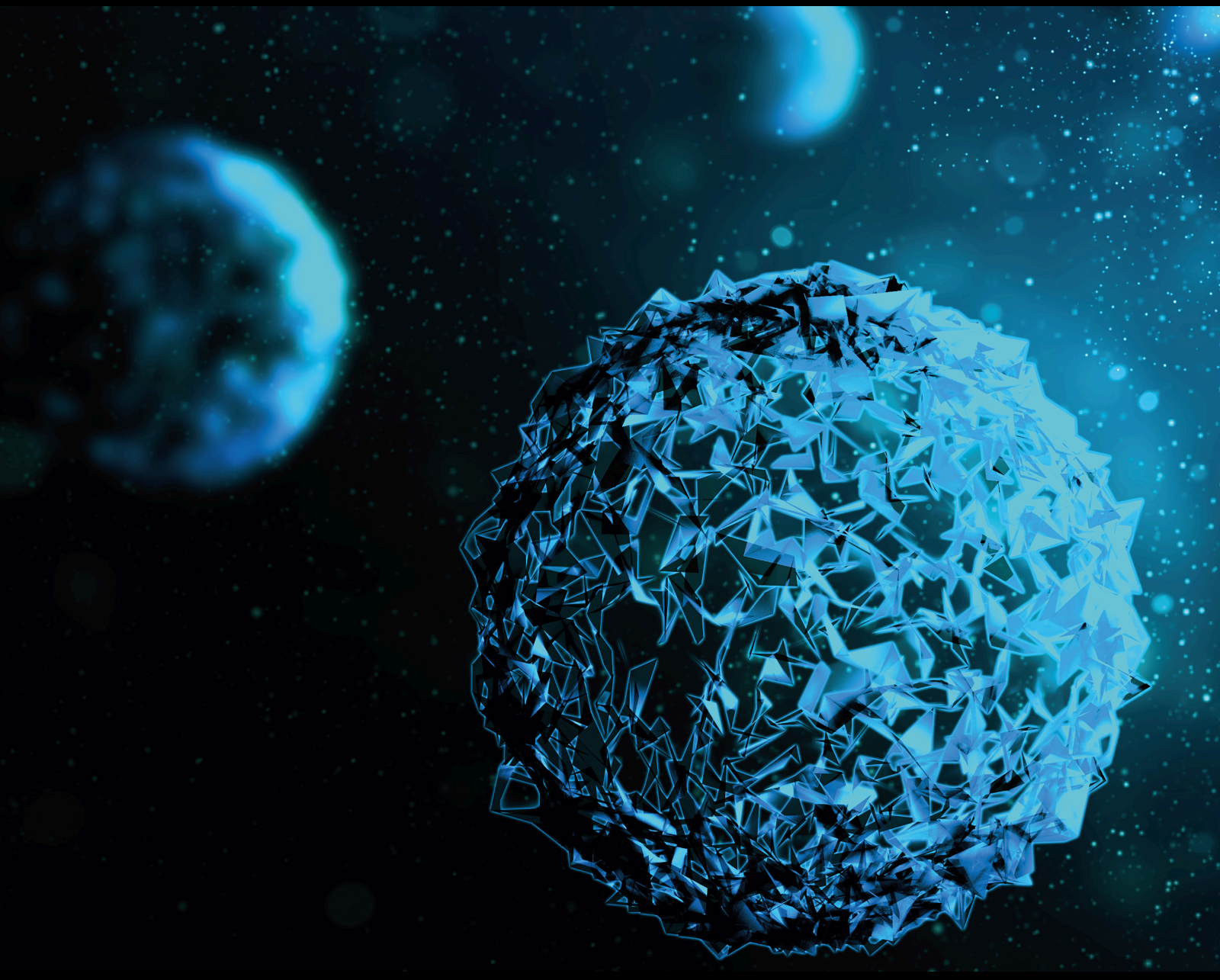
BioMed Research International

Interplay between Immune Cells and their Microenvironment Niches (IICMN-2738)

Lead Guest Editor: Jian Song
Guest Editors: Zenghui Teng, Christopher J. Pirozzi, and Weidong Cao



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Immunology

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Retraction

Retracted: Identification of a Novel *ACTN4* Gene Mutation Which Is Resistant to Primary Nephrotic Syndrome Therapy

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] L. Meng, S. Cao, N. Lin et al., "Identification of a Novel *ACTN4* Gene Mutation Which Is Resistant to Primary Nephrotic Syndrome Therapy," *BioMed Research International*, vol. 2019, Article ID 5949485, 7 pages, 2019.

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Review Article

Significance of LL-37 on Immunomodulation and Disease Outcome

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Guest Editor: Jian Song

LL-37, also called cathelicidin, is an important part of the human immune system, which can resist various pathogens. A plethora of experiments have demonstrated that it has the multifunctional effects of immune regulation, in addition to antimicrobial activity. Recently, there have been increasing interest in its immune function. It was found that LL-37 can have two distinct functions in different tissues and different microenvironments. Thus, it is necessary to investigate LL-37 immune functions from the two sides of the same coin. On the one side, LL-37 promotes inflammation and immune response and exerts its anti-infective and antitumor effects; on the other side, it has the ability to inhibit inflammation and promote carcinogenesis. This review presents a brief summary of its expression, structure, and immunomodulatory effects as well as brief discussions on the role of this small peptide as a key factor in the development and treatment of various inflammation-related diseases and cancers.

1. Introduction

Antimicrobial peptides (AMPs) are important elements of the immune system which are capable of combating a broad spectrum of organisms and transformed or cancerous cells [1, 2]. Mammalian AMPs belong to the defensin and cathelicidin families. So far, there is a unique cathelicidin peptide found in 1995 and called human cationic antimicrobial peptide (hCAP18) [2]. Its active part starts with double leucine and consists of 37-amino acids at the C-terminus, so which is called LL-37. Not only human but also only one cathelicidin the analogue of human cathelicidin was found in mice named cathelicidin-related antimicrobial protein (CRAMP).

This small number of AMPs is expressed in some cell types that may encounter pathogens. Cathelicidin is involved in the innate immune system; after infection, LL-37 bridges the innate and acquired immunity through recruiting immune cells to the infected site and stimulates and/or modulates adaptive immunity through specific activation of the receptors of the immune cells as well [3]. Moreover, LL-37 regulates the production of chemokines and pro- and anti-inflammatory cytokines, in order to maintain the fine balances between pro- and anti-inflammatory responses. This ability to maintain equilibrium plays a very important role in resisting pathogens while maintaining the stability of the immune system. If defects in the expression or processing of LL-37 break this balance, it will result in abnormalities of the body.

