and chronic wounds due to delayed T-cell infiltration, decreased chemokine production, and reduced macrophage phagocytic capacity, in addition to delayed reepithelization, collagen synthesis, and angiogenesis [28].

Alterations of the peripheral nervous system with decreased protective sensation and foot deformities enhance the risk of chronic skin ulcerations on the lower extremities of diabetic subjects, mainly on the foot, that affect 15% of diabetic patients [29], and five-year postamputation, the mortality rate is 50–59% [30].

Chronic inflammation, insufficient angiogenic response, production of ROS greater than antioxidant capacity, collagen accumulation, dysfunction of migration and proliferation of fibroblast and keratinocytes, and an imbalance between the accumulation and degradation of ECM are some of the mechanisms responsible for poor healing in diabetic patients [1, 28].

These scenarios show the necessity for studies that investigate possible therapies that accelerate tissue repair, reducing the susceptibility to infections [31].

2. Omega-6 Fatty Acids: From Inflammation to Regeneration

For decades, nutritional supplementation was mainly used to avoid nutritional deficiency. However, currently it is being recognized that adequate levels of essential nutrients can prevent as well as treat some disturbances [32].

Fatty acids are carboxylic acids formed by hydrogen and carbon atoms [33]. Based on the presence of double bonds, fatty acids are classified as saturated (no double bonds) or unsaturated (with double bonds). Among the unsaturated fatty acids, there is also a classification that takes into account the number of unsaturations: monounsaturated fatty acids (MUFAs) present with one double bond on acyls (the main food source is olive oil) and polyunsaturated fatty acids (PUFAs) contain two or more double bonds [33].

PUFAs are classified by the position of the first double bound counting from the methyl terminal. Then, when the first double bond is at the 6th carbon atom from the methyl terminal, PUFAs are called omega-6, ω-6, or n-6. They include linoleic acid (LA, C18 : 2 ω-6), an essential fatty acid because it cannot be synthesized by the human body. LA can be "stretched" and desaturated into other ω-6 fatty acids, such as γ-linolenic (GLA, 18 : 3 ω-6) and the arachidonic acid (AA, 20 : 4 ω-6). Moreover, biohydration of linoleic acid by bacteria (anaerobic bacteria such as *Butyrivibrio fibrisolvens*) in the gut of animals and the action of Δ9 desaturation of 18 : 1 *trans*-11 in animal tissue can generate conjugated linoleic acid (CLA) [34].

Fatty acids alter skin structural and immunological status since they constitute the stratum corneum, and they can alter the permeability of the skin. They also interfere with maturation and differentiation of the stratum corneum and inhibit production of proinflammatory eicosanoids, reactive species (ROS and RNS), and cytokines, thus influencing the inflammatory response and possibly wound healing [35–38].

The objective of this study was to review the scientific literature on the relationship between omega-6 fatty acids (linoleic, conjugated linolenic, gamma-linolenic, and arachidonic acid) and the wound healing process.

2.1. Linoleic Acid. Linoleic acid (LA, 18 : 2 ω-6, *cis*-9, 12-octadecadienoic acid) is the PUFA most commonly consumed in the human diet, being mainly found in safflower, corn, and sunflower oils, present in medium quantities in soy, sesame, and almond oils, and in smaller quantities in canola, olive, coconut, and palm oils [39].

2.1.1. Effects of LA on Tissue Repair. In developing countries, creams with linoleic acid are used to treat wounds. One of the first studies in this area described that topical application of a solution containing 1.6 g of essential fatty acid (mainly linoleic acid) prevented pressure ulcers in hospitalized patients [40]. This improvement was related to better hydration and elasticity. In this study, the control group received a solution with 1.6 g of mineral oil, which is a liquid paraffin. Although frequently used in baby creams to maintain hydration, in the present study, only 22% of the patients in the control group presented a hydrated skin. On the other hand, 98% of the patients treated with a linoleic solution showed a hydrated skin. These results indicate that maintenance of hydration is a mechanism by which linoleic acid improves wound healing.

In 2008, Magalhães et al. [41] did not observe any effects of topical application of medium chain triglycerides (caprylic, capric, caproic, and lauric acids), linoleic acid, soy lecithin, or a vitamin A and E mixture on wound healing in rats. The main problem with this protocol is the composition of the mixture tested and the control used, since the control group received a 0.9% NaCl solution without antioxidant vitamins or fatty acids.

Other studies described positive effects of oils rich in linoleic acid such as lucuma nut oil (LNO) and pumpkin oil on wound healing [42, 43]. The major constituents of LNO are LA (38.9%), oleic acid (27.9%), and palmitic and stearic acids (18.6 and 8.9%, resp.). Rojo et al. [42] used two different models to prove the beneficial effects of LNO: the zebrafish (*Danio rerio*) model and a CD-1 murine model. At first, LNO (20–100 μg/mL) was added to a zebrafish larva plate after a tail primordial cut. Through fluorescence microscopy and image analysis of fluorescent endothelial cells from zebrafish, it was observed that LNO accelerated the regeneration. This prohealing effect was attributed to increases in angiogenesis. However, the authors evaluated the number of fluorescent endothelial cells and not the number of new vessels formed. Angiogenesis is a complex process that involves release of proangiogenic factors, such as VEGF, PDGF, and FGF, as well as proteolytic degradation of basement membrane, and the migration, proliferation, and organization of endothelial cells in a tube form [44]. Angiogenesis is a crucial step for proper wound healing since it reestablishes the oxygen and nutrient supply.

LNO was also tested in a CD-1 murine model. For this, a wound was induced in the back region of mice and topically treated with 200, 500, or 100 μg of LNO daily. Corroborating the results with zebrafish, LNO (500 and 1000 μg) also induced a more rapid wound closure in CD-1 mice. The authors hypothesize that this effect could be related to the anti-inflammatory actions of the fatty acids present in the LNO [42]; however, they did not show any results that could support this hypothesis.

Linoleic acid has been thought to be behind the effects of pumpkin oil on wound healing. Pumpkin oil is constituted, mainly by LA (50%), oleic acid (25%), and palmitic acid (15%) [43]. This high content of LA was correlated with improvement in wound closure, since it shortened bleeding time, suggesting a stabilization of fibrin and consequent migration of fibroblast; it augmented hydroxyproline content, possibly due to fibroblast activation; and it reduced the number of infiltrating macrophages in wound tissue 11 days after lesion induction. Altogether, these results indicate that topical treatment with pumpkin oil accelerates tissue repair, mainly due to the effects of LA [43].

Considering these results with LA-rich oils, some groups have tested the effects of pure LA on wound healing. The use of isolated fatty acids ensures that the observed effects are not due to minor oil compounds or a combination of fatty acids.

In this context, it was also reported that there were beneficial effects of pure LA topically applied into wounds. BALB/c mice treated with pure LA (30 μM) for 20 days exhibited accelerated tissue repair 48 hours after wound induction [45]. This result was related to increased production of nitric oxide (NO). NO is a free radical derived from L-arginine oxidation through the nitric oxide synthase (NOS) activity. After an inflammatory insult, inducible nitric oxide synthase (iNOS) is expressed in immune cells and produces a large amount of NO that will generate other free radicals, expanding the inflammatory response [46]. NO plays important roles such as activation of macrophages and fibroblasts, induction of collagen synthesis, and the proliferation of keratinocytes during wound healing, thus accelerating reepithelization [47].

However, in Wistar rats, topical treatment with pure LA did not alter the wound area, although there was an increase in wet wound weight (oedema) and in neutrophil numbers, indicating a positive effect on the migratory response during the inflammatory phase [48].

Another approach used to investigate LA effects on wound healing is oral supplementation.

Wistar rats orally supplemented with pure LA (0.22 g per kg body weight) by gavage during the 5 days prior to wound induction had an increased inflammatory response 1 hour (initial stage of inflammation) after skin injury. This proinflammatory effect was characterized by an increase in inflammatory cell influx into the wound site due to elevations in hydrogen peroxide (H_2O_2) production and chemokine release. On the other hand, 24 hours later, LA reduced the activation of nuclear transcription factor (NF-κB) and then diminished the production of proinflammatory cytokines such as IL-1β and IL-6. At the same time, there was an elevation in AP-1 (activator protein-1) activation. AP-1 is a transcription factor that induces the expression of genes related to proliferation of keratinocytes and fibroblasts, which are two important cells involved in the proliferative phase of wound healing. Therefore, LA accelerated the inflammatory phase of wound healing, allowing the next phase (proliferation) to start early and accelerating wound healing over a period of 7 days [49].

More recently, the same protocol was tested in diabetic Wistar rats; the results showed that LA positively modulates tissue repair not only by accelerating the inflammatory phase but also by inducing angiogenesis. During the proliferative phase (7 days), it was observed that LA increased the number of vessels in the wound tissue, which was related to an elevation in VEGF concentration and ANGPT-2 (angiopoietin-2) expression [38]. VEGF and ANGPT-2 are proangiogenic factors essential for new vessel formation. VEGF induces ANGPT-2 expression, which primes endothelial cells to respond to inflammatory cytokines, thereby augmenting the migration and proliferation of endothelial cells [50].

Taken together, these studies demonstrate that linoleic acid can improve wound healing due to its mechanical properties and by modulating the cellular response, increasing the migration and functions of inflammatory and endothelial cells as well as inducing angiogenesis at the wound site.

2.1.2. Mechanisms of Action of LA. The mechanisms described so far to explain the effects of LA on wound healing involve inflammatory responses of neutrophils and macrophages.

Neutrophils are the first cell type recruited to the inflammatory site, being determinants for the healing process [51]. To analyse the effects of LA on neutrophil migration, an air pouch was induced into the dorsal region of Wistar rats treated with LA (100 μM), and 4 hours later, the exudate was collected and the cells were counted. LA increased neutrophil influx to the pouches [48], corroborating the results described in wound tissue. This effect on migration can be explained by the induction of adhesion molecules such as L-selectin on neutrophil surfaces [52]. Neutrophil recruitment is a highly regulated process that involves at least four steps: rolling, activation, adhesion, and transmigration. Through the intravital microscopy assay, it was observed that LA also elevated leukocyte-endothelium interactions (rolling and adhesion) [52].

Once in the injured site, neutrophils produce cytokines, chemokines, ROS, and other molecules to expand the inflammatory response. Measuring intra or extracellular ROS production, Hatanaka et al. [53] demonstrated that LA increased anion superoxide and H_2O_2 in a dose-dependent manner. The authors tested 5 different techniques (luminol- and lucigenin-amplified chemiluminescence, cytochrome c, hydroethidine, and phenol red reduction) and described that LA interfered with luminol and cytochrome c reactions, jeopardizing the ROS results [53]. In the wound healing context, ROS production is the first event that occurs after tissue disruption due to hypoxia [54]. Low concentrations of H_2O_2 are important to support wound healing [55] since ROS not only disinfects the injured area but also acts as signalling messengers regulating gene expression [56] and cellular function such as migration [57] and cytokine production [58].

Inflammation control is crucial to tissue repair, since chronic inflammation can worsen the wound. In this sense, LA has also shown a beneficial effect since it increases the release of proinflammatory mediators in the initial inflammation phase (1–4 hours) and reduces them in the resolution phase (18–48 hours) [52].

Another important cell type that is involved in inflammatory responses is the macrophage. As observed with neutrophils, LA reduced the production of IL-1β, IL-6, and VEGF in the absence of LPS, although it accelerated IL-1β release and decreased IL-10 synthesis when cells were stimulated with LPS. However, LA did not affect ROS production (superoxide anion, hydrogen peroxide, and NO) as well as the lipid mediators, prostaglandin E_2 (PGE2), leukotriene B_4 (LTB_4), and 15(S)-hydroxyeicosatetraenoic acid (15[2]-HETE) [59]. Lipid mediators are a class of inflammatory molecules derived from the metabolization of arachidonic [60], eicosapentaenoic (EPA), or docosahexaenoic (DHA) acids. Classes 2 and 4 are derived from AA and exhibit more proinflammatory effects, increasing migration, production of cytokines, and ROS. On the other hand, classes 3 and 5 are derived from EPA and DHA and are related to anti-inflammatory effects. More recently, a new class of lipid mediators derived from omega-3 fatty acids (EPA and DHA) were described, the maresins, resolvins, and

protectins that exert proresolution effects, resolving inflammation [61]. During the inflammatory response, it is important that there is a shift between proinflammatory molecules to proresolution to limit the damage induced by exacerbated inflammation.

During the proliferation and remodelling phases, fibroblasts, endothelial cells, and keratinocytes play important roles in producing growth factors that orchestrate the reconstruction of vessels and induce wound contraction [62]. In this context, Rojo et al. [42] described a promigratory effect of LNO (60 μg/mL) on human fibroblasts, which was related to an increase in vinculin expression. Vinculin is a focal adhesion protein essential for fibroblasts-ECM interactions [63] involved in wound contraction.

One important aspect not fully clarified is if LA must be metabolized to exert its effects on cellular functions or if it acts as an effector molecule. To answer this question, some studies have described G-protein coupled receptors (GPCR or GPR) as responsible for fatty acid effects [64, 65]. GPR is a class of seven transmembrane receptors involved in a broad spectrum of cellular responses [64]. Among GPR, GPR40 has been described as a sensor for LA, oleic acid (OA), CLA, and other long chain fatty acids [65, 66]. In HaCaT cells (keratinocyte cell line), once activated, it reduced the production of cytokines (CCL-5 and CCL17) and suppressed allergic inflammation in skin [67], and then, it could be involved in the effects of LA on wound healing. These results indicate that LA can modulate immune response by acting as an effector molecule. However, considering the importance of LA to cellular membranes, it is possible that the results observed are due to its metabolization as well. More studies are needed to clarify this point.

In conclusion, it has been shown that LA-rich oil or pure LA modulates cellular functions such as migration, production of ROS, cytokines, and chemokines, expression of adhesion molecules, and interaction with ECM. These alterations seem to be related to improvements in tissue repair.

2.2. Conjugated Linoleic Acid (CLA). The presence of conjugated linoleic acid (CLA) was first reported in 1930 [68], but only in the 1980s was CLA described as a bioactive dietary constituent, and the interest in CLA's effects has increased due its anticarcinogenic properties and reduction of adipose tissue mass observed in mice [69].

CLA comprises a mix of positional and geometric isomers of linoleic acid with a single pair of conjugated double bonds. CLA is formed during LA biohydration by bacteria in the gut of ruminant animals, and thus, the main natural sources of CLA are ruminant meats (beef and lamb) and dairy products (milk and cheese) [69, 70].

At least 28 CLA isomers are known, but the *cis*-9, *trans*-11 (c9, t11) is the most abundant form of CLA in nature, and nutritional supplements are a mixture of c9, t11 and *trans*-10, *cis*-12 (t10, c12) CLA [71, 72]. Initially, it was thought that the effects of CLA were global, and the results were due to interactions between its two main isomers: c9, t11 and t10, c12. However, later evidence suggested that the physiological effects of CLA may be different between the isomers, animal species (rats and mice), and cell types [73].

The last decade has seen a plethora of claims, supported by animal and cell lineage models, that dietary CLA intake is associated with potential health benefits [70]. These include reduction in fat deposition, protection from atherosclerosis and cancer, and enhanced immunity [69, 74].

Although preclinical data suggest benefits of CLA supplementation, clinical findings in humans have yet to show evidence of a positive effect, and even the findings in animals are still controversial [73].

Some studies revealed that CLA can induce adverse effects such as fatty liver, insulin resistance, and lipodystrophy [75]. Thus, it is recommended that ingestion of a balanced diet with natural sources of CLA be followed.

2.2.1. Effects of CLA on Tissue Repair. Mice fed a diet supplemented with 0.5% or 1% CLA (38% c9, t11 CLA; 39% t10, c12 CLA; 3% c9, c11 CLA; and 1% t9, t11 CLA) for 2 weeks presented a reduction in wound area (1% CLA) that was related to an increase in antioxidant defences [76]. ROS are essential to protect the organism against invading bacteria and other microorganisms; moreover, they are important to intracellular signalling. However, excessive production of ROS or impaired detoxification of these molecules causes oxidative stress [54]. To understand the prohealing effect, the authors measured malondialdehyde (MDA) content in the liver, a marker of lipid peroxidation, and the expression of antioxidant enzymes at the wound site. Mice supplemented with CLA had a reduced MDA content and increased CuZn superoxide dismutase (SOD) and MnSOD protein expression, showing an antioxidant effect of this fatty acid, which can explain its benefit on wound healing. At the same time, they described a reduction of phosphorylated inhibitor kappa B alpha (p$I\kappa B\alpha$) protein expression at the end of the inflammatory phase of wound healing [76]. In the cytoplasm, NF-κB is found complexed with IκB. Once phosphorylated, IκB releases NF-κB that translocates to the nucleus and induces the expression of genes related to inflammatory responses [77]. Therefore, the reduction in p$I\kappa B\alpha$ indicates that NF-κB is in the cytoplasm, and the expression of proinflammatory genes is reduced. To show this, the expression of cyclooxygenase-2 (COX-2) and HO-1 was evaluated. CLA reduced the protein expression of these inflammatory genes, confirming its inhibitory effect on NF-κB activation [76].

In the carcinogenic context, topical application of CLA to hairless mouse skin also reduced COX-2 expression due to inhibition of NF-κB activation in the skin [78]. To elucidate the CLA effects on the NF-κB pathway, it was described that this fatty acid downregulated the catalytic activity of IκB kinase (IKKα/β), mitogen-activated protein kinase (p38 MAPK), and protein kinase B (Akt) [78]. We suggest Zhang et al. [77] for a comprehensive review of the NF-κB signalling pathway.

2.2.2. Mechanisms of Action of CLA. The mechanisms by which CLA modulates immune function are not completely elucidated, but they include regulation of prostaglandin and cytokine production, since it has been observed that CLA reduces COX-2 expression and modulates NF-κB activation [76, 78, 79].

Peripheral blood mononuclear cells (PBMC) treated with t10, c12 CLA (100 μM) for 24 hours diminished TNF-α production. This effect seems to be isomer-specific since treatment with c9, t11 CLA (100 μM) or LA (100 μM) had no effect on TNF-α concentration [80].

Cho et al. [81] suggested that t10, c12 CLA has a priming effect on polymorphonuclear (PMN) and mononuclear cells isolated from dogs. PMN or mononuclear cells directly treated with CLA did not alter TNF-α production. Thus, they took this preconditioned medium and added it to a new cell culture. This preconditioned medium increased TNF-α concentrations and augmented the oxidative burst activity and phagocytic capacity of PMN and mononuclear cells [81]. When the recombinant anti-TNF-α antibody was added to this preconditioned medium, the effects were abolished, suggesting that the effects of CLA are mediated by TNF-α released from PBMC.

Taken together, these results showed that dietary administration of CLA can improve wound healing due to antioxidant and anti-inflammatory effects in the later inflammatory phase of tissue repair.

2.3. Gamma Linolenic Acid (GLA). Gamma-linolenic acid (GLA, 18 : 3 ω-6) is an omega-6 fatty acid formed through LA metabolization, due to delta-6-desaturase action [82]. It is found in plant seed oils, such as borage, black current seed, and primrose oil [83]. The most common form of GLA consumption is through oral supplementation with GLA-rich oil capsules, mainly from evening primrose oil (EPO) [84].

GLA has been investigated in chronic inflammatory diseases such as rheumatoid arthritis [83, 85, 86], atopic dermatitis, acne vulgaris, and psoriasis [87–89] due to its anti-inflammatory effects. GLA can be converted into dihomo-γ-linoleic acid (DGLA), which is metabolized into prostaglandin E1 (PGE1) or 15-hydroxyeicosatrienoic acid (15-HETrE) [82, 89]. These eicosanoids have anti-inflammatory and immunoregulatory effects [85].

2.3.1. Effects of GLA on Tissue Repair. GLA ingestion was also used to treat patients with acne vulgaris [88]. In this study, 45 patients received 2 capsules of borage oil (400 mg of GLA) for 10 weeks, and acne lesion number and severity were assessed as well as inflammation by histological analysis. The GLA group had a reduction in the lesion number and severity, which could be associated with a reduction in inflammation and interleukin-8 (IL-8) staining demonstrated by histologic analysis [88]. Although the authors speculate that two mechanisms (modulation of inflammation and improvement of skin quality) could explain their results, no other analyses were made of their samples. Therefore, it is not possible to affirm how GLA had beneficial effects on acnes vulgaris.

Ingestion of GLA-rich oil capsules was also related to clinical improvement of atopic dermatitis (AD) [89]. The clinical effect was positively correlated with plasma GLA and DGLA concentrations after 4 weeks of capsule consumption.

2.3.2. Mechanisms of Action of GLA. Considering the relevance of macrophages in inflammatory processes such as arthritis and wound healing, it is of great value to investigate the effects of GLA on their functions.

In the RAW 264.7 macrophage cell line, GLA concentrations (100 to 200 μM) reduced the expression of inducible nitric oxide synthase (iNOS) and consequently the NO concentration [90]. GLA also inhibited the expression of COX-2 and prointerleukin-1, suggesting a reduction in inflammatory responses. To explain these results, the authors evaluated the expression of proteins involved in the NF-κB pathway. GLA diminished IκB phosphorylation and degradation, blocking the transmigration of NF-κB to the nucleus, which was confirmed by the reduction in nuclear p65 protein expression. Altogether, these results explain the reduced activation of NF-κB in GLA-treated macrophages [90].

More studies are necessary to prove the beneficial effects of GLA on wound healing.

2.4. Arachidonic Acid. Arachidonic acid (AA, 20 : 4 ω-6) is the second most abundant fatty acid in injured tissue after a tissue lesion [91]. Once released from membrane phospholipids by phospholipase A_2, AA is metabolized by cyclooxygenases and lipoxygenases and produces the eicosanoids [92].

Eicosanoids are a wide variety of 20-carbon bioactive lipid products that include prostaglandins (PGs) and thromboxanes (TXs) of series 2 and leukotrienes (LTs) of series 4, lipoxins (LXs), hydroxyeicosatetraenoic acids (HETEs), and epoxyeicosatrienoic acids (EETs) [93] that modulate inflammatory responses. They are highly potent, short-lived molecules that act locally and have been strongly associated with a variety of physiological and pathological processes including cancer, inflammatory diseases, and wound healing [92]. In the wound healing process, the effects of AA are associated mainly with the production of eicosanoids, because they are abundant in the wound bed [94].

The AA metabolites are predominantly proinflammatory because they stimulate the chemotaxis of inflammatory cells, increase the activity of elastase that degrades extracellular proteins, and impair the formation and remodelling of healing tissue [94].

2.4.1. Effects of AA on Tissue Repair. Considering that AA generates eicosanoids and that these molecules modulate tissue repair, an AA-enriched diet was tested in an intestinal ischaemia-injured model [95]. The diets were enriched with 0.5 or 5% of AA and administered over 10 days to pigs. After this period, blockage of the mesenteric blood vessels induced an ischaemic ileum injury, and the protective and reparative effects of AA administration were analysed. It was observed there was a protective effect of AA (5%) since the percentage of denuded villus area was reduced in relationship to the control. At the same time, the AA group presented an improvement in recovery since these animals showed a reduction in mucosal-to-serosal flux of ^{3}H-mannitol and ^{14}C-inulin when compared with the control group (0% of AA), suggesting that the epithelial barrier is more preserved in the AA group [95]. Although AA-enriched diet (5%) does not alter COX-2 mRNA expression, it was observed that there was an increase in PGE_2 concentration after ischaemic injury [95]. This effect

Table 1: Effects of linoleic (LA) fatty acid.

Fatty acid	Condition	Study model	Treatment time	Dose/concentration	Molecules associated	Effect in tissue repair	Reference
LA	Wound healing	Diabetic Wistar rats	18 days	0.22 g/Kg bw (oral administration)	Increased VEGF and ANGPT-2	Accelerated the inflammatory phase and angiogenesis	[38]
	Pressure ulcers	Healthy humans	21 days	1.6 g EFA with LA extracted from sunflower oil (topical application)	NA	Increased hydration and elasticity.	[40]
	Wound healing	Healthy rats	12 days	0.14 g solution with TGs, LA, vitamins A and E, and soy lecithin (topical application)	NA	No effects	[41]
	Wound healing	Zebrafish	48 hours	10–100 μg/mL of lucuma nut oil	NA	Improved regeneration (100 μg/mL)	[42]
		CD-1 mice	11 days	200, 500, or 1000 μg of lucuma nut oil (topical application)	NA	Improved wound healing and formation of new blood vessels (500 and 1000 μg)	
	Wound healing	Healthy rats	11 days	0.52 μL/mm^2 of pumpkin oil (topical application)	Increased hydroxyproline content	Accelerated wound closure and bleeding time, improved fibrin stabilization, increased migration of fibroblasts, and reduced infiltration of macrophages	[43]
	Wound healing	Healthy BALB/c mice	20 days	30 μM of pure LA (topical application)	Increased NO production	Accelerated tissue repair	[45]
	Wound healing	Healthy Wistar rats	24 hours	300 μL of pure LA (topical application)	Increased total protein and DNA contents and elevated VEGF-α and IL-1	No effect on wound area	[48]
	Wound healing	Healthy Wistar rats	5 days	0.22 g/Kg bw of pure LA (oral administration)	Increased H_2O_2 and AP-1 and reduced NF-κB, IL-1β, and IL-6	Accelerated the inflammatory phase	[49]
	Neutrophil functions	Intraperitoneal neutrophils from healthy Wistar rats	10 days	0.11, 0.22, and 0.44 g/kg of bw (oral administration)	Increased L-selectin, IL-1β, and CINC-2$\alpha\beta$	Increased leukocyte-endothelium interactions	[52]
	Neutrophil functions	Intraperitoneal neutrophils from healthy Wistar rats	20 minutes	0, 10, 25, 50, 100, and 200 μM *(in vitro)*	Increased O_2^- and H_2O_2 (50 μM)	Increased ROS	[53]
	Macrophage functions	Macrophages from healthy Wistar rats	10 days	0.22 g/Kg bw (oral administration)	Reduced IL-6, VEGF, and IL-10	Modulated cytokine production by macrophages	[59]

Essential fatty acids (EFA); triglycerides (TGs); nitric oxide (NO); Deoxyribonucleic acid (DNA); vascular endothelial growth factor (VEGF); interleukin-1β (IL-1β); body weight (bw); hydrogen peroxide (H_2O_2); activator protein-1 (AP-1); nuclear transcription factor (NF-κB); interleukin-6 (IL-6); angiopoietin-2 (ANGPT-2); cytokine-induced neutrophil chemoattractant-2 (CINC-2$\alpha\beta$); reactive oxygen species (ROS); lipopolysaccharides (LPS); interleukin-10 (IL-10); not analysed (NA).

Table 2: Effects of conjugated linoleic acid (CLA).

Fatty acid	Condition	Study model	Treatment time	Dose/concentration	Molecules associated	Effect in tissue repair	Reference
CLA	Wound healing	Healthy mice	2 weeks	0.5 or 1% of CLA (diet)	Increased CuZnSOD, and MnSOD and reduced pIkBα, COX-2, HO-1, and MDA	Increased the antioxidant defences and reduced the wound area (1%)	[76]
	Hairless skin	Mice	6 hours	0.25 or 1 mg (topical application)	Reduced NF-κB, COX-2, IKKα/β, MAPK, and Akt	Antitumor (1 mg)	[78]
	Inflammatory diseases	Bovine PBMC	24 hours	100 μM *(in vitro)*	Decreased TNF-α	Additional studies are needed	[80]
	Inflammatory diseases	Blood phagocytes isolated from dogs	24 hours	10 μM *(in vitro)*	Increased TNF-α	Increased oxidative burst activity and phagocytic capacity	[81]

CuZn superoxide dismutase (CuZnSOD); Mn superoxide dismutase (MnSOD); cicloxigenase-2 (COX-2); malondialdehyde (MDA); nuclear transcription factor (NF-κB); IκB kinase (IKKα/β); mitogen-activated protein kinase (MAPK); protein kinase B (Akt); tumor necrosis factor α (TNF-α); peripheral blood mononuclear cells (PBMC); not analysed (NA).

Table 3: Effects of gamma linolenic (GLA) fatty acid.

Fatty acid	Condition	Study model	Treatment time	Dose/concentration	Molecules associated	Effect in tissue repair	Reference
GLA	Acne vulgaris	Healthy humans	10 weeks	400 mg (oral administration)	Reduced IL-8	Reduced lesion number, severity, and inflammation	[88]
	Atopic dermatitis (AD)	Humans	12 weeks	320 or 480 mg (oral administration)	NA	Improvement of clinical signs of AD	[89]
	Macrophage functions	RAW 264.7 macrophages		100 to 200 μM *(in vitro)*	Reduced iNOS, NO, COX-2, pro-IL-1, pIκB, and NF-κB	Decreased inflammation	[90]

Interleukin-8 (IL-8); inducible nitric oxide synthase (iNOS); oxide nitric (NO); cicloxigenase-2 (COX-2); prointerleukin-1 (pro-IL-1); nuclear transcription factor (NF-κB); not analysed (NA)

Table 4: Effects of arachidonic (AA) fatty acid.

Fatty acid	Condition	Study model	Treatment time	Dose/concentration	Molecules associated	Effect in tissue repair	Reference
AA	Wound healing	hUCB-MSC	24 hours	5 or 10 μM *(in vitro)*	Increased mTOR^{ser2481}, Akt^{ser407}, PKCζ, and MMPs	Increased cell migration and angiogenesis (10 μM)	[91]
	Intestinal ischemic injury	Pigs	10 days	0.5 or 5% of AA (diet)	Increased PGE_2	Preservation of epithelial barrier (5%)	[95]
	IBD	Rats	8 weeks	0, 5, 35, or 240 mg/Kg of bw (oral administration)	Increased COX-2, LTB_4, TXB_2, and MPO	Increased inflammation and macrophage infiltration	[98]
	Angiogenesis	Porcine endothelial cells	24 hours	0, 20, 50, 60, and 80 μM *(in vitro)*	NA	Increased cell spreading (20 μM) and reduced cell spreading (80 μM)	[101]

Prostaglandin E2 (PGE_2); inflammatory bowel disease (IBD); body weight (bw); cicloxigenase-2 (COX-2); leukotriene B_4 (LTB_4); thromboxane (TXB_2); myeloperoxidase (MPO); human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC); mammalian target of rapamycin complex 1 phosphorylation (mTOR^{ser2481}); protein kinase B (Akt^{ser407}); phosphorylates protein kinase Cζ (PKCζ); matrix metalloproteinases (MMPs); not analysed (NA).

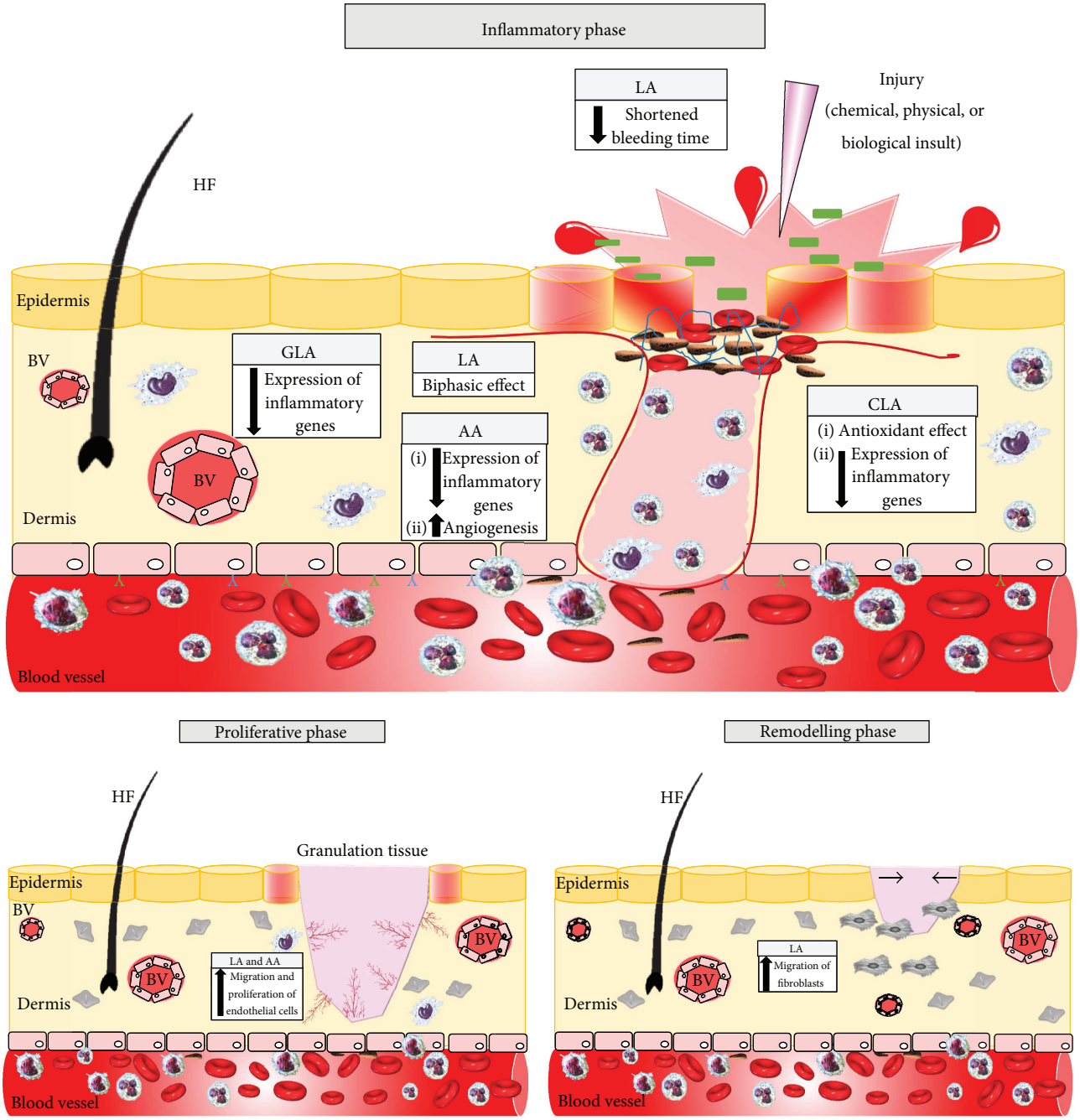


Figure 2: Effects of linoleic acid (LA), conjugated linoleic acid (CLA), gamma linolenic acid (GLA), and arachidonic acid on wound healing phases.

was abolished when animals received indomethacin, a nonselective COX inhibitor. PGE_2 has been described to be a protective factor that stimulates the recovery of gut injury [96]. One of the mechanisms involved is the induction of angiogenesis due to an increase in VEGF content [97].