The purpose of this review is to offer a concise general view of the expression, structure, and immunomodulatory effects of antimicrobial peptides LL-37 on immunocompetent cells and briefly discuss the role of this small peptide as a key factor in the development and treatment of various inflammation-related diseases and cancers.

2. Structure

The human cathelicidin preprotein hCAP18, which is encoded on chromosome 3p21.3 [2], the genes that consist of 4 exons and 3 introns, is cleaved into active type LL-37 by protease-3 extracellularly under specific conditions [4]. The sequence of LL-37 is LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES. At neutral pH, this peptide is amphipathic and cationic which net charge is +6 [5]. Although a state of random coil in pure water, in solutions with millimolar concentrations of salts or membrane-like environment, little cathelicidin peptide forms a α-helical structure with cationic amphipathic [1]. Conditions encouraging tend to favor α-helical structure rely on the solution condition including NaCl concentration and pH, membrane-like environment, or higher peptide concentrations [5].

There are three parts of the linear cationic α-helix of LL-37 structure, two α-helices of N-terminus and C-terminus which are from residues 2~30 of peptide and one unstructured C-terminal tail that consists of the C-terminal residues 31 to 37 [6]. The cationic hydrophobic surface of LL-37 enables to interact with negatively charged elements, like bacterial cell walls, lipopolysaccharide (LPS), and nucleotide, because of being formed by four aromatic phenylalanine side chains and the border by predominately positively charged residues [7]. The N-terminal helix is responsible for peptide oligomerization, proteolytic resistance, chemotaxis, and hemolytic activity, while the C-terminal helix is involved in antipathogen effect [8]. Above these two structures are needed in peptide aggregation [9]. The C-terminal tail is the critical structure to form a tetramer of peptide, which facilitates its ability to activate effect cells and favors to interact with components of serum and the bacterial outer wall that result peptide sequestration and decrease its antipathogen potential [9].

3. Expression

LL-37 is expressed in many types of tissue cells such as keratinocytes, differentiated epithelial cells in the colon, airway, ocular surface, genitals, in eccrine glands, Brunner glands in the duodenum [10], myelocytes [11], mesenchymal stromal cells (MSCs) [12, 13], and cells of testes [11]. Expression in most epithelia is constitutive, while injury can induce peptide expression in keratinocytes, where the precursor localizes in granules of the superficial epidermis and partially resides in lamellar bodies [14]. Chakraborty et al. indicated that the constitutive expression in epithelial cells is regulated through cAMP-signaling pathways, and some complexes are required which are formed by cAMP-responsive element-binding protein (CREB) and activator protein 1 (AP-1) to bind to the cathelicidin peptide promoter sequence and induce transcription [15].

As an important part of the immune system, cathelicidin protein constitutive expression is produced in natural killer (NK) cells, neutrophils, T cells, and mast cells [10, 16]. As the first line cells confront the pathogens, neutrophils are the main source of this peptide. The neutrophil synthesizes inactive hCAP18 precursor and store in secondary particles. Active toll-like receptors (TLRs) by damage-associated molecular pattern molecules (DAMPs) or pathogen-associated molecular pattern molecules (PAMPs), and/or changes in cytokine types and levels, can promote cell to degranulate. Upon stimulation, the cathelicidin protein precursor will be degranulated and released extracellularly, where the active cathelicidin proteolytically processed by specific proteases 3 was unleashed [4]. Furthermore, monocytes, dendritic cells (DCs), and macrophages of the immune system are proved to express LL-37 [10]. Peripheral blood-derived cells are proved to express a gradient dose: high levels in neutrophils; low levels in lymphocytes, while different types of lymphocytes of low level produce the same amount, and the expression of monocytes is moderate [17].

Different factors by different incentives from the body and outside can influence this peptide expression, for example, interferon γ (IFN-γ), interleukin 6 (IL-6), glucocorticoids, transmigration across activated endothelium, bacterial exotoxins, certain bacteria, entitystat, and calcipotriol are found to be the downregulated factors [10], while the upregulation of expression was found to be tumor necrosis factor α (TNF-α), IL-17A, toll-like receptor agonists, insulin-like growth factor 1 (IGF-1), vitamin D receptor agonists, hormonal 1,25dihydroxyvitamin D, phenyl butyrate, sodium butyrate, MUC2 mucin, simvastatin, injury and wounding, and endoplasmic reticulum stress [10, 18, 19].

As this small peptide plays an important role involved in the innate immune system and adaptive immune system, overexpression or low expression will break the immune balance to cause some diseases. In the inflamed mucosa of ulcerative colitis (UC) and Crohn's disease (CD), the expression of LL-37 mRNA was reported that has increased significantly [20, 21]. These researches display that the TLR3 ligand, double-stranded RNA (mimicked by polyinosinic-polycytidylic acid (poly(I:C))), and induced LL-37 mRNA overexpress in colonic subepithelial myofibroblasts (SEMFs) that proved self-nucleic acids break innate tolerance [20, 21], while overexpressed LL-37 found in psoriatic epidermis activates DCs to produce cytokines like TNF-α, IL-23, and IL-17 and become an autoantigen which can trigger the T cell and adaptive immune system [22–24]. In autoimmune diseases, overexpression of LL-37 excessively exerts its immune regulation, thus destroying the homeostasis of the immune system. On the other hand, lower expression also causes more severe infections, for instance, periodontitis caused by bacteria [25], tuberculosis [26, 27], oral squamous cell carcinoma (OSCC), and so on. Interestingly, because of functions of LL-37 in different tissue are pleiotropic, some changes in the expression of LL-37 appear in different tumors. Overexpression is found in ovarian cancer, lung cancer, breast cancer, malignant melanoma, and prostate cancer, but gastrointestinal cancer (colon cancer and gastric cancer) and hematological malignancies were reported lower than normal [28].