In the dextran sodium sulphate-induced inflammatory bowel disease (IBD) model, oral administration of AA (240 mg/kg of body weight) for 8 weeks aggravated inflammation since it increased COX-2, LTB_4, and TXB_2 concentrations in colonic tissue. AA also elevated myeloperoxidase (MPO) activity and macrophage infiltration, which reinforces its proinflammatory effect [98].

Epoxyeicosatrienoic acids (EETs) are metabolites produced from AA due to cytochrome P450 (CYP450) activity, predominantly in the endothelium. EETs can stimulate angiogenesis and organ or tissue regeneration [21, 99]. Local application of 11,12- or 14,15-EETs (10 μM/methylcellulose discs) accelerated wound healing due to the increase in MMP2 and MMP7 and reduction in TIMP-1 and TNF-α during the proliferative phase of wound healing

(12 days). These results indicate that EETs favoured extracellular matrix degradation and endothelial cell migration, two important steps in the angiogenesis process [21]. These positive effects were also confirmed in transgenic animals that exhibit high or low EET. In this model, the wound healing was accelerated in high EET animals due to vascularization [100].

To better explain the roles of lipid mediators during impaired wound healing, a lipidomic approach was carried out in transgenic animals ($LIGHT^{-/-}$) that exhibited an exacerbated inflammatory response characterized by high levels of oxidative stress and cytokines and imbalance between production and degradation of ECM [99]. These animals had increased concentrations of 11-, 12-, and 15-hydroxyeicosatetraenoic acid, leukotrienes (LTD_4 and LTE), prostaglandins (PGE_2 and PGF_2), thromboxane (TXA_2/TXB_2), and prostacyclins in early stages (1 day) of wound healing. These results were associated with enhanced coagulation and infiltration of neutrophils in $LIGHT^{-/-}$ when compared with wild type mice [99].

The factors that lead to colitis, ischemia-reperfusion damage, and skin wound are physiologically different as well as the responses observed in these conditions. Although a general inflammatory response is usually observed, which is characterized by recruitment and activation of inflammatory cells, production of cytokines and growth factors as well as the repair of the damaged tissue, there are specificities inherent to each tissue that can change the effect of determined fatty acid.

2.4.2. Mechanisms of Action of AA. In agreement with the *in vivo* studies, AA induced endothelial cell adhesion *in vitro*. Once again, this modulatory effect presented a biphasic pattern as also observed with other fatty acids, such as LA. In the first phase, the positive effect on cell spreading was independent of AA concentration. However, in the later phase, there was an inverse correlation between AA concentration and spreading [101]. Low AA concentrations (20 μM) increased cell spreading, and high AA concentrations (80 μM) reduced it. This effect could be associated with the metabolites generated from AA oxidation. At a low concentration, AA is totally metabolized, and the products can induce cell adhesion. On the other hand, at high concentrations, the reaction is saturated in AA, and the enzymes involved are not sufficient to metabolize all AA available. Then, there is a reduction in AA metabolites and consequently a reduction in cell spreading [101]. AA also randomizes the migration of endothelial cells. This action is related to the loss of direction during migration due to the presence of AA and seems to be inversely correlated with AA concentration [101].

Some of these AA effects, observed in endothelial cells, were also described in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs), and once again, AA concentration was inversely related with the migratory response. The mechanism behind this effect seems to involve GPR40 [91], a membrane receptor for fatty acids [102].

In a very detailed study, Oh et al. [91] demonstrated that AA binds to GPR40 and then induces mammalian target of rapamycin complex 1 (mTORC2) phosphorylation ($mTOR^{ser2481}$) that activates Akt^{ser407}, which phosphorylates protein kinase Cζ (PKCζ). pPKCζ activates p38, through Sp1 phosphorylation, and increases the expression of matrix metalloproteinases (MMPs). MMP degrades fibronectin, an extracellular matrix component, promoting the migration of hUCB-MSCs.

Altogether, the studies demonstrate that AA and its metabolites promote wound healing due to induction of cell migration and angiogenesis. However, these positive effects are closely related with the concentrations used.

3. Summary

Wound healing is an evolutionarily conserved process essential for species' survival. An investigation of factors that improve wound healing is of crucial interest. Experimental and clinical studies indicate that LA improves wound healing due to its biphasic effects on the inflammatory phase of tissue repair (Table 1). CLA seems to have antioxidant and anti-inflammatory effects on the later inflammatory phase of tissue repair, favouring the beginning of the proliferative phase (Table 2). Although less studied, GLA presented positive effects controlling inflammation (Table 3). Studies investigating the effects of AA demonstrated that AA and its metabolites promoted wound healing due to induction of cell migration and angiogenesis (Table 4).

In general, omega-6 fatty acids positively modulate all phases of wound healing, but more studies are necessary to clarify the mechanisms involved (Figure 2).

Clinical studies are essential to establish the strategies of fatty acid administration (topically or orally), the optimal concentrations, and their safety.

Conflicts of Interest

None of the authors have any conflict of interest or anything to disclose.

Authors' Contributions

All authors did the literature search. Jéssica R. Silva and Beatriz Burger wrote the manuscript and contributed equally to this paper. Hosana G. Rodrigues also wrote the manuscript and critically revised the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2013/06810-4).

References

[1] H. Brem and M. Tomic-Canic, "Cellular and molecular basis of wound healing in diabetes," *Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1219–1222, 2007.

[2] Y.-S. Wu and S.-N. Chen, "Apoptotic cell: linkage of inflammation and wound healing," *Frontiers in Pharmacology*, vol. 5, p. 1, 2014.

[3] J. M. Loree, A. A. L. Pereira, M. Lam et al., "Classifying colorectal cancer by tumor location rather than sidedness highlights a continuum in mutation profiles and consensus molecular subtypes," *Clinical Cancer Research*, vol. 24, no. 5, pp. 1062–1072, 2018.

[4] C. Dunnill, T. Patton, J. Brennan et al., "Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process," *International Wound Journal*, vol. 14, no. 1, pp. 89–96, 2017.

[5] T. Nauta, V. van Hinsbergh, and P. Koolwijk, "Hypoxic signaling during tissue repair and regenerative medicine," *International Journal of Molecular Sciences*, vol. 15, no. 11, pp. 19791–19815, 2014.

[6] D. G. Menter, S. Kopetz, E. Hawk et al., "Platelet "first responders" in wound response, cancer, and metastasis," *Cancer Metastasis Reviews*, vol. 36, no. 2, pp. 199–213, 2017.

[7] M. Xue and C. J. Jackson, "Extracellular matrix reorganization during wound healing and its impact on abnormal scarring," *Advances in Wound Care*, vol. 4, no. 3, pp. 119–136, 2015.

[8] S. A. Eming, P. Martin, and M. Tomic-Canic, "Wound repair and regeneration: mechanisms, signaling, and translation," *Science Translational Medicine*, vol. 6, no. 265, article 265sr6, 2014.

[9] L. E. Tracy, R. A. Minasian, and E. J. Caterson, "Extracellular matrix and dermal fibroblast function in the healing wound," *Advances in Wound Care*, vol. 5, no. 3, pp. 119–136, 2016.

[10] N. X. Landen, D. Li, and M. Ståhle, "Transition from inflammation to proliferation: a critical step during wound healing," *Cellular and Molecular Life Sciences*, vol. 73, no. 20, pp. 3861–3885, 2016.

[11] W. Raphael and L. Sordillo, "Dietary polyunsaturated fatty acids and inflammation: the role of phospholipid biosynthesis," *International Journal of Molecular Sciences*, vol. 14, no. 10, pp. 21167–21188, 2013.

[12] P. C. Calder, "Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology?," *British Journal of Clinical Pharmacology*, vol. 75, no. 3, pp. 645–662, 2013.

[13] T. J. Shaw and P. Martin, "Wound repair at a glance," *Journal of Cell Science*, vol. 122, no. 18, pp. 3209–3213, 2009.

[14] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, "Wound repair and regeneration," *Nature*, vol. 453, no. 7193, pp. 314–321, 2008.

[15] S. A. Eming, T. A. Wynn, and P. Martin, "Inflammation and metabolism in tissue repair and regeneration," *Science*, vol. 356, no. 6342, pp. 1026–1030, 2017.

[16] O. Chow and A. Barbul, "Immunonutrition: role in wound healing and tissue regeneration," *Advances in Wound Care*, vol. 3, no. 1, pp. 46–53, 2014.

[17] J. Li, J. Chen, and R. Kirsner, "Pathophysiology of acute wound healing," *Clinics in Dermatology*, vol. 25, no. 1, pp. 9–18, 2007.

[18] F. Chilton, R. Murphy, B. Wilson et al., "Diet-gene interactions and PUFA metabolism: a potential contributor to health disparities and human diseases," *Nutrients*, vol. 6, no. 5, pp. 1993–2022, 2014.

[19] J. M. Reinke and H. Sorg, "Wound repair and regeneration," *European Surgical Research*, vol. 49, no. 1, pp. 35–43, 2012.

[20] A. S. MacLeod and J. N. Mansbridge, "The innate immune system in acute and chronic wounds," *Advances in Wound Care*, vol. 5, no. 2, pp. 65–78, 2016.

[21] A. L. Sander, K. Sommer, T. Neumayer et al., "Soluble epoxide hydrolase disruption as therapeutic target for wound healing," *Journal of Surgical Research*, vol. 182, no. 2, pp. 362–367, 2013.

[22] K. Järbrink, G. Ni, H. Sönnergren et al., "Prevalence and incidence of chronic wounds and related complications: a protocol for a systematic review," *Systematic Reviews*, vol. 5, no. 1, p. 152, 2016.

[23] C. K. Sen, G. M. Gordillo, S. Roy et al., "Human skin wounds: a major and snowballing threat to public health and the economy," *Wound Repair and Regeneration*, vol. 17, no. 6, pp. 763–771, 2009.

[24] R. G. Frykberg and J. Banks, "Challenges in the treatment of chronic wounds," *Advances in Wound Care*, vol. 4, no. 9, pp. 560–582, 2015.

[25] K. Copeland and A. R. Purvis, "A retrospective chart review of chronic wound patients treated with topical oxygen therapy," *Advances in Wound Care*, vol. 6, no. 5, pp. 143–152, 2017.

[26] K. Heyer, M. Augustin, K. Protz, K. Herberger, C. Spehr, and S. J. Rustenbach, "Effectiveness of advanced versus conventional wound dressings on healing of chronic wounds: systematic review and meta-analysis," *Dermatology*, vol. 226, no. 2, pp. 172–184, 2013.

[27] C. Wicke, A. Bachinger, S. Coerper, S. Beckert, M. B. Witte, and A. Königsrainer, "Aging influences wound healing in patients with chronic lower extremity wounds treated in a specialized wound care center," *Wound Repair and Regeneration*, vol. 17, no. 1, pp. 25–33, 2009.

[28] S. Guo and L. A. Dipietro, "Factors affecting wound healing," *Journal of Dental Research*, vol. 89, no. 3, pp. 219–229, 2010.

[29] K. Anderson and R. L. Hamm, "Factors that impair wound healing," *Journal of the American College of Clinical Wound Specialists*, vol. 4, no. 4, pp. 84–91, 2012.

[30] U. Okonkwo and L. DiPietro, "Diabetes and wound angiogenesis," *International Journal of Molecular Sciences*, vol. 18, no. 7, p. 1419, 2017.

[31] W. J. Ennis, A. Sui, and A. Bartholomew, "Stem cells and healing: impact on inflammation," *Advances in Wound Care*, vol. 2, no. 7, pp. 369–378, 2013.

[32] L. Das, E. Bhaumik, U. Raychaudhuri, and R. Chakraborty, "Role of nutraceuticals in human health," *Journal of Food Science and Technology*, vol. 49, no. 2, pp. 173–183, 2012.

[33] P. C. Calder, "Fatty acids and inflammation: the cutting edge between food and pharma," *European Journal of Pharmacology*, vol. 668, Supplement 1, pp. S50–S58, 2011.

[34] C. R. Kepler, K. P. Hirons, J. McNeill, and S. B. Tove, "Intermediates and products of the biohydrogenation of linoleic acid by *Butyrinvibrio fibrisolvens*," *Journal of Biological Chemistry*, vol. 241, no. 6, pp. 1350–1354, 1966.

[35] J. W. Alexander and D. M. Supp, "Role of arginine and omega-3 fatty acids in wound healing and infection," *Advances in Wound Care*, vol. 3, no. 11, pp. 682–690, 2014.

[36] P. C. Calder, "Omega-3 fatty acids and inflammatory processes," *Nutrients*, vol. 2, no. 3, pp. 355–374, 2010.

[37] M. M. McCusker and J. M. Grant-Kels, "Healing fats of the skin: the structural and immunologic roles of the ω-6 and

ω-3 fatty acids," *Clinics in Dermatology*, vol. 28, no. 4, pp. 440–451, 2010.

[38] H. G. Rodrigues, M. A. R. Vinolo, F. T. Sato et al., "Oral administration of linoleic acid induces new vessel formation and improves skin wound healing in diabetic rats," *PloS One*, vol. 11, no. 10, article e0165115, 2016.

[39] N. Kaur, V. Chugh, and A. K. Gupta, "Essential fatty acids as functional components of foods-a review," *Journal of Food Science and Technology*, vol. 51, no. 10, pp. 2289–2303, 2014.

[40] V. Declair, "The usefulness of topical application of essential fatty acids (EFA) to prevent pressure ulcers," *Ostomy/Wound Management*, vol. 43, no. 5, pp. 48–52, 1997.

[41] M. S. F. Magalhães, F. V. Fechine, R. N. de Macedo et al., "Effect of a combination of medium chain triglycerides, linoleic acid, soy lecithin and vitamins A and E on wound healing in rats," *Acta Cirurgica Brasileira*, vol. 23, no. 3, pp. 262–269, 2008.

[42] L. E. Rojo, C. M. Villano, G. Joseph et al., "Wound-healing properties of nut oil from *Pouteria lucuma*," *Journal of Cosmetic Dermatology*, vol. 9, no. 3, pp. 185–195, 2010.

[43] S. Bardaa, N. Ben Halima, F. Aloui et al., "Oil from pumpkin (*Cucurbita pepo* L.) seeds: evaluation of its functional properties on wound healing in rats," *Lipids in Health and Disease*, vol. 15, no. 1, p. 73, 2016.

[44] P. Carmeliet, "Mechanisms of angiogenesis and arteriogenesis," *Nature Medicine*, vol. 6, no. 4, pp. 389–395, 2000.

[45] C. R. B. Cardoso, M. A. Souza, E. A. V. Ferro, S. Favoreto, and J. D. O. Pena, "Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds," *Wound Repair and Regeneration*, vol. 12, no. 2, pp. 235–243, 2004.

[46] J. Víteček, A. Lojek, G. Valacchi, and L. Kubala, "Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges," *Mediators of Inflammation*, vol. 2012, Article ID 318087, 22 pages, 2012.

[47] S. Frank, H. Kämpfer, C. Wetzler, and J. Pfeilschifter, "Nitric oxide drives skin repair: novel functions of an established mediator," *Kidney International*, vol. 61, no. 3, pp. 882–888, 2002.

[48] L. M. Pereira, E. Hatanaka, E. F. Martins et al., "Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats," *Cell Biochemistry and Function*, vol. 26, no. 2, pp. 197–204, 2008.

[49] H. G. Rodrigues, M. A. R. Vinolo, J. Magdalon et al., "Oral administration of oleic or linoleic acid accelerates the inflammatory phase of wound healing," *Journal of Investigative Dermatology*, vol. 132, no. 1, pp. 208–215, 2012.

[50] O. Gealekman, A. Burkart, M. Chouinard, S. M. Nicoloro, J. Straubhaar, and S. Corvera, "Enhanced angiogenesis in obesity and in response to PPARγ activators through adipocyte VEGF and ANGPTL4 production," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 295, no. 5, pp. E1056–E1064, 2008.

[51] T. N. Mayadas, X. Cullere, and C. A. Lowell, "The multifaceted functions of neutrophils," *Annual Review of Pathology*, vol. 9, no. 1, pp. 181–218, 2014.