4. Chemotaxis

As previously stated, the infected epithelial cells expressed LL-37 which can directly recruit immunocompetent cells, including neutrophils, monocytes, and DCs. While the neutrophils are attracted, that is the first line dealing with microbes, as a main source of cathelicidin neutrophils that continue to be released at the infected sites. Furthermore, LL-37 induce monocyte [29], fibroblasts [30], epithelial cells [29], and human airway smooth muscle (HASM) cells [31] to secrete IL-8, which further indirectly attracts immune cells, then these cells together with LL-37 against the infection. In addition, other chemokines which have the ability to attract more immunocompetent cells like neutrophils, monocytes, DCs, and T cells, that are released by the cells encounter infections upon treatment with LL-37, for instance, CCL4, CCL20, and C-X-C motif ligand (CXCL) 1 proved to be produced by primary monocytes [32], C-C motif ligand (CCL) 2 released by endothelial cells [33], CCL3 and CCL2 by mast cells [34], and a synergistic increase in CCL20, CXCL1, CXCL8 (IL-8), and CCL2 secretion upon LL-37 exposure to keratinocyte-fibroblast cocultures [35]. After the activation of the innate immunity system, antigen-presenting cells carry the antigen to the specific T cells. Then, the cells of the adaptive immune system are directly attracted [1]. Current researches show that LL-37 plays a chemotactic role from the initial stage of infection to the adaptive immune response, promotes the anti-infective inflammatory response, and plays a bridging role from innate immunity to adaptive immunity. Not only chemotactic to immune cells, previous studies have shown that LL-37 recruit multipotent mesenchymal stromal cell (MSC) migration to tumor through formyl peptide receptor (FPR) 2 [36]. Using anti-LL-37 antibody to neutralize LL-37 in vivo can notably partly decrease the implantation of MSCs into ovarian cancer modeled by OVCAR-3 ovarian cancer cells, causing suppression of tumor growth and breakdown of the fibrovascular network. These findings consistent with that LL-37 treatment enhanced the proliferation and migration of human adipose-derived stromal/stem cells (ASCs) [37]. It is indicated that the LL-37-mediated recruiting MSCs could promote tumor progression.

5. Immunomodulation

5.1. Neutrophils. Neutrophil constitutes an important part of the innate immune system and is the front line to resist bacterial infection. Pathogen identification and subsequent recruitment of granulocytes into the infected site are key factors for host defense against bacterial diseases. This process includes the recognition of PAMPs by host pattern recognition receptors (PRRS), and the production of a variety of pro-inflammatory cytokines and chemokines at the site of infection. These chemical attractants promote the recruitment of neutrophils to infection and inflammation sites and produce variable potent mediators, including chemokines, cytokines, colony-stimulating factors, fibrous factors, and angiogenic factors, and then ingest and kill invading microorganisms. The effective antibacterial activity of neutrophils is the synergistic action between high protein hydrolase and degrading enzyme, cation molecule and active oxygen, which enables the immune system to successfully protect the host from various bacterial pathogens [38]. Under steady-state conditions, neutrophils undergo structural (spontaneous) apoptosis to end their short life (about 4-5 days). After neutrophil necrosis or apoptosis, neutrophil extractor traps (NETs) will be formed in the inflammatory site, which can provide high concentration of antibacterial molecules in the local area and quickly control the infection of bacteria in vivo. However, in abnormal circumstances, such as the induction of abnormal somatic cells, pathogens, or cancer cells, the secondary necrosis of apoptotic neutrophils happened, and the release of active molecules is continuously produced, forming an abnormal number of NETs, damaging healthy host cells, causing inflammation expansion and tissue damage [39–41].

As the main source of LL-37, neutrophils, stimulated by TLR ligands, not only can release antimicrobial peptides but also influenced by antimicrobial peptides and change physiological functions. It was reported that LL-37 induces neutrophil migration and chemotaxis mediated via FPR molecules in vitro [42, 43]. But neutrophil chemotaxis induced by serum amyloid A (SAA) almost competes inhibited by LL-37 [44]. SAA is an acute phase response protein produced by the body, which can be used to determine the severity of infection and inflammation. When SAA rises, it indicates that the body has produced a wide range of inflammation. We think that LL-37 may play an important role in immune regulation, limit the further expansion of SAA-induced inflammation, and maintain the stability of the immune system. The same condition happened in sepsis-induced acute lung injury, LL-37 and its analogy sLL-37 through the focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), and P38 pathways which inhibit neutrophil infiltration and migration after the severe infection [45]. Interestingly, the cathelicidin can depress the expression of C-X-C chemokine receptor type 2 (CXCR2), which is a neutrophil surface receptor that mediates neutrophil migration to the sites of inflammation [46, 47].

These two sides function as well as reflect regulation of LL-37 for neutrophil releasing active substances. Some data indicated that cathelicidins directly activate neutrophils to mediator release. Human cathelicidin, at a concentration of 20 μg/ml, stimulates neutrophils to the synthesis of proinflammatory CXCL8 under the control of p38 mitogen-activated protein kinase (MAPK) and ERK [47, 48]. It is well known that this chemokine, acting via CXCR2, induces chemotaxis of not only neutrophils but also other granulocytes and stimulates neutrophils to phagocytosis. On the other hand, interesting data suggest that LL-37 inhibits SAA-induced CXCL8 production and causes dramatic inhibition of ERK and p38 MAPK activities [44].

LL-37 is not only involved in regulating the physiological function of neutrophils when they are alive but also can influence neutrophil apoptosis and the physiological function of forming nets after death. LL-37 induces secondary necrosis of apoptotic neutrophils through the increased expression of antiapoptotic protein Bcl-XL and by blocking the

activation of caspase-3 [49, 50] via the activation of FPR and purinergic receptor P2X ligand-gated ion channel 7 (P2X7 receptor) on these cells [46]. NETs which are involved in a variety of chronic inflammatory pathologies can release LL-37 in vivo and ex vivo, and LL-37 also promoted peripheral neutrophils to form NETs in a dose-dependent manner ex vivo [51]. And NETs via C1q/LL-37 specifically inhibited interleukin IL-6 secretion by LPS-activated macrophages [52].

5.2. Monocytes/Macrophages/Dendritic Cells. Monocytes and macrophages are the immediate arm of the immune system and play an important role in immunomodulatory and tumor immunity via producing both inflammatory mediators and antigen processing. Monocytes are called adult stem cells; it can be differentiated into different cell types: macrophages, DCs, monoosteophils, osteoclast, endothelial cells, etc. For example, monocyte can differentiate into inflammatory macrophages or DCs during inflammation effected by the inflammatory milieu and pathogen-associated pattern-recognition receptors. Macrophages may be characterized as M1- and M2-polarized subtypes; M1 macrophages exhibit inflammatory and antitumor functions via the release of soluble enzyme and cytokines, whereas M2 macrophages have anti-inflammatory functions, may promote tumor cell proliferation, and participate in tissue remodelling. DCs are the most powerful antigen-processing and antigen-presenting cells which can efficiently uptake, process, and present antigens and stimulate the proliferation of nonsensitized cells, be equipped with high phagocytic activity as immature cells and high cytokine-producing capacity as mature cells. It can stimulate the proliferation and activation of nonsensitized T cells, and is the central link to initiate, regulate, and maintain specific antigen induction in vivo. Tumor-specific DC can stimulate specific long-lasting tumor immunity induction.

5.2.1. Monocytes. LL-37 has been shown to be involved in monocyte/macrophage differentiation. It was reported that LL-37 enhances the GM-CSF/IL-4-driven differentiation of blood monocytes into immature DCs [53]. LL-37-derived DCs preserve the basic DC phenotype; LL-37 appropriately promotes maturation of DC and changes the expression of chemokine receptors that facilitate mDC migration to T cell areas. LL-37 in synergy with Peptidoglycan (PGN) can induce monocytes from the peripheral blood of healthy individuals polarized toward the $CD14^{high}$ $CD16^{+}$ subset, and LL-37 further induced PGN-driven differentiated monocytes into immature dendritic cells (iDC), as evident by the increased expression of CD1a, CD86, and HLA-DR markers, resulting in the induction of T cell proliferation and Th17 polarization [54]. It displayed that LL-37 can influence monocyte differentiation, induce PGN-driven monocytes polarized to DCs, and promote proinflammation and adaptive immune response. Other than this, monocytes from the blood sample can differentiate into the population of monocyte-derived bone-forming cells (monosteophils) and accelerate bone repair treating with an effective dose of LL-37 which are uptaken via CXCR2-specific endocytosis of monocytes [55–57].

Monocytes are stimulated by LL-37 to upregulate the release of proinflammatory chemokines (CXCL1, CCL2, and CCL7) and cytokines (IL-8 and IL-6) with IL-1β synergistically or not and transcript the genes encoding anti-inflammatory cytokines (IL-10 and IL-19) [58, 59]. Not only upregulate pro- or anti-inflammatory cytokines, LL-37 which inhibit monocytes express some cytokines CXCR2, TNF-α, and IL-6 with IL-1β synergistically or not. In addition, LL-37 strongly inhibits the synthesis of TNF-α and IL-12 by monocytes stimulated with IFN-γ [60].

5.2.2. Macrophages. Meantime, LL-37 can regulate the activity of macrophages of the immune system. In macrophages, LL-37 upregulates or downregulates different genes to influence cell functions; the genes predicted to be upregulated by LL-37 were including those encoding chemokines chemokine receptor (CCR) 2, CCL7, IL-8, anti-inflammatory cytokine IL-10, and M-CSF [29]. Contrariwise, this peptide can downregulate another 20 genes, including gene encoding proinflammatory IL-12 [29]. In line with this, cathelicidin and its derivative wildly restrain the production of TNF-α and IL-1β by IL-32-driven macrophage, promoting to produce the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1RA) without changes in chemokine production [61].

While some studies focus on LL-37 regulation of the active macrophages by bacterial components, LL-37 obviously decreases Neisseria meningitides endotoxin lipooligosaccharide (LOS), LPS, or lipoteichoic acid (LTA) which induced both TNF-α and nitric oxide (NO) release from macrophages [62–65]. Among them, LOS, LPS, and LTA are bacterial endotoxin. In addition, it was reported that cathelicidin causes significant suppression of producing TNF-α by macrophages inducing with arabinosylated lipoarabinomannan (AraLAM). LAM is an important component of the cell wall of *Mycobacterium tuberculosis* [66]. What is more, Hu et al. showed that cathelicidin inhibits pyroptosis of macrophages and proinflammatory cytokine synthesis (IL-1β, IL-6, and TNF-α) by activated macrophages induced by LPS/ATP in vitro or cecal ligation puncture (CLP) in CLP septic mice; thus, the peptide not only neutralizes the action of LPS but also inhibits the response of P2X7 [67, 68]. Consist with this, exogenous LL-37 decreased TNF-α and IL-17 while inducing IL-10 and transforming growth factor β (TGF-β) production that was also independent of the P2X7 receptor and did not reduce antimycobacterial activity during infection [69]. Thus, LL-37 has a more comprehensive immune regulation function without affecting anti-infection. Interestingly, Ruan et al. uncovered that LTA upregulated the concentration of peptide located mostly in the cytoplasm of macrophages [63]. It indicated that pathogens stimulate body release LL-37 firstly and then exert their anti-infection and immunomodulatory function.

Unlike the proinflammatory and anti-infective functions in an inflammatory environment, LL-37 can overexpress in certain tumors and promote the differentiation of macrophages to M2, which in turn promotes tumorigenesis. As

cathelicidin of mice, CRAMPs are proved to promote tumorigenesis via macrophage. With prostate cancer CRAMP$^{(+)}$, more immature myeloid progenitors (IMPs) polarize into protumorigenic M2 macrophages than CRAMP$^{(-)}$ TME. Meanwhile, in vitro experiments confirmed that CRAMP can mediate autocrine signaling to promote M-CSF and monocyte chemotactic protein (MCP) 1 overexpressing in TRAMP-C1 cells (prostate cancer cell lines without CRAMP expression) to regulate macrophage differentiation to M2 by activating STAT3 [70]. Furthermore, the human cathelicidin is proudly expressed by tumor-associated macrophages (TAMs) present within the pancreatic ductal adenocarcinoma (PDAC) stroma and induced by CSC-secreted tumor growth factor-β (TGF-β) family members Nodal and ActivinA. The synthesis human cathelicidin enhances the ability of pancreatic cancer stem cell (CSC) to invasion, self-renewal, and tumorigenesis, via the G protein-coupled receptor (GPCR), FPR2 and P2X7 receptor [71]. Thus, LL-37 as a tumor microenvironment factor plays a critical role in tumorigenesis. Similarly, human macrophages were cocultured with colorectal cancer cells SW480 or HCT116 to mimic the tumor microenvironment; the mimic TAMs enhance the production and release of antimicrobial peptides LL-37 to promote the proliferation of colorectal cancer cells via the Wnt/β-catenin pathway [72].

Different environments or stimuli can affect the function and differentiation of monocytes/macrophages. And LL-37 plays two opposite roles in it. In an inflammatory or infective environment, LL-37 can promote the proinflammatory and anti-infective functions of monocytes/macrophages, while also carefully monitoring the trend of inflammation, promoting the synthesis of anti-inflammatory factors, preventing the situation from expanding, and maintaining the overall immune balance of the body. Tumor-derived LL-37, an overexpressed peptide in the tumor environment, helps tumor cells to polarize macrophages into M2 type of TAMs, inhibit immune function, and promote tumorigenesis.