[52] H. G. Rodrigues, M. A. R. Vinolo, J. Magdalon et al., "Dietary free oleic and linoleic acid enhances neutrophil function and modulates the inflammatory response in rats," *Lipids*, vol. 45, no. 9, pp. 809–819, 2010.

[53] E. Hatanaka, A. C. Levada-Pires, T. C. Pithon-Curi, and R. Curi, "Systematic study on ROS production induced by oleic, linoleic, and γ-linolenic acids in human and rat neutrophils," *Free Radical Biology and Medicine*, vol. 41, no. 7, pp. 1124–1132, 2006.

[54] M. Schafer and S. Werner, "Oxidative stress in normal and impaired wound repair," *Pharmacological Research*, vol. 58, no. 2, pp. 165–171, 2008.

[55] S. Roy, S. Khanna, K. Nallu, T. K. Hunt, and C. K. Sen, "Dermal wound healing is subject to redox control," *Molecular Therapy*, vol. 13, no. 1, pp. 211–220, 2006.

[56] M. J. Morgan and Z.-g. Liu, "Crosstalk of reactive oxygen species and NF-κB signaling," *Cell Research*, vol. 21, no. 1, pp. 103–115, 2011.

[57] N. Tobar, M. Cáceres, J. F. Santibáñez, P. C. Smith, and J. Martínez, "RAC1 activity and intracellular ROS modulate the migratory potential of MCF-7 cells through a NADPH oxidase and NF-κB-dependent mechanism," *Cancer Letters*, vol. 267, no. 1, pp. 125–132, 2008.

[58] D. Han, M. D. Ybanez, S. Ahmadi, K. Yeh, and N. Kaplowitz, "Redox regulation of tumor necrosis factor signaling," *Antioxidants & Redox Signaling*, vol. 11, no. 9, pp. 2245–2263, 2009.

[59] J. Magdalon, M. A. R. Vinolo, H. G. Rodrigues et al., "Oral administration of oleic or linoleic acids modulates the production of inflammatory mediators by rat macrophages," *Lipids*, vol. 47, no. 8, pp. 803–812, 2012.

[60] J. Frieder, D. Kivelevitch, C. T. Fiore, S. Saad, and A. Menter, "The impact of biologic agents on health-related quality of life outcomes in patients with psoriasis," *Expert Review of Clinical Immunology*, vol. 14, no. 1, pp. 1–19, 2017.

[61] C. N. Serhan, "Discovery of specialized pro-resolving mediators marks the dawn of resolution physiology and pharmacology," *Molecular Aspects of Medicine*, vol. 58, pp. 1–11, 2017.

[62] I. Pastar, O. Stojadinovic, N. C. Yin et al., "Epithelialization in wound healing: a comprehensive review," *Advances in Wound Care*, vol. 3, no. 7, pp. 445–464, 2014.

[63] S. Liu, X. Shi-wen, L. Kennedy et al., "FAK is required for TGFβ-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype," *Molecular Biology of the Cell*, vol. 18, no. 6, pp. 2169–2178, 2007.

[64] M. A. R. Vinolo, S. M. Hirabara, and R. Curi, "G-protein-coupled receptors as fat sensors," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 15, no. 2, pp. 112–116, 2012.

[65] C. P. Briscoe, M. Tadayyon, J. L. Andrews et al., "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids," *Journal of Biological Chemistry*, vol. 278, no. 13, pp. 11303–11311, 2003.

[66] T. Sartorius, A. Drescher, M. Panse et al., "Mice lacking free fatty acid receptor 1 (GPR40/FFAR1) are protected against conjugated linoleic acid-induced fatty liver but develop inflammation and insulin resistance in the brain," *Cellular Physiology and Biochemistry*, vol. 35, no. 6, pp. 2272–2284, 2015.

[67] T. Fujita, T. Matsuoka, T. Honda, K. Kabashima, T. Hirata, and S. Narumiya, "A GPR40 agonist GW9508 suppresses CCL5, CCL17, and CXCL10 induction in keratinocytes and attenuates cutaneous immune inflammation," *Journal of*

Investigative Dermatology, vol. 131, no. 8, pp. 1660–1667, 2011.

[68] P. W. Parodi, "Conjugated linoleic acid: the early years," in *Advances in Conjugated Linoleic Acid Research Volume I*, M. P. Yuramecz, M. M. Mossoba, J. K. G. Kramer, M. W. Pariza, and G. J. Nelson, Eds., pp. 1–11, AOCS Press, Champaing, IL, 1999.

[69] M. H. Cooper, J. R. Miller, P. L. Mitchell, D. L. Currie, and R. S. McLeod, "Conjugated linoleic acid isomers have no effect on atherosclerosis and adverse effects on lipoprotein and liver lipid metabolism in apoE$^{-/-}$ mice fed a high-cholesterol diet," *Atherosclerosis*, vol. 200, no. 2, pp. 294–302, 2008.

[70] A. Bhattacharya, J. Banu, M. Rahman, J. Causey, and G. Fernandes, "Biological effects of conjugated linoleic acids in health and disease," *The Journal of Nutritional Biochemistry*, vol. 17, no. 12, pp. 789–810, 2006.

[71] Y. Kim, J. Kim, K. Y. Whang, and Y. Park, "Impact of conjugated linoleic acid (CLA) on skeletal muscle metabolism," *Lipids*, vol. 51, no. 2, pp. 159–178, 2016.

[72] R. Wall, R. P. Ross, G. F. Fitzgerald, and C. Stanton, "Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids," *Nutrition Reviews*, vol. 68, no. 5, pp. 280–289, 2010.

[73] M. Viladomiu, R. Hontecillas, and J. Bassaganya-Riera, "Modulation of inflammation and immunity by dietary conjugated linoleic acid," *European Journal of Pharmacology*, vol. 785, pp. 87–95, 2016.

[74] A. Roy, J. M. Chardigny, D. Bauchart et al., "Butters rich either in *trans*-10-C18:1 or in *trans*-11-C18:1 plus *cis*-9, *trans*-11 CLA differentially affect plasma lipids and aortic fatty streak in experimental atherosclerosis in rabbits," *Animal*, vol. 1, no. 3, pp. 467–476, 2007.

[75] J. Oleszczuk, L. Oleszczuk, A. K. Siwicki, and E. Skopińska-Skopińska, "Biological effects of conjugated linoleic acids supplementation," *Polish Journal of Veterinary Sciences*, vol. 15, no. 2, pp. 403–408, 2012.

[76] N. Y. Park, G. Valacchi, and Y. Lim, "Effect of dietary conjugated linoleic acid supplementation on early inflammatory responses during cutaneous wound healing," *Mediators of Inflammation*, vol. 2010, Article ID 342328, 8 pages, 2010.

[77] F. Zhang, R. Zhong, S. Li et al., "Acute hypoxia induced an imbalanced M1/M2 activation of microglia through NF-κB signaling in Alzheimer's disease mice and wild-type littermates," *Frontiers in Aging Neuroscience*, vol. 9, p. 282, 2017.

[78] D.-M. Hwang, J. K. Kundu, J.-W. Shin, J.-C. Lee, H. J. Lee, and Y.-J. Surh, "*cis*-9, *trans*-11-conjugated linoleic acid down-regulates phorbol ester-induced NF-κB activation and subsequent COX-2 expression in hairless mouse skin by targeting IκB kinase and PI3K-Akt," *Carcinogenesis*, vol. 28, no. 2, pp. 363–371, 2006.

[79] T. L. Hwang, Y. C. Su, H. L. Chang et al., "Suppression of superoxide anion and elastase release by C_{18} unsaturated fatty acids in human neutrophils," *Journal of Lipid Research*, vol. 50, no. 7, pp. 1395–1408, 2009.

[80] M. C. Perdomo, J. E. Santos, and L. Badinga, "*trans*-10, *cis*-12 conjugated linoleic acid and the PPAR-γ agonist rosiglitazone attenuate lipopolysaccharide-induced TNF-α production by bovine immune cells," *Domestic Animal Endocrinology*, vol. 41, no. 3, pp. 118–125, 2011.

[81] M. H. Cho, J. H. Kang, and M. P. Yang, "Immunoenhancing effect of *trans*-10, *cis*-12 conjugated linoleic acid on the phagocytic capacity and oxidative burst activity of canine peripheral blood phagocytes," *Research in Veterinary Science*, vol. 85, no. 2, pp. 269–278, 2008.

[82] J. Lee, H. Lee, S. B. Kang, and W. Park, "Fatty acid desaturases, polyunsaturated fatty acid regulation, and biotechnological advances," *Nutrients*, vol. 8, no. 1, 2016.

[83] C. Dawczynski, U. Hackermeier, M. Viehweger, R. Stange, M. Springer, and G. Jahreis, "Incorporation of n-3 PUFA and γ-linolenic acid in blood lipids and red blood cell lipids together with their influence on disease activity in patients with chronic inflammatory arthritis - a randomized controlled human intervention trial," *Lipids in Health and Disease*, vol. 10, no. 1, p. 130, 2011.

[84] S. Taweechaisupapong, N. Srisuk, C. Nimitpornsuko, T. Vattraphoudes, C. Rattanayatikul, and K. Godfrey, "Evening primrose oil effects on osteoclasts during tooth movement," *The Angle Orthodontist*, vol. 75, no. 3, pp. 356–361, 2005.

[85] B. C. Olendzki, K. Leung, S. Van Buskirk, G. Reed, and R. B. Zurier, "Treatment of rheumatoid arthritis with marine and botanical oils: influence on serum lipids," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 827286, 9 pages, 2011.

[86] D. Vasiljevic, M. Veselinovic, M. Jovanovic et al., "Evaluation of the effects of different supplementation on oxidative status in patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 35, no. 8, pp. 1909–1915, 2016.

[87] R. H. Foster, G. Hardy, and R. G. Alany, "Borage oil in the treatment of atopic dermatitis," *Nutrition*, vol. 26, no. 7-8, pp. 708–718, 2010.

[88] J. Y. Jung, H. H. Kwon, J. S. Hong et al., "Effect of dietary supplementation with omega-3 fatty acid and gamma-linolenic acid on acne vulgaris: a randomised, double-blind, controlled trial," *Acta Dermato Venereologica*, vol. 94, no. 5, pp. 521–5, 2014.

[89] D. Simon, P. A. Eng, S. Borelli et al., "Gamma-linolenic acid levels correlate with clinical efficacy of evening primrose oil in patients with atopic dermatitis," *Advances in Therapy*, vol. 31, no. 2, pp. 180–188, 2014.

[90] C. S. Chang, H. L. Sun, C. K. Lii, H. W. Chen, P. Y. Chen, and K. L. Liu, "Gamma-linolenic acid inhibits inflammatory responses by regulating NF-κB and AP-1 activation in lipopolysaccharide-induced RAW 264.7 macrophages," *Inflammation*, vol. 33, no. 1, pp. 46–57, 2010.

[91] S. Y. Oh, S. J. Lee, Y. H. Jung, H. J. Lee, and H. J. Han, "Arachidonic acid promotes skin wound healing through induction of human MSC migration by MT3-MMP-mediated fibronectin degradation," *Cell Death & Disease*, vol. 6, no. 5, article e1750, 2015.

[92] S. Tuncer and S. Banerjee, "Eicosanoid pathway in colorectal cancer: recent updates," *World Journal of Gastroenterology*, vol. 21, no. 41, pp. 11748–11766, 2015.

[93] A. C. Kendall and A. Nicolaou, "Bioactive lipid mediators in skin inflammation and immunity," *Progress in Lipid Research*, vol. 52, no. 1, pp. 141–164, 2013.

[94] R. K. Sivamani, "Eicosanoids and keratinocytes in wound healing," *Advances in Wound Care*, vol. 3, no. 7, pp. 476–481, 2014.

[95] S. K. Jacobi, A. J. Moeser, B. A. Corl, R. J. Harrell, A. T. Blikslager, and J. Odle, "Dietary long-chain PUFA enhance acute repair of ischemia-injured intestine of suckling pigs," *The Journal of Nutrition*, vol. 142, no. 7, pp. 1266–1271, 2012.

[96] A. T. Blikslager, A. J. Moeser, J. L. Gookin, S. L. Jones, and J. Odle, "Restoration of barrier function in injured intestinal mucosa," *Physiological Reviews*, vol. 87, no. 2, pp. 545–564, 2007.

[97] K. Takeuchi, M. Tanigami, K. Amagase, A. Ochi, S. Okuda, and R. Hatazawa, "Endogenous prostaglandin E2 accelerates healing of indomethacin-induced small intestinal lesions through upregulation of vascular endothelial growth factor expression by activation of EP4 receptors," *Journal of Gastroenterology and Hepatology*, vol. 25, pp. S67–S74, 2010.

[98] Y. Naito, X. Ji, S. Tachibana et al., "Effects of arachidonic acid intake on inflammatory reactions in dextran sodium sulphate-induced colitis in rats," *British Journal of Nutrition*, vol. 114, no. 5, pp. 734–745, 2015.

[99] S. Dhall, D. S. Wijesinghe, Z. A. Karim et al., "Arachidonic acid-derived signaling lipids and functions in impaired healing," *Wound Repair and Regeneration*, vol. 23, no. 5, pp. 644–656, 2015.

[100] D. Panigrahy, B. T. Kalish, S. Huang et al., "Epoxyeicosanoids promote organ and tissue regeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 33, pp. 13528–33, 2013.

[101] N. S. Rossen, A. J. Hansen, C. Selhuber-Unkel, and L. B. Oddershede, "Arachidonic acid randomizes endothelial cell motion and regulates adhesion and migration," *PLoS One*, vol. 6, no. 9, article e25196, 2011.

[102] T. Tomita, K. Hosoda, J. Fujikura, N. Inagaki, and K. Nakao, "The G-protein-coupled long-chain fatty acid receptor GPR40 and glucose metabolism," *Frontiers in Endocrinology*, vol. 5, p. 152, 2014.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 2845352, 5 pages
https://doi.org/10.1155/2018/2845352

Research Article

Rooming-in Reduces Salivary Cortisol Level of Newborn

Giuseppe De Bernardo,[1] Marina Riccitelli,[2] Maurizio Giordano,[1] Fabrizio Proietti,[2] Desiree Sordino,[3] Mariangela Longini,[2] Giuseppe Buonocore,[2] and Serafina Perrone[2]

[1]*Department of Emergency, NICU AORN Santobono-Pausilipon, Naples, Italy*
[2]*Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy*
[3]*Neonatology and Obstetrics Nursing, C.G. Ruesch, Naples, Italy*

Correspondence should be addressed to Serafina Perrone; saraspv@yahoo.it

Received 23 November 2017; Revised 9 January 2018; Accepted 15 January 2018; Published 8 March 2018

Academic Editor: Francisco Rios

Background. Rooming-in practice improves breastfeeding and reduces newborn stress reactivity. When this modality is not available, partial rooming-in after birth can be considered. Salivary cortisol levels (SCLs) are considered reliable biomarkers to indicate stress. *Objective*. To test the hypothesis that rooming-in duration impacts neonatal stress response in hospitalized newborns. *Design/methods*. Forty term newborns, enrolled in the Neonatology and Obstetrics Nursing, C.G. Ruesch, Naples, Italy, were divided, according to the mother's choice, into the study (SG; $n = 20$) and control (CG; $n = 20$) groups if they received full (24 hs) or partial (14 hs) rooming-in care, respectively. Saliva samples were collected from all babies between 7:00 a.m. and 8:00 a.m. of the 3rd day of life by using oral swab. Salivary cortisol levels were measured using an enzyme immunoassay kit (Salimetrics LLC, PA, USA). *Results*. A statistically significant difference in the SCLs between SG and CG was found (median: 258 ng/dl versus 488.5 ng/dl; $p = 0.048$). *Conclusions*. Data support the practice of full rooming-in care compared with partial rooming-in. The rooming-in duration clearly reduces SCLs and likely neonatal stress. These lower SCLs may have long-term positive effects reducing the risk of metabolic syndrome, high blood pressure, and cognitive and behavioural changes.

1. Introduction

According to clinical managements, newborns are often exposed to painful and stressful situations. The concept of infant pain and effects of pain exposure during hospitalization of infants has progressed greatly in the past 25 years [1]. Grunau et al. and Miller and Ferriero investigated whether neonatal procedural pain/stress impacts the developing brain, discovering that greater exposure to procedural pain-related stress was associated with reduced development of white matter and subcortical gray matter [2, 3]. Therefore, the knowledge of socioemotional stress reactivity during the early period of neonatal life is crucial to understand the brain neurodevelopmental trajectory [4]. When the infant faces up to a stressful situation, the developing hypothalamic-pituitary-adrenal axis is activated: the hypothalamus secretes the corticotropin-releasing hormone, which starts the release of adrenocorticotropic hormone by the pituitary gland. This hormone induces, in the adrenal gland cortex, the secretion of glucocorticoids as cortisol, considered a stress indicator hormone since its plasma levels will be high as a result of the psychological and physical stress response [5]. Long-term side effects of high concentration of cortisol can result in insulin resistance, hyperlipidaemia, immunologic deficiencies, and destructive changes in the hippocampus [6]. Salivary cortisol has been reported as a useful biomarker of psychological stress and related mental or physical diseases [7]. Since Francis et al. in 1987 [8] showed a correlation between infants' plasma and saliva cortisol, only few studies investigated salivary cortisol levels (SCL) due to the complexity of methodology measurement. Morelius et al. published an easy method to collect and analyse cortisol concentration in small amount of saliva [9]. Some studies performed with this method detected patterns of high hormonal stress response to heel lance for infants aged between 34 and 41 weeks of gestation [10]. Infants aged

under three months showed increased cortisol levels from baseline in response to painful interventions (e.g., heel lance and inoculation) as well as routine handling interventions (e.g., physical examination, diaper changing, and removal from bath) [11]. Conversely, mother-infant proximity and interactions reduce infants' and mothers' stress during the early postpartum period. Rooming-in practice allows mothers and babies to stay together in the same room during the hospitalization time. Separate care consists in keeping the baby in the hospital nursery and bringing him to the mother only for breastfeeding. These two different approaches may influence mother-infant interaction and neonatal stress response [12].