5.2.3. Dendritic Cells. As a potent modifier of DC differentiation, LL-37 is bridging innate and adaptive immune responses at sites of inflammation, where high levels of LL-37 secreted by recruited neutrophils and resident epithelial cells chemoattract pre-DC. The addition of peptide suppressed mature DC (stimulated by LPS) release of IL-6 and TNF-α; the expression of HLA-DR, CD80, CD83, and CD86; and the chemokine receptor CCR7. This suppression is a concentration-dependent manner; up to 20 μg/ml of LL-37 will result in a total inhibition of secretion [73]. Not only for DCs but also influence for T cells via DCs, DC exposure with LL-37 and LPS resulted in a prominently reduced capacity of DCs to stimulate CD4$^+$ T cells, as decreased IFN-γ and IL-2 secrete and their proliferation [73]. On the contrary, another study using LPS stimulate DCs derived from monocytes by incubation with IL-4, GM-CSF, and LL-37; the results displayed upregulated endocytic capacity, modified phagocytotic receptor expression and function, upregulated costimulatory molecule expression, enhanced the secretion of T helper cell (Th) 1 cytokines, and then promoted Th1 responses in vitro [53]. In like manner, it is demonstrated that the addition of LL-37 (without LPS or other antigen) is internalized by immature dendritic cells derived from human peripheral blood monocytes (MDDC) with subsequent localization primarily in the cytoplasmic compartment and then could also be transported into the nucleus of MDDC, caused phenotypic changes, and characterized by an increased expression of the antigen-presenting molecule HLA-DR and the costimulatory molecule CD86 [74]. Above data look like in contradiction, but the sequence of stimulation between LL-37 and LPS is different. LL-37 can restrict the proinflammation of LPS and control immunity development. Addition to this, LL-37 can influence the function of DCs by internalization and manipulate T cell polarization by DC. Taken together, these findings suggest that, after triggering of the innate immunity, LL-37 would be released to affect sequent cellular adaptive immune.

Besides itself of LL-37, Hurtado and Peh suggested that LL-37 can bind to bacterial DNA (CpG oligodeoxynucleotides) which brings about a significant reduction in the time through sensing the presence of bacterial DNA via TLR9 by B cells and plasmacytoid DCs (pDCs) [75]. Since LL-37 is a cationic charge, a stable LL37/nucleic acid complex is formed. The latter enters the endosomal compartment of pDCs and stimulates TLR9 and TLR7. LL-37 combine with extracellular autonuclei to form an effective trigger for the release of IFN from pDCs [76]. Furthermore, LL-37/RNA complexes can also induce the activation of IFN-conditioned myeloid DCs (mDCs) by TLR7/8 stimulation [77]. Thus, these results proved that LL-37 play a crucial role in the formation of psoriasis.

5.3. Lymphocytes. Lymphocytes include T cells (thymocytes), B cells (bone marrow or bursa-derived cells), and NK cells. T cells and B cells are mainly involved in the adaptive immune system. The primary T cells migrate within the secondary lymphoid organs where they encounter and interact with the DCs and further differentiate into T cell subsets. For example, some T cells (also known as CD4$^+$ cells) called Th cells can produce cytokines that direct immune responses, for example, Th1, Th17, and Th2. Th1 cells are differentiated induced by cytokines such as IL-12; secrete IL-2, IFN-γ, TNFβ, and other cytokines; and participate in regulating cellular immunity, assist in cytotoxic T cell differentiation, and participate in delayed-type hypersensitivity reactions, while Th17 is differentiated under the stimulation of IL-6 and IL-23 and mainly secretes IL17, IL1, IL-6, and TNF-α. These cytokines can collectively mobilize, recruit, and activate neutrophils; thus, like Th1, polarized Th17 cells have the capacity to cause inflammation and autoimmune disease. Th2 helper cells are mainly immune responses against extracellular multicellular parasites, which are mainly induced by IL-4, and mainly secretes IL-4, IL-5, and IL-10. Regulatory T cell (Tregs) was named as suppressor T cell in the 1970s. Tregs is a key cell in the negative regulation of the body's immune response, playing a paramount role in maintaining self-tolerance and immune homeostasis and participating in tumor cells to escape the body's immune surveillance [78]. There are many types of Tregs, such as CD4$^+$ CD25$^+$ T cells. With the help of activated Th cells and antigen-presenting

cells (APCs), B cells become activated B cells by antigen stimulation and then differentiate into plasma cells to synthesize and secrete various antibodies, mainly performing humoral immunity of the body. NK cells are part of the innate immune system that distinguishes between abnormal cells (infected cells and tumor cells) and normal cells by recognizing changes of molecules known as Major Histocompatibility Complexes (MHC) class I on the cell surface and via releasing cytotoxicity (cells-killing) granules which then destroy the altered cells and plays an important role in protecting the host from tumor and viral infections.

5.3.1. T Cells. Early research shows LL-37-derived mDC maturation with LPS produces a characteristic Th1-inducing cytokine profile (significantly an increase of IL-12, IL-6, and TNF-α and significantly a decrease of IL-4). Furthermore, significantly increased synthesis of IFN-γ by T cells was tested which is stimulated by LL-37-derived mDC. Thus, LL-37 appears to act as a bridge between the innate and adaptive immune systems [53], while LL-37 not only produces an enhanced Th1 response but also produces an adjuvant that enhances the Th17 response in the oral mucosa, where mFPR2 on M cells interacts with LL-37-Ag and is recognized by APC near the M cell, mature to $CD11c^{+}CD70^{+}$ APC, which subsequently produces a Th17-biased environment by increasing IL-17, and leads to an increase in the formation of germinal centre (GC) B cells and GC; thus, LL-37 mediates an Ag-specific immune response through regulating the mucosal immune environment [79]. Interestingly, by assessing the role of LL-37 in peripheral blood mononuclear cells (PBMC), the researchers found that LL-37 also promotes the production of regulatory T cells, while LL-37 does not affect T cell activation; in the context of inflammation (PHA activation), peptides can induce resting T cell proliferation, significantly increasing Tregs production and decreasing proinflammatory factor expression (INF-γ, TNF-α) of PBMC; indicating that when the peptide plays its own anti-infective property, the control proinflammatory responses are always accompanied, in order to protect the body against severe inflammatory response [80]. These results show the two sidedness of antimicrobial peptides. In the face of different immune cell populations and different microenvironments, antibacterial peptides will exhibit different states, even diametrically opposite immune responses, so the microenvironment obviously plays a pivotal role in determining how T cells respond to LL-37.

5.3.2. NK Cells. As an important part of the innate immune system, NK cells can be used for immune surveillance of certain tumor and virus-infected cells. Cathelicidin was observed to be abundant in tumor-infiltrating $NK1.1^{+}$ cells in mice. Functional in vitro analyses found that NK cells derived from cathelicidin knockout mice ($Camp^{-/-}$) versus wild-type mice showed impaired cytotoxic activity toward tumor targets. Moreover, $Camp^{-/-}$ permitted faster tumor growth than wild-type controls in two different xenograft tumor mouse models (murine B16 melanoma and RMA-S lymphoma) that exclude an observed perforin deficiency [16]. The findings indicate the significance of cathelicidin to NK cell function and in vivo tumor defense. In addition, LL-37 improves CpG delivery to intracellular TLR9 results in the enhanced proliferation and activation of NK cells, to prevent relapse in the case of ovarian cancer [81]. However, the details of mechanism that LL-37 interact with NK cells are needed to be clarified further.

5.4. Mast Cells. Mast cells are an important component of host defense pathogens and can affect both innate and acquired immune responses. Mast cells participate in the entire process of inflammation, such as promoting inflammation and limiting inflammation, through the production of mediators, including cytokines, chemokines, and biologically active mediators. Various endogenous and exogenous mediators can activate cells through different receptors expressed by mast cells, and activated cells rapidly release relevant mediators stored in cytoplasmic granules to participate in inflammatory responses such as histamine, protease, and cytokines (IL-3, IL-4, IL-6, IL-8, IL-10, TNF-α, etc.) [82, 83]. In addition, activated mast cells can release a variety of newly produced lipid mediators including leukotriene (LT), prostaglandin (PG), thromboxane (TX), and platelet-activating factor (PAF) [82, 84].

In mast cells, this host-defense peptide causes degranulation assessed by histamine or β-hexosaminidase release by intracellular Ca^{2+} mobilization [34, 85] but also the production and release cytokines (IL-1β, IL-2, IL-4, IL-6, IL-31, TNF-α, and GM-CSF) and chemokines (CCL 2 and CCL3) stimulated with 10 μg/ml LL-37 for 3-24 h [34] or 5-20 μg/ml LL-37 for 6 h [86] in a dose-dependent and time-dependent fashion [34, 86]. In addition, this peptide activates mast cells to produce strong proinflammatory mediators (LTC4 and PGD2) [86]. These factors play a critical role involved in inflammatory and anti-inflammatory responses. In line with this, LAD2 cells were treated with 1 μg/ml cathelicidin which tended to increase the level of TLR4 expression, Th1 cytokines IL-2, proinflammatory cytokines TNF-α and IL-1β and significantly induced Th2 cytokines, IL-4 and IL-5 release; however, significantly Th2 cytokines could be inhibited by LPS, although IL-1β production was not diminished [87]. Thus, these data reveal that LL-37 synergism the bacterial components may skew the mast cell toward innate immunity and adaptive immunity [87].

While studying the functional effects of LL-37 on mast cells, some literature also explored and described its mechanism of action. LL-37 may bind the negatively charged cell surface molecules, rapidly internalize into the cells via clathrin-mediated endocytosis, and interact with Mas-related gene X2 (MrgX2) to activate mast cell (LAD2 cell) degranulation and release of de novo synthesized mediator function primarily; this effect is associated with the activation of the Gi protein, PLC/PKC/Calcium/NFAT, PI3K/Akt, and MAPK signaling pathways [34, 88, 89]. Notwithstanding the foregoing, LL-37 (10 μM) enable to permeabilize both nuclear and plasma membranes to enhance the export of nucleic acids of mast cells, total protein, and lactate dehydrogenase (LDH) [90]. Thus, it was proposed that LL-37-induced release of nucleic acids from mast cells may be another mechanism of LL-37 moderating the immune response.

5.5. MSCs. MSCs are pluripotent stem cells that share all the commonalities of stem cells, namely, self-renewal and multidirectional differentiation. MSCs regulate the innate immune system and adaptive immune system function through direct contact between cells and secretion of mesenchymal stem cells. MSCs can induce immunomodulatory effects on various cells associated with carcinogenesis via producing a variety of cytokines and growth factors [91, 92].

LL-37 augments the promoting tumorigenesis properties of MSCs by recruiting them to ovarian tumors through FPR2 [37]. Follow-up researches in vitro show MSCs, after 48 h of LL-37 treatment, were stimulated to release significantly more angiogenic and inflammatory molecules compared with untreated cells, including IL-1 receptor antagonist, IL-6, IL-10, CCL5, vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) [37, 93]. Besides LL-37 treatment enhanced the proliferation and migration of human adipose-derived stromal/stem cells (ASCs), it also promotes expressing FPR2, early growth response (EGR) 1 expression, and MAPK activation, and that preconditioning of ASCs with LL-37 has a strong potential to promote cell proliferation, cell migration, and paracrine actions, which may be useful in terms of implantation for tissue regeneration [37]. However, research in vivo was indicated that LL-37-mediated recruitment of MSCs can also facilitate ovarian tumor progression through secreting proangiogenic factors which resulted in a significant increasing number of vascular channels in nude mice and some cytokines including IL-1β, IL-6, IL-8, IL-10, and TNF-α (and the reduction of IL-12 expression). Consistently, in vitro endothelial cell formation by MSCs is enhanced by LL-37 presence with a positive effect on tumor growth [37]. Moreover, LL-37 modulates TLR3 expression, promotes higher levels of anti-inflammatory factors (indoleamine2,3-dioxygenase (IDO), IL-10, and TGF-β), and boosts the suppressive function of pMSCs over stimulated T cells; thus, LL-37 may offer protection against opportunist microorganisms, meanwhile ensuring the maintenance of MSCs in their highest anti-inflammatory state [94]. Therefore, the LL-37 boost proliferation, immunosuppressive, and migratory potential of MSCs to promoting tumorigenesis or anti-inflammation.

6. Diseases

Defects in the expression or processing of immunomodulatory peptide, resulting in abnormalities in immune regulation, lead to inflammation-related diseases such as inflammatory bowel disease (IBD), psoriasis, periodontal disease, or cancers. Thus, detailed knowledge about the associated molecular mode of action on tissues and their various cells is necessary to understand the pathogenesis of these diseases.