We tested the hypothesis that full-time rooming-in (for 24 hs) is better than partial rooming-in (for 14 hs) to reduce neonatal stress response in hospitalized newborns.

2. Materials and Methods

2.1. Enrollment and Group Composition Participants. The study was carried out from January to September 2016, in Neonatology and Obstetrics Nursing, C.G. Ruesch, and Gynaecology and Obstetrics of the Villa Cinzia Hospital in Naples, Italy. Healthy newborns from families that were Italians for more than 3 generations were consecutively recruited while written informed consent was obtained from the families. Eligibility criteria for newborns included patients with gestational age > 37 weeks, 3 days of life, body weight > 2500 gr. Exclusion criteria were patients who underwent painful, stressful procedures, patients born from smoking mothers, mothers with maternal diabetes, placenta abruption, chronic pathologies or using drugs/alcohol during pregnancy, or infants with foetal growth restriction, foetal malformations, chromosomopathy, metabolic disorders, or clinical signs of maternal or foetal infection. Newborns' mothers were <35 years old and primiparous. They had a body mass index between 19 and 24. Infants were divided according to the mother's choice, into the study (SG; $n = 20$) and control (CG; $n = 20$) groups if they received rooming-in care for 24 hs or 14 hs (7:00 a.m.–9:00 p.m.), respectively. The control group (CG) was enrolled from January to June 2016 in Villa Cinzia Hospital and assisted in rooming-in care for 14 hours (7:00 a.m.–9:00 p.m.) as required by the mother. The study group (SG) was enrolled from June to September 2016 in Neonatology and Obstetrics Nursing, C.G. Ruesch, and treated with rooming-in care for 24 hours.

All babies received the same clinical management, the only difference was the rooming-in length. Both hospitals had 20 beds for newborns. Parents could observe clinical bedside round and hold meetings with physicians in the places for rooming-in at 9:00 a.m. in both hospitals. If necessary, families could hold meetings with paediatric nurses every 3 hours. Newborns were not subjected to painful procedures in the first 3 days of life, and during this period, parents could change the diaper, medicate the umbilicus, and breastfeed their children in both hospitals; only the wrapping was performed by paediatric nurses. The environment of the childbirth centres was strictly controlled; the temperature did not have to rise above 25°C, while the luminosity was set at 2000 lux and the noises did not get over 45 dB. The detection of the stress level of the newborns was performed by sampling saliva from 7:00 a.m. to 8:00 a.m. on the 3rd day of life by a paediatric nurse, who was the same throughout the study, to exclude interoperator sampling collecting bias. An oral swab was positioned in the oral cavity for 60–90 sec. to obtain 200–1000 microl. of saliva. The oral swabs were stored in a test tube (stored at a temperature < 20°C) and brought, after two weeks, to the analytical laboratory.

2.2. Instruments. During hospital stay of the newborns, one of the authors, aware of the study, recorded clinical features (nationality, parents' residence, gestational age, body weight at birth, days of life in a database (Excel 2007)).

Paediatric nurses who were on duty measured the body weight of the newborns with a seca 354 baby scale. The oral swabs used for this study were SalivaBio Infant's Swab (Salimetrics) while the test tubes were SalivaBio Storage Tube (Salimetrics). The correct collection of the salivary sample considered that patients did not have to eat 60 min before the sampling, the documentation of the oral injury, and vigorous physical activity, as well as, 10 min before the sampling, the cleaning of the oral mucosa with water to remove food leftovers. The cortisol levels in salivary samples were quantified with the Salimetrics Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics LLC, 101 Innovation Boulevard, Suite 302, State College, PA 16803). The method is based on a competitive immunoassay. Cortisol levels were measured in duplicate. Cortisol in standards and samples compete with cortisol conjugated to horseradish peroxidase for the antibody binding sites on a microtiter plate. Bound cortisol enzyme conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). The optical density is read on a standard plate reader at 450 nm. In brief, the thawed salivary samples were vortexed and centrifuge at 1500 ×g for 15 minutes to remove mucins ad debris. 25 μl of samples and standard solution with concentration ranging from 0.012 to 3 μg/dl were mixed in a pipette into the plate wells. After, 200 μl of enzyme conjugate cortisol diluted 1 : 1600 in assay diluent was added to all the wells. After an incubation of 1 hour at room temperature, the plate was washed four times with washing buffer. The enzymatic reaction was started by adding 200 μl of TMB Substrate Solution. Mixed on a plate rotator for 5 minutes at 500 rpm and incubated in the dark at room temperature for an additional 25 minutes. The reaction was stopped by adding 50 μl of stop solution and rapidly read at 450 nm with a plate reader. The cortisol levels were determinate by interpolating the optical density on the standard curve obtained with 4-parameter nonlinear regression curve fit. The intra-assay coefficient of variation was 4% at 2.07 μg/dl, and the interassay coefficient of variation was 3% at 1.99 μg/dl. The analytical sensitivity was 0.007 μg/dl.

2.3. Statistical Analysis. Analyses of the differences in clinical outcomes were performed by a statistician who was aware of the study aims using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Data with a normal

Table 1: Clinical characteristics of study population.

	Control group ($n = 20$)	Study group ($n = 20$)
	Partial rooming-in practice (14 hs)	Full rooming-in practice (24 hs)
Nationality	Italian	Italian
Parents' residence	Campania	Campania
Type of delivery	Elective caesarean section	Elective caesarean section
Maternal age (yrs)	32 ± 2	33 ± 1.5
Maternal BMI	22 ± 2	20 ± 1
Parity	0	0
Gestational age (wks)	38.31 ± 0.63	38.89 ± 1
Birth weight (g)	3072 ± 406	3294.5 ± 357
Days of life at the time of sampling	3	3
Salivary cortisol levels (ng/dl)	727.4 ± 797.18	323 ± 231.8

Data are expressed as mean ± SD. BMI: body mass index.

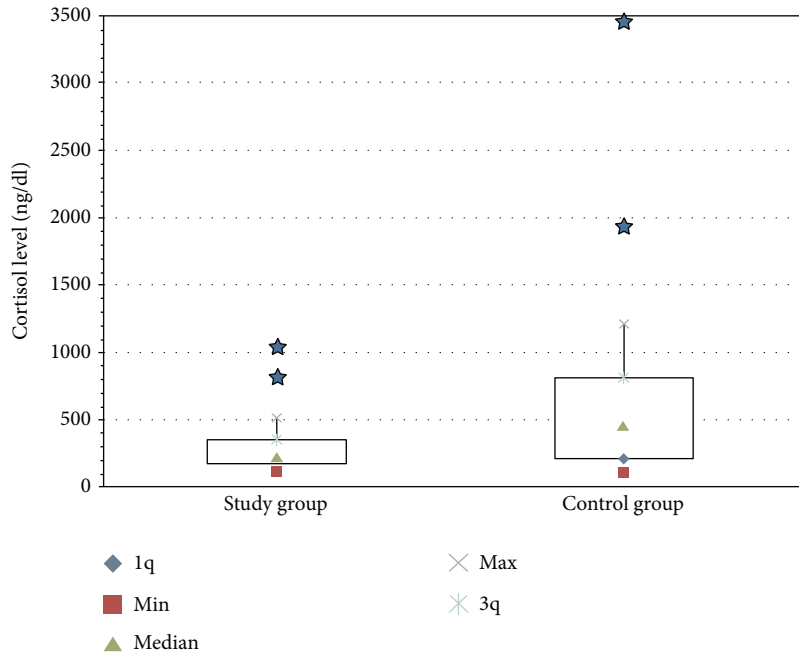


Figure 1: Salivar cortisol levels in the study group and the control group.

distribution were analysed using the Kolmogorov–Smirnov test. Homogeneity of data groups was assessed using an unpaired t-test while the different SCLs between the two groups were analysed by Mann–Whitney U test.

3. Results

A total of 20 newborns as control group and 20 newborns as study group were recruited. Preliminary data analysis validated the homogeneity of the samples. As showed in Table 1, all newborns and their families were Italian and resided in Campania. There was no difference between the case and study groups for the following parameters: body weight at birth, gestational age, and type of delivery. The sampling of saliva obtained on the 3rd day of life revealed that SCLs were lower in the study group than in the control group (Table 1).

There was a statistically significant difference in the SCLs ($p = 0.048$) between the study group (median: 258 ng/dl) and the control group (median: 488.5 ng/dl) (Figure 1).

4. Discussion

Keeping the mother and infant together (rooming-in) or separating them after birth are both traditional practices seen in many cultures. Mother-infant proximity during after birth may influence neonatal stress response: this time, shortly after birth represents a sensitive or critical period for programming future physiology and behaviour [13, 14]. According to mammalian neuroscience, rooming-in practice allows the intimate contact that evokes neurobehaviours ensuring

fulfilment of basic biological needs [15]. Als suggested that the neurodevelopmental subsystem interaction between the neonate's internal functioning, the environment, and caregivers was the foundation of the neonatal developmental process: if a lack of equilibrium occurs within one subsystem, all other subsystems are affected [16]. Recently, rooming-in has been encouraged also for its benefits both for infants at risk of neonatal abstinence syndrome [17] and for implementation of the duration of breastfeeding [12]. The present paper aims to assess if the presence of the family, using different modalities of rooming-in care, reduces recovery stress by measuring SCLs in hospitalized newborns.

Usually, the procedures practiced are continuous rooming in (for 24 hs), which seems to be preferable, or partial. In literature are no evidence-based reports on which of these two practices is the best. Our work has the purpose to light up this lack of information about these procedures, to minimize pain, and to identify which interventions in hospital reduce stress in infants and to prioritize this intervention in everyday care.

Stress can be defined as a reaction to any real or perceived threat that leads to a disorder in the dynamic equilibrium between the organism and its environment [18, 19]. Newborns are often living and developing in an environment that can be stressful in many ways, starting from the time of birth. An organism's capacity to respond to stressors and maintain homeostasis is regulated by the hypothalamic-pituitary-adrenal (HPA) axis through the cortisol production. Since the beginning of the second trimester, the HPA axis functions and organizes in response to the environment [20]. Different life events, including trauma events, have been shown to influence the HPA, that is, cortisol secretion; therefore, cortisol is considered a major biomarker of stress, also among infants [21]. Recently, it has been showed that prenatal exposure to maternal stress could affect the subsequent cortisol reactivity of the infant [22]. Consequently, we excluded from our study all infants born from a nonphysiological pregnancy or from mothers with chronic pathologies or using drugs/smoke/alcohol. As Taylor et al. affirmed, also the mode of delivery could influence neonatal stress response: they found a lower cortisol reactivity during a procedure among infants delivered by caesarean section compared to those vaginally born [23]. Relying on this, we considered appropriate selecting only newborns born from a caesarean section, to exclude another source of external stress.

Secretion of the cortisol is essential for lung maturation at birth; hence, there is a corresponding increase in cortisol levels with increasing gestational age [21, 24]. Our population consists of full-term newborns, to equalize basal levels of cortisol in all our neonates.

Recently, some authors presented cortisol reference intervals for infants during the first twelve months of life, but only one month after birth, full-term healthy infants develop a circadian rhythm of salivary cortisol with increasing morning levels and corresponding decreasing evening levels [25]. The detection of the stress level in newborns was performed during the morning, in all patients at the same time. There are no cortisol reference values during the first hours of life; thus, it has been possible to establish which of the practice between continuous and partial rooming-in was less stressful only by comparing the cortisol levels between the two groups. Since 1987, cortisol in saliva has been used as a valid method to assess adrenocortical activity in newborns and their response to stressful stimuli [8]: it is an easy and painless method to collect cortisol samples, to analyse neonatal stress. Nowadays, it is possible to collect enough saliva without disturbing the infant, for instance, during sensitive situations, such as mother-infant interaction [6].

In this pilot study, performed in a limited number of newborns, we observed a statistically significant difference between the SG and CG groups in SCLs that strongly suggested that full-time rooming-in is a better practice than partial rooming care in reducing neonatal stress response.

Our data strongly support the practice of rooming-in for 24 hours compared with partial rooming-in. We hypothesize that lower SCLs may have long-term positive effects in reducing the risk of metabolic syndrome, high blood pressure, and cognitive and behavioural changes. The results of this study pave the way for longer RCTs to confirm these preliminary results. Moreover, long-term follow-up studies are needed to verify the long-term effects on brain neurodevelopment.

Disclosure

The paper was represented as a poster in Conference: PAS San Francisco 2017.

Conflicts of Interest

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

Acknowledgments

The authors thank EURAIBI (Europe Against Infant Brain Injury) foundation for its partial grants.

References

[1] R. E. Grunau, "Neonatal pain in very preterm infants: long-term effects on brain, neurodevelopment and pain reactivity," *Rambam Maimonides Medical Journal*, vol. 4, no. 4, article e0025, 2013.

[2] R. E. Grunau, L. Holsti, D. W. Haley et al., "Neonatal procedural pain exposure predicts lower cortisol and behavioral reactivity in preterm infants in the NICU," *Pain*, vol. 113, no. 3, pp. 293–300, 2005.

[3] S. P. Miller and D. M. Ferriero, "From selective vulnerability to connectivity: insights from newborn brain imaging," *Trends in Neurosciences*, vol. 32, no. 9, pp. 496–505, 2009.

[4] L. Provenzi, L. Giusti, M. Fumagalli et al., "Pain-related stress in the Neonatal Intensive Care Unit and salivary cortisol reactivity to socio-emotional stress in 3-month-old very preterm infants," *Psychoneuroendocrinology*, vol. 72, pp. 161–165, 2016.

[5] D. M. Cabral, S. R. Antonini, R. J. Custódio, C. E. Martinelli, and C. A. B. da Silva, "Measurement of salivary cortisol as a marker of stress in newborns in a neonatal intensive care unit," *Hormone Research in Paediatrics*, vol. 79, no. 6, pp. 373–378, 2013.

[6] E. Mörelius, E. Theodorsson, and N. Nelson, "Salivary cortisol and mood and pain profiles during skin-to-skin care for an unselected group of mothers and infants in neonatal intensive care," *Pediatrics*, vol. 116, no. 5, pp. 1105–1113, 2005.

[7] D. H. Hellhammer, S. Wüst, and B. M. Kudielka, "Salivary cortisol as a biomarker in stress research," *Psychoneuroendocrinology*, vol. 34, no. 2, pp. 163–171, 2009.

[8] S. J. Francis, R. F. Walker, D. Riad-Fahmy, D. Hughes, J. F. Murphy, and O. P. Gray, "Assessment of adrenocortical activity in term newborn infants using salivary cortisol determinations," *The Journal of Pediatrics*, vol. 111, no. 1, pp. 129–133, 1987.

[9] E. Morelius, N. Nelson, and E. Theodorsson, "Salivary cortisol and administration of concentrated oral glucose in newborn infants: improved detection limit and smaller sample volumes without glucose interference," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 64, no. 2, pp. 113–118, 2004.

[10] T. C. Castral, F. Warnock, C. B. Dos Santos et al., "Maternal mood and concordant maternal and infant salivary cortisol during heel lance while in kangaroo care," *European Journal of Pain*, vol. 19, no. 3, pp. 429–438, 2015.

[11] M. R. Gunnar, N. M. Talge, A. Herrera et al., "Stressor paradigms in developmental studies: what does and does not work to produce mean increases in salivary cortisol," *Psychoneuroendocrinology*, vol. 34, no. 7, pp. 953–967, 2009.

[12] S. H. Jaafar, J. J. Ho, and K. S. Lee, "Rooming-in for new mother and infant versus separate care for increasing the duration of breastfeeding," *Cochrane Database of Systematic Reviews*, vol. 8, 2016.

[13] N. Császár and N. Bókkonac, "Mother-newborn separation at birth in hospitals: a possible risk for neurodevelopmental disorders?," *Neuroscience & Biobehavioral Reviews*, vol. 84, pp. 337–351, 2018.

[14] J. Kaufman, P. M. Plotsky, C. B. Nemeroff, and D. S. Charney, "Effects of early adverse experiences on brain structure and function: clinical implications," *Biological Psychiatry*, vol. 48, no. 8, pp. 778–790, 2000.

[15] E. R. Moore, N. Bergman, G. C. Anderson, N. Medley, and Cochrane Pregnancy and Childbirth Group, "Early skin-to-skin contact for mothers and their healthy newborn infants," *Cochrane Database of Systematic Reviews*, vol. 11, 2016.

[16] H. Als, "A synactive model of neonatal behavioral organization: framework for the assessment and support of the neurobehavioral development of the premature infant and his parents in the environment of the neonatal intensive care unit. In JK Sweeney (ed.), The high risk neonate: developmental therapy perspectives," *Physical & Occupational Therapy in Pediatrics*, vol. 6, no. 3, pp. 3–53, 1986.

[17] S. McKnight, H. Coo, G. Davies et al., "Rooming-in for infants at risk of neonatal abstinence syndrome," *American Journal of Perinatology*, vol. 33, no. 5, pp. 495–501, 2016.

[18] S. Levine, "Developmental determinants of sensitivity and resistance to stress," *Psychoneuroendocrinology*, vol. 30, no. 10, pp. 939–946, 2005.

[19] R. Gitau, N. M. Fisk, and V. Glover, "Human fetal and maternal corticotrophin releasing hormone responses to acute stress," *Archives of Disease in Childhood - Fetal and Neonatal Edition*, vol. 89, no. 1, pp. F29–F32, 2004.

[20] R. J. Bolt, M. M. Van Weissenbruch, C. Popp-Snijders, F. G. J. Sweep, H. N. Lafeber, and H. A. Delemarre-van de Waal, "Maturity of the adrenal cortex in very preterm infants is related to gestational age," *Pediatric Research*, vol. 52, no. 3, pp. 405–410, 2002.

[21] B. E. Murphy, "Cortisol and cortisone in human fetal development," *Journal of Steroid Biochemistry*, vol. 11, no. 1, pp. 509–513, 1979.

[22] C. Schuller, N. Känel, O. Müller et al., "Stress and pain response of neonates after spontaneous birth and vacuum-assisted and cesarean delivery," *American Journal of Obstetrics and Gynecology*, vol. 207, no. 5, p. 416, 2012.

[23] A. Taylor, N. M. Fisk, and V. Glover, "Mode of delivery and subsequent stress response," *Lancet*, vol. 355, no. 9198, p. 120, 2000.

[24] B. Stevens, J. Yamada, A. Ohlsson et al., "Sucrose for analgesia in newborn infants undergoing painful procedures," *Cochrane Database of Systematic Reviews*, vol. 7, 2004.

[25] K. Ivars, N. Nelson, and A. Theodorsson, "Development of salivary cortisol circadian rhythm and reference intervals in full-term infants," *PLoS One*, vol. 10, no. 6, article e0129502, 2015.

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 1574928, 9 pages
https://doi.org/10.1155/2018/1574928

Research Article

Neutrophils Release Metalloproteinases during Adhesion in the Presence of Insulin, but Cathepsin G in the Presence of Glucagon

Natalia V. Fedorova,[1] Alexander L. Ksenofontov,[1] Marina V. Serebryakova,[1] Vladimir I. Stadnichuk,[2] Tatjana V. Gaponova,[3] Ludmila A. Baratova,[1] Galina F. Sud'ina,[1] and Svetlana I. Galkina[1]

[1]*A. N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119991, Russia*
[2]*Physical Department, Lomonosov Moscow State University, Moscow 119991, Russia*
[3]*FGBU Hematology Research Center, Russian Federation Ministry of Public Health, Moscow 125167, Russia*

Correspondence should be addressed to Svetlana I. Galkina; galkina@genebee.msu.su

Received 27 October 2017; Accepted 19 December 2017; Published 14 February 2018

Academic Editor: Joilson O. Martins

In patients with reperfusion after ischemia and early development of diabetes, neutrophils can attach to blood vessel walls and release their aggressive bactericide agents, which damage the vascular walls. Insulin and 17β-estradiol (E2) relieve the vascular complications observed in metabolic disorders. In contrast, glucagon plays an essential role in the pathophysiology of diabetes. We studied the effect of hormones on neutrophil secretion during adhesion to fibronectin. Amino acid analysis revealed that proteins secreted by neutrophils are characterized by a stable amino acid profile enriched with glutamate, leucine, lysine, and arginine. The total amount of secreted proteins defined as the sum of detected amino acids was increased in the presence of insulin and reduced in the presence of glucagon. E2 did not affect the amount of protein secretion. Proteome analysis showed that in the presence of insulin and E2, neutrophils secreted metalloproteinases MMP-9 and MMP-8 playing a key role in modulation of the extracellular matrix. In contrast, glucagon induced the secretion of cathepsin G, a key bactericide protease of neutrophils. Cathepsin G can promote the development of vascular complications because of its proinflammatory activity and ability to stimulate neutrophil adhesion via the proteolysis of surface receptors.

1. Introduction

Neutrophils, or polymorphonuclear leukocytes, play an important role in host defense against bacterial or fungal pathogens due to their ability to penetrate into infected tissue and phagocytose and kill microbes. To kill microbes, bactericide agents that are localized in the neutrophil secretory granules are released into the formed phagosomes and outwards [1]. Bactericides, being released outside, cause the destruction of surrounding host tissues and the development of inflammation.

Neutrophils can release their bactericides to the outside also in the absence of infection. In patients with certain metabolic disorders, neutrophils that normally roll in the bloodstream can attach to the walls of the blood vessels and secrete their aggressive products. Adherence of neutrophils to endothelial cells is an early and requisite event in ischemia/reperfusion-induced inflammatory injury [2, 3]. The authors of many modern studies consider the integrin-dependent adhesion of neutrophils and associated secretion of neutrophil bactericidal agents to be the cause of the early stages of retinopathy [4–6] or nephropathy [7, 8] in patients with diabetes.

Insulin produced by β-cells of the pancreatic Langerhans islets plays a key role in maintaining vascular health. Clinical evidence indicates that intensive insulin therapy protects the

endothelium in cardiovascular diseases, during critical illness [9, 10], and reduces major cardiovascular events in diabetics [11]. In particular, insulin attenuates neutrophil accumulation in myocardial ischemia/reperfusion rabbit hearts and inhibits neutrophil adhesion to cultured endothelial cells subjected to simulated ischemia/reperfusion [12].

Glucagon produced by α-cells of the Langerhans islets is a physiological antagonist of insulin. In healthy patients, production of glucagon is inhibited by insulin at a high level of glucose in the blood. In patients with diabetes, an oral glucose load induced a paradoxical rise in glucagon secretion. Absolute or relative hyperglucagonaemia has been recognized for years in all experimental or clinical forms of diabetes [13]. Glucagon seems to play an essential role in the pathophysiology of diabetes. Knockout of the glucagon receptor or administration of a monoclonal specific glucagon receptor antibody makes insulin-deficient type 1 diabetic rodents thrive without insulin [14–16]. However, the mode of glucagon action remains to be elucidated. Despite the active search, a glucagon antagonist, which can be effective in treating diabetes, has not yet been found. In the treatment of type 2 diabetes, there are largely used drugs that act, in part, by inhibiting the secretion or action of glucagon, such as glucagon-like peptide-1 (GLP-1) and GLP-1 receptor agonists, dipeptidyl peptidase-4 inhibitors (DPP-4), and metformin [17–19]. Inhibition of glucagon secretion and stimulation of insulin release equally contribute to a decrease in glucose with respect to the action of GLP-1 [17].

The action of GLP-1 is transient, since the peptide is rapidly inactivated by DPP-4. DPP-4 inhibitors are used to prolong the action of GLP-1. Metformin has a glucose-lowering effect, inhibiting hepatic gluconeogenesis and counteracting the action of glucagon [19]. GLP-1 receptor agonists, DPP-4 inhibitors, and metformin have beneficial effects on cardiovascular complications in patients with type 2 diabetes, as well as in patients with reperfusion after ischemia [20–22]. Prevention of vascular adhesion of monocytes contributes to the cardiovascular protective effect of GLP-1 analogs [23, 24]. Metformin also could reduce vascular complication of diabetes by decreasing leukocyte oxidative stress and undermining adhesion molecule levels and leukocyte-endothelium interactions [25].

Female hormones play an important role in maintaining vascular health, as evidenced by an increase in the incidence of cardiovascular disease in women after menopause [26]. Pretreatment with estrogen prior to myocardial ischemia and reperfusion causes a decrease in neutrophil infiltration into the irreversibly injured myocardium [27]. Traditionally, estrogen acts via classical nuclear estrogen receptors. In addition to this "genomic" signalling pathway, a "rapid, nonnuclear" signalling pathway mediated by cell membrane-associated estrogen receptors also has been recognized. This nonnuclear signalling appears to be critical for the protective effects of estrogen in the cardiovascular system [28–30].

In this work, we studied the effect of insulin, glucagon, and 17β-estradiol (E2) on secretion of human neutrophils upon adhesion to fibronectin. We used scanning electron microscopy to study the morphology of the attached neutrophils, as well as amino acid analysis and mass spectrometry to study the amount and composition of secreted proteins.

2. Material and Methods

2.1. Materials. Ficoll-Paque for neutrophil isolation was obtained from Pharmacia (Uppsala, Sweden). Fibronectin was from Calbiochem (La Jolla, USA). Bicarbonate-free Hank's solution, Ca^{+}-free Dulbecco PBS, insulin, glucagon, and 17β-estradiol were purchased from Sigma. Trypsin was from Promega, and Coomassie Brilliant Blue G-250 was from Serva.

2.2. Neutrophil Isolation. Neutrophils were isolated from the blood of healthy volunteers, who did not take any medication for 2 weeks, using experimental procedures approved by the Ethics Committee of A. N. Belozersky Institute. Erythrocytes were precipitated with 3% dextran T-500 at room temperature. Neutrophils were isolated from the plasma by centrifugation via Ficoll-Paque (density 1.077 g/mL). The remaining red blood cells were eliminated by hypotonic lysis. After washing, neutrophils (purity 96-97%, viability 98-99%) were stored before the experiment in Dulbecco's PBS containing 1 mg/mL glucose (without $CaCl_2$).

2.3. Adhesion of Neutrophils to Fibronectin-Coated Cover Slips. For scanning electron microscopy (SEM), neutrophils were plated onto fibronectin-coated glass coverslips. The coverslips were coated with fibronectin for 2 hours by incubation in Hank's solution containing 5 μg/mL of fibronectin at room temperature and washed with buffer. Neutrophils adhered to the fibronectin-coated coverslips (3×10^6 cells in 2 mL per well) for a 20 min incubation in Hank's solution containing 10 mM HEPES (pH 7.35) at 37°C. Insulin, glucagon, or 17β-estradiol (0.1 μM) was added to the cells before plating. After that, the cells were fixed for SEM.

2.4. Sampling of the Extracellular Medium to Determine the Amino Acid and Protein Composition of Neutrophil Secretion. Six-well culture plates were coated with fibronectin for 2 hours of incubation in Hank's solution containing 5 μg/mL fibronectin at room temperature and washed. Neutrophils adhered to the protein-coated wells (3×10^6 cells in 1.3 mL per well) in Hank's solution containing 10 mM HEPES (pH 7.35) for 20 minutes at 37°C. Insulin (0.1 μM), glucagon (0.1 μM), or 17β-estradiol (0.1 μM) was added to the cells prior to plating. After the incubation, the extracellular medium was sampled from the control and hormone-treated cells. Aliquots were collected from six similar wells and combined. Inhibitors of metalloproteinase, serine and cysteine proteinases, and myeloperoxidase (EDTA, 5 mM; PMSF, 200 μM; E64, 10 μM; and sodium azide, 0.025%, resp.) were immediately added to the samples. Unattached neutrophils were removed by centrifugation at 2000g.

2.5. Extraction, Concentration, and Hydrolysis of Proteins. Proteins from the collected EM samples were extracted with an equal volume of a chloroform-methanol mixture (2 : 1, v/v). The mixture was vortexed for 1-2 minutes and

stirred in a shaker at 4°C for 30 minutes. The chloroform and methanol phases were then separated by centrifugation at 11,000g for 20 minutes, and the solvents were evaporated. The chloroform phase contained almost all of the proteins detected. The water-methanol fractions contained trace amounts of protein. Concentrated extracts of chloroform phases were subjected to electrophoresis. For amino acid analysis, the same extracts were prehydrolysed, as described by Tsugita and Scheffler [31].

2.6. Quantitative Determination of Amino Acids with Ninhydrin. The amino acid profile of protein hydrolysates was determined by an L-8800 amino acid analyser (Hitachi, Tokyo, Japan) with an electronic heating bath and two single-channel colorimeters according to the manufacturer's user manual (Hitachi High-Technologies Corporation, Japan, 1998). The protein hydrolysates were separated on a 2622SC-PF ion-exchange column (Hitachi Ltd., P/N 855-4507, 4, 6 × 60 mm) by a step gradient of four Li-citrate buffers at a flow rate of 0.35 mL/min and a thermostating column at 30–70°C. Postcolumn derivatization (136°C, flow rate 0.35 mL/min) was performed using a mix of equal volumes of ninhydrin buffer R2 and ninhydrin solution R1 (Wako Pure Chemical Industries, P/N 298-69601). Colored products were detected by measuring the absorbance at 570 nm for all amino acids except proline and at 440 nm for proline. Data were processed using MultiChrom for Windows software (Ampersand Ltd., Moscow, Russia).

The total amount of proteins released by control cells was defined as the sum of detected amino acids (Table 1). Insulin and glucagon are protein hormones with a molecular mass of 5800 and 3482, respectively. When used at 0.1 μM, they contribute 0.58 or 0.35 μg of protein per millilitre. Bearing in mind that the total volume of each sample is 7.8 mL (1.3 mL × 6), we subtracted 4.5 or 2.7 μg, respectively, from the total amount of proteins secreted by insulin- or glucagon-treated cells.

2.7. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Protein separation was performed using one-dimensional sodium dodecyl sulfate electrophoresis on a 15% polyacrylamide gel under nonreducing conditions in the Mini-PROTEAN 3 Cell (Bio-Rad) [32]. Prior to electrophoresis, aliquots of the preparations were boiled for 3 minutes in lysis buffer (Tris-HCl 30 mM, pH 6.8; SDS 1%; urea 3 M; glycerin 10%; bromophenol blue 0.02%). Gels were stained with Coomassie Brilliant Blue G-250 0.22% (Serva).

2.8. Mass Spectrometry Identification of Proteins and Preparation of Samples. A MALDI-time of flight (ToF)-ToF mass spectrometer (Ultraflex II Bruker, Germany) equipped with a neodymium-doped (Nd) laser was used for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) analysis of proteins. Proteins separated by electrophoresis were subjected to trypsin hydrolysis directly in the gel. To this end, after electrophoresis, 1 × 1 mm slices of gel were cut from each Coomassie-stained protein band. Gel pieces were washed twice with 100 μL acetonitrile 40% in NH_4HCO_3 100 mM (pH 7.5) for 30 min at 37°C, dehydrated with 100 μL acetonitrile, and air-dried. Then, they were incubated with 4 μL modified trypsin 12 μg/mL (Promega) in NH_4HCO_3 50 mM for 6 h at 37°C. The resulting peptides were recovered through incubation with 6 μL trifluoroacetic acid solution 0.5% in acetonitrile 10% for 30 min. For mass spectrometric analysis, sample aliquots (1 μL) were mixed on a steel target with 0.3 μL 2,5-dihydroxybenzoic acid (20 mg/mL in acetonitrile 20% and trifluoroacetic acid 0.5%) and air-dried at room temperature. The $[MH]^+$ molecular ions were measured in reflector mode; the accuracy of mass peak measurement was within 0.005%. Identification of proteins was carried out by a peptide fingerprint search using Mascot software (http://www.matrixscience.com) through the Unipro+ (SwissPro+) mammalian protein database with the indicated accuracy. The search allowed for possible oxidation of methionine by environmental oxygen and modification of cysteine with acrylamide, and where a score was >71, protein matches were considered significant ($p < 0.05$).

Table 1: Effect of hormones on the amount of protein secreted by neutrophils in adhesion to fibronectin.

Treatment	Average amount of secreted proteins, $\mu g/18 \times 10^6$ cells
Control	64.6 ± 1.8
E2	62.5 ± 3.2
Insulin	76.1 ± 2.0*
Glucagon	50.0 ± 1.9*

Proteins were extracted from extracellular medium samples collected from neutrophils that were attached to fibronectin during a 20 min incubation in the presence of 0.1 μM E2, insulin, or glucagon. After acid hydrolysis, the protein hydrolysates were subjected to amino acid analysis. The amount of secreted proteins was determined as the sum of the amounts of the detected amino acids. *$p < 0.05$ when compared to the control value.