6.1. Inflammatory Bowel Disease. IBD, including UC and CD, is an idiopathic enteritis disease involving the ileum, rectum, and colon. It is unclear about the etiology and pathogenesis [83]. As far as we know, an abnormal reaction of the intestinal mucosal immune system results in inflammatory response of IBD. It is difficult to cure using current treatments, and new therapies are needed. More than one document has reported increased expression of LL-37 on the intestinal mucosa of patients with IBD. Moreover, this high expression occurs simultaneously in the inflammatory and uninflamed colonic mucosa of UC patients [95]. Nowadays, the regulatory mechanism of LL-37 induction was investigated that in human colonic SEMFs, the expression of LL-37 upregulating was probably induced by TLR-3 stimulation via poly(I:C) [20, 96]. Then, the increasing complex of LL-37-bacDNA may further promote more expression of LL-37 in primary human monocytes by activating the TLR9-ERK1/2 pathway and the differentiation of T cells towards Th1, Th2, and Th17 to huge scope inflammation [97–99]. Perhaps precisely because of this, bacteria may make a milieu by releasing bacDNA to utilize and resist host antimicrobial peptides as a "trojan horse" in IBD to evade immune elimination [99]. Moreover, LL-37 levels may be a marker to reflect intestinal stricture in CD patients, low levels presage a significant elevated risk of intestinal stricture, and high levels relate to good prognosis [98, 100].

Although current studies show that LL-37 appears to be an accomplice of bacterial mucosal inflammation in the etiology of IBD, there are still many studies that show that LL-37 is a new direction for the treatment of IBD. In the inflamed mucosa of IBD, LL-37 still might exert antibacterial and neutralization of LPS activities to defend the intestine from pathogen invasion and superabundance inflammation [20]. This result is consistent with other reports that administration of cathelicidin and analog is effective in UC and CD models. mCRAMP (an analog of LL-37) could attenuate dextran sulfate sodium- (DSS-) induced colitis in a murine model and relieved neutrophil infiltration in colitis tissues [101]. Cathelicidin-BF (C-BF), a snake cathelicidin-derived antimicrobial peptide, which has antibacterial activity, mitigates inflammation and ameliorates damaged barrier of DSS-induced ulcerative colitis in vivo via inhibited phosphorylation of NF-κB (p65) [102, 103]. Moreover, a short-term treatment with 2000 IU/day vitamin D significantly increased 25(OH)D levels in blood which facilitate to elevate the level of LL-37 to exert immune-modulatory and anti-inflammatory effects to prolong remission in CD [104].

Recently, cathelicidin gene and/or recombinant protein therapy for UC and CD seems to be popular. The mCRAMP-encoding plasmid may reverse increased levels of cytokines and apoptosis, promote mucus protein expression and secretion, and prevent ulcerative colitis by regulating inflammation and mucus secretion in exacerbated colitis cnlp$^{-/-}$ mice through the intrarectal administration [105]. Another study oral administration of mCRAMP-transformed *Lactococcus lactis* effectively produce mCRAMP and alleviated the degree of inflammation reflected by the decrease of the number of apoptotic cells, myeloperoxidase activity, and malondialdehyde level; then, the clinical symptoms were improved, crypt integrity is maintained, and the mucus content is preserved [106]. Additionally, injection of cathelicidin-overexpressing lentiviruses induced collagen expression to efficiently attenuate colitis-associated intestinal fibrosis through inhibiting transforming growth factor-1 (TGF-1) and IGF-1 [107]. Therefore, cathelicidin might be useful for patients with IBD via regulating the intestinal

mucosal immune system. LL-37 seems to be both morbific and treatable; as mentioned above, the reasons might be the microenvironmental impact or the difference between endogenous or exogenous; however, the true face of LL-37 needs further exploration.

6.2. Psoriasis. Psoriasis is a long-lasting autoimmune, chronic inflammatory skin disease; disturbances in the innate and adaptive cutaneous immune responses lead to uncontrolled keratinocyte proliferation and dysfunctional differentiation, characterized by patches of abnormal skin [108]. Many studies have shown that psoriasis is associated with abnormal expression and activity of cathelicidin.

Even though the exact role of LL-37 in the pathogenesis of psoriasis remains unclear, it was found LL-37 were significantly increased in psoriatic plaques and LL-37 play an important role in psoriasis. LL-37 can affect keratinocyte, activate innate and adaptive cutaneous immune responses, and maintain the autoinflammatory cascade [109]. It was indicated that cathelicidin not only induces keratinocyte migration and proliferation [110] but also stimulates keratinocytes to release different effect cytokines (including IL-1β, IL-6, IL-18, IL-20, and GM-CSF), chemokines (i.e., CCL2, CCL5, CCL20, CXCL8, and CXCL10), and anti-inflammatory cytokine IL-10 via EGFR, G protein, and PLC signaling pathways [110–112]. In addition, LL-37 enhance UV-induced IL-1β secretion and inflammasome activation via acting on the P2X7 receptor on keratinocytes [113].

Both LL-37 or the complex LL-37 and nucleic acid regulate immune responses in psoriasis. LL-37 stimulate mDCs to secrete TNF-α and IL-6, and mDCs are able to activate naïve-T cells and induce their polarization to Th1/Th17 cells in psoriasis [53, 79, 114, 115]. Meanwhile, LL-37 isolated from lesioned psoriatic skin scavenges can form complexes with human self-nucleic acid from dying cells. The LL-37/self-DNA complexes are sensed by dermal pDCs via endocytosis and stimulate IFN-α response via the TLR9/MyD88/IRF7 signaling pathway [116, 117] whereas LL-37/self-RNA complexes can activate TLR7 to release IFN-α [77]. Then, large amounts of IFN-α and activated pDCs and mDC activate downstream self-reactive T cells, which mediate immune responses and result in psoriatic lesion formation [76, 117]. Thus, LL-37 converts inert self-nucleic acid into a potent trigger of interferon production by pDCs in psoriatic skin [76].

Interestingly, researcher found that two-thirds of patients with moderate-to-severe plaque psoriasis harbor $CD4^+$ and/or $CD8^+$ T cells specific for LL-37 infiltrating lesioned skin, which produce IFN-γ (Th1 cytokines), and $CD4^+$ T cells also produce Th17 cytokines (IL-17, IL-21, and IL-22) [23]. The subsequent silico docking study further predicted the high binding affinities of multiple 9-mer peptides derived from LL-37 to the *HLA-C** 06:02 molecule to propose a mechanism of the interaction between this complex and T cells via TCRs LL-37-*HLA-C** 06:02 [118]. Thus, this study provides evidence for a role of LL-37 in psoriasis.