2.9. Scanning Electron Microscopy Technique. Neutrophils that were attached to fibronectin were fixed in 2.5% glutaraldehyde in Hanks buffer, which did not contain Ca^{2+} or Mg^{2+} ions, but contained inhibitors of metalloproteinases and serine proteases (5 mM EDTA and 0.5 mM PMSF, resp.) and 10 mM HEPES at pH 7.3. The cells were additionally fixed with 1% solution of osmium tetroxide in 0.1 M sodium cacodylate containing 0.1 M sucrose at pH 7.3. The samples were then dehydrated in an acetone series (10–100%) and dried at a critical point with liquid CO_2 as the transition liquid in the Balzers apparatus. The samples were sputter-coated with gold/palladium and observed at 15 kV using a Camscan S-2 scanning electron microscope.

3. Results and Discussion

3.1. Effect of Insulin, E2, and Glucagon on the Morphology of Human Neutrophils Attached to a Fibronectin-Coated Substrate. The adhesion of resting neutrophils (control neutrophils) to a glass or polystyrene itself leads to cell activation [33]. We studied the secretion of neutrophils in the process of adhesion to substrates coated with fibronectin, the extracellular matrix protein, since neutrophils exhibit

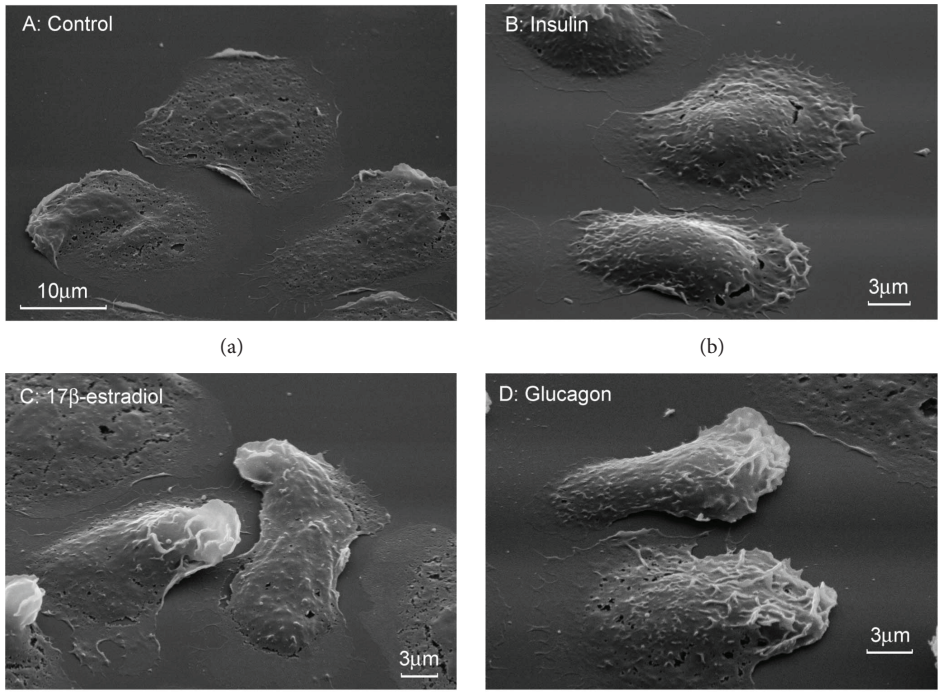


Figure 1: Effect of hormones on the morphology of human neutrophils attached to fibronectin. Scanning electron microscopy images of neutrophils that were attached to fibronectin for 20 min in control conditions (a) or in the presence of 0.1 μM insulin (b), 0.1 μM E2 (c), or 0.1 μM glucagon (d). Pictures represent typical images observed in two independent experiments.

only a priming activation when adhered to fibronectin. We compared the morphology of neutrophils that were attached to fibronectin-coated substrata in the presence 0.1 μM of insulin, glucagon, or E2. Scanning electron microscopy showed that the control cells and insulin-, glucagon-, or E2-treated cells were attached and spread on fibronectin-coated substrates (Figures 1(a)–1(d)). At the concentration used in the experiment, the hormones contribute only minor features to the morphology of the cells.

3.2. Effect of Insulin, Glucagon, and E2 on the Amount and Amino Acid Composition of Proteins Released by Neutrophils in Adhesion to Fibronectin. Neutrophils secrete their products via a variety of the mechanisms, including fusion of secretory granules with the plasma membrane [34] and shedding of membrane vesicles (ectosomes) from the plasma membrane [35–39]. Secreted proteins enter the extracellular medium as separate molecules or as part of membrane structures. To analyse all secreted proteins, we extracted proteins from the extracellular medium using a chloroform-methanol mixture. Earlier, we were convinced that practically all proteins are in the chloroform fraction [40]. After evaporation of the solvent, the proteins in the chloroform fraction were separated by electrophoresis or subjected to acid hydrolysis and subsequent amino acid analysis.

We used amino acid analysis to determine the total amount of protein secreted by neutrophils during adhesion to fibronectin under various conditions. After acid hydrolysis, the quantitative content of the individual amino acids in the protein hydrolysate was determined using an amino acid analyser (Figure 2).

In the process of acid hydrolysis, tryptophan was destroyed, and glutamine and asparagine were converted into glutamic and aspartic acid, respectively. Therefore, tryptophan is absent from the histogram, and the columns corresponding to glutamic acid and aspartic acid represent the sum of asparagine and aspartic acid or glutamine and glutamic acid, respectively. Amino acid analysis revealed a stable amino acid profile of proteins released by neutrophils that were attached to fibronectin under control conditions. The protein hydrolysate was enriched with serine, glutamate/glutamine, leucine, lysine, and arginine (Figure 2(a)). Summarizing the number of all detected amino acids, we determined the total amount of protein secreted by the cells under different experimental conditions (Table 1). The amount of secreted proteins increased by 18% in the presence of insulin but reduced in the presence of glucagon. The female hormone E2 had little effect on the amount of protein secreted by neutrophils.

A comparison of the percentage of individual amino acids in hydrolysates of proteins showed that the amino acid profile of protein secretion was generally retained for neutrophils that were attached to fibronectin in the presence of insulin, E2, or glucagon (Figure 2(b)). The amino acid profile of secretion products can serve as a type of "fingerprint" of human neutrophil secretion.

3.3. Effect of E2 on the Protein Composition of Neutrophil Secretion in Adhesion to Fibronectin. To determine which

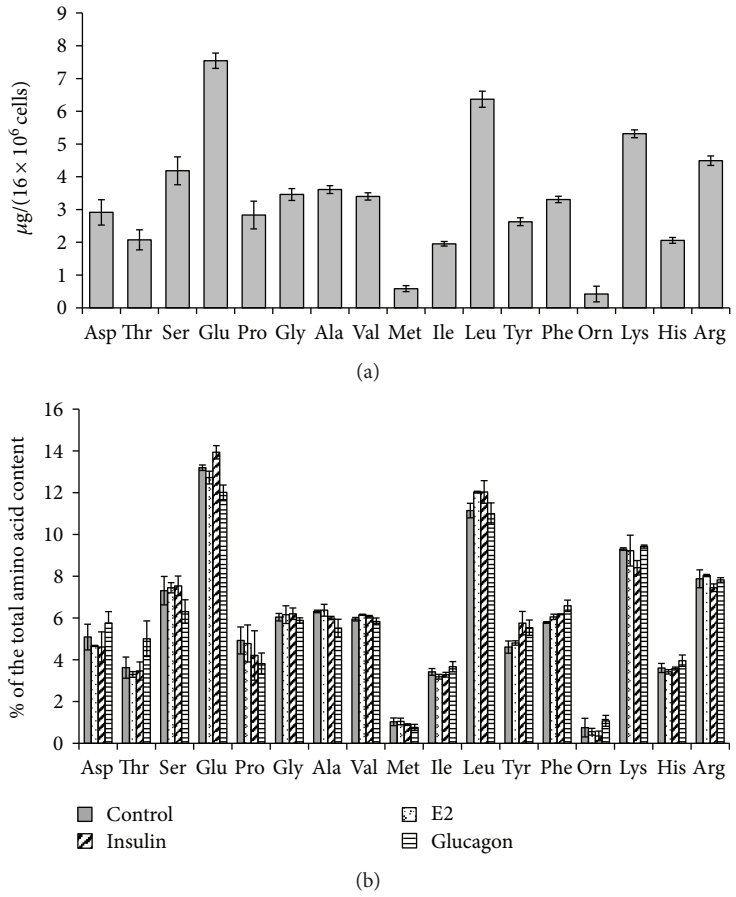


Figure 2: Effect of hormones on the amino acid composition of proteins secreted by neutrophils in adhesion to fibronectin. Human neutrophils were attached to a substrate coated with fibronectin for 20 min under control conditions or in the presence of 0.1 μM E2, insulin, or glucagon. The proteins were extracted from the extracellular medium, concentrated, and after acid hydrolysis subjected to amino acid analysis. (a) The amount of detected amino acids in the hydrolysate of proteins secreted by control neutrophils (mean ± SEM) is presented. (b) Comparison of the percentage of individual amino acids in hydrolysates of proteins secreted by neutrophils under control conditions and in the presence of hormones. Amino acid profiles were obtained by summing the results of three independent experiments.

proteins are secreted by neutrophils under the action of hormones, protein extracts of extracellular media after removal of chloroform were separated by electrophoresis (Figure 3). Identification of proteins represented by separate bands in the gel was carried out by mass spectrometry after trypsin hydrolysis directly in the gel (Table 2).

Previously, we have shown that control neutrophils during 20 min of adhesion to fibronectin secreted lactoferrin (LF), neutrophil gelatinase-associated lipocalin (NGAL), lysozyme, and myeloperoxidase (MPO), as well as albumin and cytosolic S100A8 and S100A9 proteins [40, 41]. Neutrophils contain secretory granules of four types [1]. The composition of granules of different types largely overlaps [42]. Nevertheless, LF and NGAL are considered components of the secondary granules, MPO is contained mainly in the primary granules, and albumin belongs to secretory vesicles.

When neutrophils adhere to fibronectin in the presence of E2, they release the same proteins as the control cells (LF, albumin, NGAL, S100A8, and S100A9) and, in addition, metalloproteinase 9 (MMP-9) (Figure 3(a), Table 2). MMP-9 is localized presumably in the tertiary or gelatinase granules of neutrophils [43]. Metalloproteinases are involved in the adhesion and migration of neutrophils because of their ability to modulate the extracellular matrix and remove the barriers of the basement membranes [44]. The balance between MMPs and their tissue inhibitors (TIMPs) tightly regulates physiological and pathological processes characterized by the degradation and accumulation of the extracellular matrix [45]. We assume that the ability of E2 to initiate the secretion of MMP-9 in adherent neutrophils can alter the result of the interaction of neutrophils with the walls of the blood vessel and contribute to the protective effect of E2 in metabolic diseases.

3.4. Effect of Insulin on the Protein Composition of Neutrophil Secretion in Adhesion to Fibronectin. Neutrophils that adhered to fibronectin in the presence of insulin secreted the same proteins as the control cells and, in addition, MMP-9 and matrix metalloproteinase 8 (MMP-8) (Figure 3(b); Table 2). MMP-8, or neutrophil collagenase, is involved in degradation of all structural components of extracellular matrix and plays a crucial role in many physiological processes including inflammatory and cardiovascular

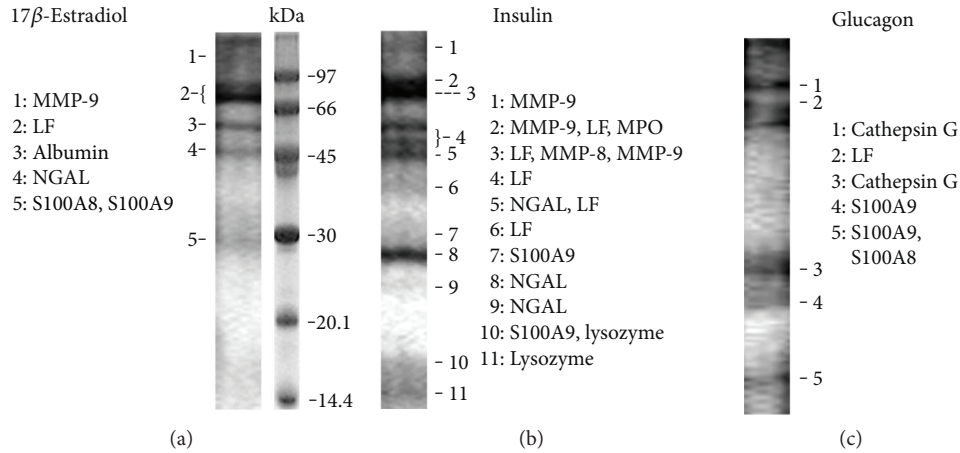


Figure 3: SDS-PAGE separation of proteins secreted by neutrophils upon adhesion to fibronectin in the presence of hormones. Human neutrophils were attached to fibronectin-coated substrata for 20 min in the presence of 0.1 μM E2 (a), 0.1 μM insulin (b), or 0.1 μM glucagon (c). Samples of extracellular medium were collected, and proteins were extracted and subjected to 15% SDS-PAGE. Pictures represent typical protein profiles observed in the three independent experiments for each hormone.

Table 2: List of proteins secreted by neutrophils in adherence to fibronectin in the presence of hormones.

	Protein name				Peptides matched/total	Coverage, %	MOWSE score
	Control	Insulin	E2	Glucagon			
MMP-9_HUMAN		MMP-9	+		18/85	25	240
MMP-8_HUMAN		MMP-8			15/85	31	240
CATG_HUMAN				Cath. G	15/77	40	75
PERM_HUMAN	+	MPO			17/85	21	119
TRFL_HUMAN	+	LF	+	+	29/85	43	240
ALBU_HUMAN	+		Albumin		15/28	24	130
NGAL_HUMAN	+	NGAL	+		7/15	42	105
LYSC_HUMAN	+	Lysozyme*	+			31	98
S10A9_HUMAN	+	S100-A9	+	+	9/19	48	147
S10A8_HUMAN	+		S100-A8	+	5/30	44	84

Neutrophils were attached to fibronectin for 20 minutes under control conditions (marked +), or in the presence of 0.1 μM insulin, 0.1 μM E2, or 0.1 μg glucagon. Mass spectrometric analysis data were taken from experiments with insulin. Analogous proteins that were identified in control experiments or in experiments with E2 or glucagon are marked (+). Mass spectrometric data for cathepsin G are taken from experiments with glucagon, and those for albumin and S100A8 from experiments with E2. Proteins were separated by SDS-PAGE and identified by mass spectrometric analysis. *Protein was identified by MSMS analysis. Proteins identified in two or three analogous experiments were included in the list.

disorders [46]. Since many inflammatory processes are accompanied by increased levels of MMP-8, it is assumed that the enzyme contributes to the development of inflammation. However, modern research refutes this view. The emergence of MMP-8-deficient mice resulted in the publication of a number of studies demonstrating an anti-inflammatory role of MMP-8 during lipopolysaccharide- (LPS-) mediated acute lung injury [47, 48] or allergen-induced airway inflammation [49].

Another characteristic feature of insulin-stimulated secretion was the presence of NGAL (neutrophil gelatinase-associated lipocalin) in several electrophoretic bands (Figure 3(b), bands 5, 8, 9) corresponding to monomeric (24 kDa) or homodimeric (46 kDa) forms of the protein [50]. In secretion from control [40] or E2-treated cells (Figure 3(a), band 4), NGAL was represented by minor bands corresponding to its homodimer form. These data indicating that, in the presence of insulin, neutrophils secrete an increased amount of NGAL are consistent with previously published work demonstrating that hyperinsulinaemic induction in humans significantly increases circulating levels of lipocalin-2. Additionally, in omental adipose tissue explants, insulin caused a significant dose-dependent increase in lipocalin-2 protein production and secretion into conditioned media [51].

A characteristic feature of NGAL is the ability to form complexes with MMP-9 after these components are in the extracellular environment. According to some researchers, the complex with NGAL supports allosteric activation of MMP-9 [52]. From another point of view, the formation of complex with NGAL protects MMP-9 from degradation by TIMPs, thereby maintaining the activity of the enzyme [44, 53, 54]. In any case, insulin initiates the secretion of MMP-9 in neutrophils and, at the same time, increases

the release of lipocalin, which helps maintain the activity of MMP-9.

3.5. Effect of Glucagon on the Protein Composition of Neutrophil Secretion in Adhesion to Fibronectin. The main components of the secretion of neutrophils adherent to fibronectin in the presence of glucagon were the cathepsin G, LF, and S100A9 proteins (Figure 3(c), Table 2). Cathepsin G is one of the three serine proteases (along with proteinase-3 and elastase) that are contained in primary (azurophil) granules of neutrophils and involved in clearance of internalized pathogens, proteolytic modification of chemokines and cytokines, and shedding of cell surface receptors [55]. Cathepsin G deficiency in gene-targeted mice increases susceptibility to *Staphylococcus aureus* and fungal infections indicating the key role of the enzyme in neutrophil antimicrobial activity [56, 57].

The glucagon-induced neutrophil secretion is also enriched in LF. Recent data show that LF can serve as an allosteric enhancer of the proteolytic activity of cathepsin G [58]. LF potently increases the activity of cathepsin G at pH 7.4 and to an even higher extent at pH 5, as well as in granulocyte-derived supernatant. Furthermore, LF might induce a conformational change of cathepsin G resulting in advanced substrate selectivity. LF and cathepsin G appear to act synergistically during secretion by granulocytes augmenting the process associated with host defense. We suggest similar synergistic interactions may occur in blood vessels between cathepsin G and LF that are secreted by glucagon-treated neutrophils attached to the vessel walls in patients with metabolic disorders.

Cathepsin G secreted by neutrophils can damage the vascular walls via promotion of inflammation or disruption of the neutrophil surface receptors. Cathepsin G, for example, is able to cleave leukosialin (CD43), the predominant cell surface sialoprotein of leukocytes, and releases its extracellular domain [59]. The shedding of highly negatively charged membrane sialoglycoprotein CD43 is commonly thought to enhance neutrophil adhesion. Thus, glucagon-induced cathepsin G secretion, in turn, may further potentiate the adhesion of neutrophils and the corresponding damage to blood vessels [60, 61].

3.6. Conclusions. Our in vitro experiments revealed that insulin and E2 stimulated secretion of MMP-9 and MMP-8 by human neutrophils during adhesion to fibronectin-covered substrata. In contrast, glucagon stimulated secretion of cathepsin G. We assume that hormones can affect the state of blood vessels in diabetes and metabolic disorders, regulating the adhesion of neutrophils to the walls of blood vessels and their corresponding secretion. Extracellular matrix proteins play a crucial role in the process of cell adhesion. Insulin and E2 can alter the adhesion of neutrophils initiating the secretion of metalloproteinase, which modifies extracellular matrix proteins. Glucagon can contribute to the development of metabolic vascular disorders by initiating the secretion of cathepsin G, the key enzyme of the bactericide activity of neutrophils. Cathepsin G may promote inflammation and stimulate further neutrophil adhesion via proteolysis of cell surface receptors.

Abbreviations

SEM:	Scanning electron microscopy
MPO:	Myeloperoxidase
Lf:	Lactoferrin
NGAL:	Neutrophil gelatinase-associated lipocalin
S100A8, S100A9:	The S100 calcium-binding proteins, known also as calgranulins A and B or myeloid-related proteins MRP8 and MRP14
MMP-9:	Matrix metalloproteinase 9 or gelatinase B, 92 kD
MMP-8:	Matrix metalloproteinase 8
TIMPs:	Tissue inhibitors of metalloproteinases
E2:	17β-Estradiol
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The work was supported by a grant from the Russian Foundation of Basic Research 16-04-670-a.

References

[1] M. Faurschou and N. Borregaard, "Neutrophil granules and secretory vesicles in inflammation," *Microbes and Infection*, vol. 5, no. 14, pp. 1317–1327, 2003.

[2] Z. V. Schofield, T. M. Woodruff, R. Halai, M. C. Wu, and M. A. Cooper, "Neutrophils—a key component of ischemia reperfusion injury," *Shock*, vol. 40, no. 6, pp. 463–470, 2013.

[3] J. E. Jordan, Z. Q. Zhao, and J. Vinten-Johansen, "The role of neutrophils in myocardial ischemia-reperfusion injury," *Cardiovascular Research*, vol. 43, no. 4, pp. 860–878, 1999.

[4] F. Hirata, M. Yoshida, and Y. Ogura, "High glucose exacerbates neutrophil adhesion to human retinal endothelial cells," *Experimental Eye Research*, vol. 82, no. 1, pp. 179–182, 2006.

[5] K. Mastej and R. Adamiec, "Neutrophil surface expression of CD11b and CD62L in diabetic microangiopathy," *Acta Diabetologica*, vol. 45, no. 3, pp. 183–190, 2008.

[6] N. Patel, "Targeting leukostasis for the treatment of early diabetic retinopathy," *Cardiovascular & Hematological Disorders Drug Targets*, vol. 9, no. 3, pp. 222–229, 2009.

[7] T. Takahashi, F. Hato, T. Yamane et al., "Increased spontaneous adherence of neutrophils from type 2 diabetic patients with overt proteinuria: possible role of the progression of diabetic nephropathy," *Diabetes Care*, vol. 23, no. 3, pp. 417-418, 2000.

[8] N. J. Fardon, R. Wilkinson, and T. H. Thomas, "Abnormalities in primary granule exocytosis in neutrophils from type I diabetic patients with nephropathy," *Clinical Science*, vol. 102, no. 1, pp. 69–75, 2002.

[9] L. Langouche, I. Vanhorebeek, D. Vlasselaers et al., "Intensive insulin therapy protects the endothelium of critically ill

patients," *The Journal of Clinical Investigation*, vol. 115, no. 8, pp. 2277–2286, 2005.

[10] P. Dandona, A. Chaudhuri, H. Ghanim, and P. Mohanty, "Insulin as an anti-inflammatory and antiatherogenic modulator," *Journal of the American College of Cardiology*, vol. 53, no. 5, pp. S14–S20, 2009.

[11] D. M. Nathan, P. A. Cleary, J. Y. Backlund et al., "Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes," *The New England Journal of Medicine*, vol. 353, no. 25, pp. 2643–2653, 2005.

[12] J. Li, F. Wu, H. Zhang et al., "Insulin inhibits leukocyte-endothelium adherence via an Akt-NO-dependent mechanism in myocardial ischemia/reperfusion," *Journal of Molecular and Cellular Cardiology*, vol. 47, no. 4, pp. 512–519, 2009.

[13] P. J. Lefebvre, N. Paquot, and A. J. Scheen, "Inhibiting or antagonizing glucagon: making progress in diabetes care," *Diabetes, Obesity & Metabolism*, vol. 17, no. 8, pp. 720–725, 2015.

[14] Y. Lee, M. Y. Wang, X. Q. Du, M. J. Charron, and R. H. Unger, "Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice," *Diabetes*, vol. 60, no. 2, pp. 391–397, 2011.

[15] Y. Lee, E. D. Berglund, M. Y. Wang et al., "Metabolic manifestations of insulin deficiency do not occur without glucagon action," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 37, pp. 14972–14976, 2012.

[16] M. Y. Wang, H. Yan, Z. Shi et al., "Glucagon receptor antibody completely suppresses type 1 diabetes phenotype without insulin by disrupting a novel diabetogenic pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 8, pp. 2503–2508, 2015.

[17] K. J. Hare, T. Vilsboll, M. Asmar, C. F. Deacon, F. K. Knop, and J. J. Holst, "The glucagonostatic and insulinotropic effects of glucagon-like peptide 1 contribute equally to its glucose-lowering action," *Diabetes*, vol. 59, no. 7, pp. 1765–1770, 2010.

[18] Y. Otsuka, S. Yamaguchi, A. Furukawa, M. Kosuda, M. Nakazaki, and H. Ishihara, "Addition of sitagliptin or metformin to insulin monotherapy improves blood glucose control via different effects on insulin and glucagon secretion in hyperglycemic Japanese patients with type 2 diabetes," *Endocrine Journal*, vol. 62, no. 2, pp. 133–143, 2015.

[19] I. Pernicova and M. Korbonits, "Metformin-mode of action and clinical implications for diabetes and cancer," *Nature Reviews Endocrinology*, vol. 10, no. 3, pp. 143–156, 2014.

[20] C. Y. Chou, Y. T. Chang, J. L. Yang et al., "Effect of long-term incretin-based therapies on ischemic heart diseases in patients with type 2 diabetes mellitus: a network meta-analysis," *Scientific Reports*, vol. 7, no. 1, article 15795, 2017.

[21] M. Huang, R. Wei, Y. Wang et al., "Protective effect of glucagon-like peptide-1 agents on reperfusion injury for acute myocardial infarction: a meta-analysis of randomized controlled trials," *Annals of Medicine*, vol. 49, no. 7, pp. 552–561, 2017.

[22] S. Yandrapalli, G. Jolly, A. Horblitt, A. Sanaani, and W. S. Aronow, "Cardiovascular benefits and safety of non-insulin medications used in the treatment of type 2 diabetes mellitus," *Postgraduate Medicine*, vol. 129, no. 8, pp. 811–821, 2017.

[23] M. H. Noyan-Ashraf, E. A. Shikatani, I. Schuiki et al., "A glucagon-like peptide-1 analog reverses the molecular pathology and cardiac dysfunction of a mouse model of obesity," *Circulation*, vol. 127, no. 1, pp. 74–85, 2013.

[24] M. Arakawa, T. Mita, K. Azuma et al., "Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4," *Diabetes*, vol. 59, no. 4, pp. 1030–1037, 2010.

[25] N. Diaz-Morales, S. Rovira-Llopis, C. Banuls et al., "Does metformin protect diabetic patients from oxidative stress and leukocyte-endothelium interactions?," *Antioxidants & Redox Signaling*, vol. 27, no. 17, pp. 1439–1445, 2017.

[26] M. R. Gubbels Bupp, "Sex, the aging immune system, and chronic disease," *Cellular Immunology*, vol. 294, no. 2, pp. 102–110, 2015.

[27] E. A. Booth and B. R. Lucchesi, "Estrogen-mediated protection in myocardial ischemia-reperfusion injury," *Cardiovascular Toxicology*, vol. 8, no. 3, pp. 101–113, 2008.

[28] I. Cabas, M. C. Rodenas, E. Abellan, J. Meseguer, V. Mulero, and A. Garcia-Ayala, "Estrogen signaling through the G protein-coupled estrogen receptor regulates granulocyte activation in fish," *Journal of Immunology*, vol. 191, no. 9, pp. 4628–4639, 2013.

[29] K. Ueda and R. H. Karas, "Emerging evidence of the importance of rapid, non-nuclear estrogen receptor signaling in the cardiovascular system," *Steroids*, vol. 78, no. 6, pp. 589–596, 2013.

[30] S. Kovats, "Estrogen receptors regulate innate immune cells and signaling pathways," *Cellular Immunology*, vol. 294, no. 2, pp. 63–69, 2015.

[31] A. Tsugita and J. J. Scheffler, "A rapid method for acid hydrolysis of protein with a mixture of trifluoroacetic acid and hydrochloric acid," *European Journal of Biochemistry*, vol. 124, pp. 585–588, 1982.

[32] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[33] I. Ginis, K. Zaner, J. S. Wang, N. Pavlotsky, and A. I. Tauber, "Comparison of actin changes and calcium metabolism in plastic- and fibronectin-adherent human neutrophils," *Journal of Immunology*, vol. 149, pp. 1388–1394, 1992.

[34] R. D. Burgoyne and A. Morgan, "Secretory granule exocytosis," *Physiological Reviews*, vol. 83, no. 2, pp. 581–632, 2003.

[35] J. M. Stein and J. P. Luzio, "Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles," *The Biochemical Journal*, vol. 274, no. 2, pp. 381–386, 1991.

[36] C. Hess, S. Sadallah, A. Hefti, R. Landmann, and J. A. Schifferli, "Ectosomes released by human neutrophils are specialized functional units," *Journal of Immunology*, vol. 163, pp. 4564–4573, 1999.

[37] O. Gasser, C. Hess, S. Miot, C. Deon, J. C. Sanchez, and J. A. Schifferli, "Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils," *Experimental Cell Research*, vol. 285, no. 2, pp. 243–257, 2003.

[38] J. Dalli, T. Montero-Melendez, L. V. Norling et al., "Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties," *Molecular & Cellular Proteomics*, vol. 12, no. 8, pp. 2205–2219, 2013.

[39] C. I. Timar, A. M. Lorincz, R. Csepanyi-Komi et al., "Antibacterial effect of microvesicles released from human neutrophilic granulocytes," *Blood*, vol. 121, no. 3, pp. 510–518, 2013.

[40] S. I. Galkina, N. V. Fedorova, M. V. Serebryakova et al., "Proteome analysis identified human neutrophil membrane

tubulovesicular extensions (cytonemes, membrane tethers) as bactericide trafficking," *Biochimica et Biophysica Acta (BBA) – General Subjects*, vol. 1820, pp. 1705–1714, 2012.

[41] S. I. Galkina, N. V. Fedorova, M. V. Serebryakova et al., "Inhibition of the GTPase dynamin or actin depolymerisation initiates outward plasma membrane tubulation/vesiculation (cytoneme formation) in neutrophils," *Biology of the Cell*, vol. 107, no. 5, pp. 144–158, 2015.

[42] G. Lominadze, D. W. Powell, G. C. Luerman, A. J. Link, R. A. Ward, and K. R. McLeish, "Proteomic analysis of human neutrophil granules," *Molecular & Cellular Proteomics*, vol. 4, no. 10, pp. 1503–1521, 2005.

[43] L. Kjeldsen, O. W. Bjerrum, J. Askaa, and N. Borregaard, "Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules," *The Biochemical Journal*, vol. 287, no. 2, pp. 603–610, 1992.

[44] S. Chakraborty, S. Kaur, S. Guha, and S. K. Batra, "The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer," *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1826, pp. 129–169, 2012.

[45] K. Brew and H. Nagase, "The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity," *Biochimica et Biophysica Acta (BBA) - Molecular*, vol. 1803, pp. 55–71, 2010.

[46] E. Dejonckheere, R. E. Vandenbroucke, and C. Libert, "Matrix metalloproteinase8 has a central role in inflammatory disorders and cancer progression," *Cytokine & Growth Factor Reviews*, vol. 22, no. 2, pp. 73–81, 2011.

[47] C. A. Owen, Z. Hu, C. Lopez-Otin, and S. D. Shapiro, "Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase," *Journal of Immunology*, vol. 172, no. 12, pp. 7791–7803, 2004.

[48] P. A. Quintero, M. D. Knolle, L. F. Cala, Y. Zhuang, and C. A. Owen, "Matrix metalloproteinase-8 inactivates macrophage inflammatory protein-1α to reduce acute lung inflammation and injury in mice," *Journal of Immunology*, vol. 184, no. 3, pp. 1575–1588, 2010.

[49] M. M. Gueders, M. Balbin, N. Rocks et al., "Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation," *Journal of Immunology*, vol. 175, no. 4, pp. 2589–2597, 2005.

[50] L. Kjeldsen, A. H. Johnsen, H. Sengelov, and N. Borregaard, "Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase," *The Journal of Biological Chemistry*, vol. 268, no. 14, pp. 10425–10432, 1993.

[51] B. K. Tan, R. Adya, X. Shan et al., "Ex vivo and in vivo regulation of lipocalin-2, a novel adipokine, by insulin," *Diabetes Care*, vol. 32, no. 1, pp. 129–131, 2009.

[52] H. Tschesche, V. Zolzer, S. Triebel, and S. Bartsch, "The human neutrophil lipocalin supports the allosteric activation of matrix metalloproteinases," *European Journal of Biochemistry*, vol. 268, no. 7, pp. 1918–1928, 2001.

[53] L. Yan, N. Borregaard, L. Kjeldsen, and M. A. Moses, "The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL," *The Journal of Biological Chemistry*, vol. 276, no. 40, pp. 37258–37265, 2001.

[54] D. X. Bu, A. L. Hemdahl, A. Gabrielsen et al., "Induction of neutrophil gelatinase-associated lipocalin in vascular injury via activation of nuclear factor-κB," *The American Journal of Pathology*, vol. 169, no. 6, pp. 2245–2253, 2006.

[55] T. Burster, H. Macmillan, T. Hou, B. O. Boehm, and E. D. Mellins, "Cathepsin G: roles in antigen presentation and beyond," *Molecular Immunology*, vol. 47, no. 4, pp. 658–665, 2010.

[56] E. P. Reeves, H. Lu, H. L. Jacobs et al., "Killing activity of neutrophils is mediated through activation of proteases by K^+ flux," *Nature*, vol. 416, no. 6878, pp. 291–297, 2002.

[57] J. Tkalcevic, M. Novelli, M. Phylactides, J. P. Iredale, A. W. Segal, and J. Roes, "Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G," *Immunity*, vol. 12, no. 2, pp. 201–210, 2000.

[58] S. Eipper, R. Steiner, A. Lesner et al., "Lactoferrin is an allosteric enhancer of the proteolytic activity of cathepsin G," *PLoS One*, vol. 11, no. 3, article e0151509, 2016.

[59] A. Mambole, D. Baruch, P. Nusbaum et al., "The cleavage of neutrophil leukosialin (CD43) by cathepsin G releases its extracellular domain and triggers its intramembrane proteolysis by presenilin/γ-secretase," *The Journal of Biological Chemistry*, vol. 283, no. 35, pp. 23627–23635, 2008.

[60] C. Nathan, Q. W. Xie, L. Halbwachs-Mecarelli, and W. W. Jin, "Albumin inhibits neutrophil spreading and hydrogen peroxide release by blocking the shedding of CD43 (sialophorin, leukosialin)," *The Journal of Cell Biology*, vol. 122, no. 1, pp. 243–256, 1993.

[61] S. Lopez, S. Seveau, P. Lesavre, M. K. Robinson, and L. Halbwachs-Mecarelli, "CD43 (sialophorin, leukosialin) shedding is an initial event during neutrophil migration, which could be closely related to the spreading of adherent cells," *Cell Adhesion and Communication*, vol. 5, no. 2, pp. 151–160, 1998.