Besides DCs and T cells, polymorphonuclear leukocytes (PMNs) are abundant in psoriatic skin and are primary sources for LL-37. The human and bacterial RNA complexed with LL-37 not only stimulate PMNs from psoriasis patients that respond via TLR8 by producing TNF-α, IL-6, IL-8, and IL-1β, and NET-release; they also can be released by PMNs. The same complex and complex RNA-LL-37 were found to be highly abundant in PMNs from psoriasis patients compared to PMNs from healthy donors [119, 120]. Moreover, RNA-LL37-induced NETs propagated PMN activation and could thus fuel a PMN-mediated and self-sustaining inflammatory loop that may represent an unexpected early initiator or amplifying event in psoriasis. Therefore, in psoriatic lesions, RNA-LL37-driven PMN activation may contribute to a vicious cycle of inflammation and immune cell attraction [121].

6.3. Periodontal Diseases. Periodontal disease refers to the disease that occurs in periodontal tissue, including gingival disease with inflammation only involving gingival tissue and periodontitis involving deep periodontal tissue (periodontal membrane, alveolar bone, and cementum). Periodontal disease is a common oral disease, which is the main cause of tooth loss in adults. It is a refractory disease, which cannot be cured for a long time, and is easy to develop into chronic. The main clinical manifestations are alveolar bone absorption, periodontal bag formation, gingival bleeding and inflammation, tooth loosening, and so on. This kind of lesions is caused by dysregulation of microbiota-host homeostasis of oral cavity which can give rise to inflammation and bone loss [121].

Salivary glands, oral mucosa, and immune cells in the oral cavity can express this kind of peptide [122]. Some studies found that some diseases (like Kostmann syndrome, periodontal disease-associated bacteria, and chronic periodontitis) in humans are related to the aberrant level of cathelicidins [123–126]. Patients with Kostmann syndrome often have low levels of LL-37 in serum and saliva because of deficiency of neutrophils, with severe alveolar bone loss or even periodontal ligament infection [124, 125]. About a third of the aggressive periodontitis patients lack active cathelicidin in the gingival crevicular fluid [123]. As antimicrobial peptides, of course, inhibiting the growth of various periodontal bacteria (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*) to keep the microbiota-host homeostasis is just one function of LL-37 [127, 128]. In oral cavity and the skin, as we described previously in this review, the interaction between TLR ligands (as LPS and flagellin), self-DNA or self-RNA, and LL-37 may be involved in infection and inflammation [127, 128]. On the other hand, TLR ligands induce receptor activator of nuclear factor kappa-B ligand (RANKL) expression in osteoblasts and TNF-α production in BMMs. RANKL binds to RANK expressed in osteoclast precursors and subsequently induces osteoclast differentiation [129, 130]. Mature osteoclasts also express RANK and TLR4 [131] and promote the bone-resorbing activity of osteoclasts through TRAF6 which is a common downstream molecule [131–133], while TLR ligands also induce LL-37 expression in several different host cells including osteoblasts and immune cells. Thus, LL-37 can inhibit TLR ligands that induced

inflammation and bone loss through antimicrobe and neutralize LPS and flagellin.

However, beyond that, Kittaka et al. found for the first time that LL-37 can regulate angiogenesis and the recruitment of stem cells to promote bone regeneration [134]. It is observed that morphologically fibroblastic cells with STRO-1^+ (a marker of MSCs), at an early stage of tissue regeneration in a rat suffering from calvarial bone defect treated with cathelicidins, accumulated in the bone defect area where endothelial cells were also localized. Recently, Yu et al. further proved these findings. It is found that LL-37 promoted bone marrow stromal cell (BMSC) proliferation, migration, and osteogenic differentiation within normal and inflammatory microenvironments via P2X7 receptor and MAPK signaling pathway and can inhibit inflammation, markedly inhibiting osteoclastic bone resorption through P2X7 receptor and MAPK pathway [135].

As we all know, not only limited to the oral cavity, LL-37 plays a very important role in the promotion of bone repair in the bone-related diseases especially for inflammation-induced bone loss, osteoporosis, bone fracture, and so on. Zhang et al. first showed that LL-37 entered monocytes from blood source through the endocytosis of CXCR2 and promoted its differentiation into novel bone-forming cells (monosteophils) [55, 56]. Furthermore, Zhang et al. confirmed by experiments in vivo that LL-37 can promote bone repair in an animal model of bone injury by inducing monocytes to human monoosteophils, characterized as $CD45^+\alpha3^{+-}$ $\alpha3\beta^+CD34^-CD14^-BAP$ (bone alkaline phosphatase)$^-$ cells [57]. At the same time, Supanchart et al. also confirmed that LL-37 inhibits the in vitro osteoclastogenesis via preventing nuclear translocation of NFAT2 (the main switch of osteoclast differentiation and bone resorption) by inhibiting the calcineurin activity [136].

At present, the study demonstrates that LL-37 can be a potential candidate drug for promoting osteogenesis and for inhibiting bacterial growth and osteoclastogenesis. Liu et al. proved this peptide significantly promotes MSC differentiation, migration, and proliferation, inhibiting LPS-induced osteoclast formation and bacterial activity in vitro; so, LL-37 combined with bone morphogenetic protein 2 (BMP2) can regulate MSCs to promote calvarial repair in an osteolytic model [137]. And LL-37 had been used as modification of bone implants to optimize osteointegration ability. He et al. loaded LL-37 on Ti substrates that benefited the cell viability, recruitment, and paracrine responses of MSCs and macrophages in vitro and induced MSC and macrophage recruitments to injury sites, and the inflammatory response was positively regulated, facilitated bone formation, and improved osteointegration via the regulation of physiological functions of MSCs and macrophages in vivo [138, 139]. These studies provide a promising strategy in the design of bone repair-related oral and bone-related diseases.

6.4. Cancer. Last few years, some evidence from cancer biology studies indicates that human cathelicidin is involved in carcinogenesis. As noted above, this peptide expression is out of control and dysregulated in some cancer types. For example, LL-37 is upregulated in various ovarian tumor subtypes, compared with normal ovarian tissues [140]. In these tumors, LL-37 has been shown to promote tumor progression through its influence on mesenchymal stromal/stem cells [37, 94, 141] via some activated pathways, such as FPR2 [142, 143], IGF-1receptor and ErbB2 [144, 145], and CXCR4 [146]. At the same time, LL-37 also promotes tumor formation by affecting immune-active cells. The interaction of LL-37 with TAM is mentioned above. Overexpressed LL-37 in tumors promotes the differentiation of macrophages to M2, which in turn promotes tumorigenesis. Furthermore, the human cathelicidin is significantly expressed by TAMs present, which promote the proliferation of colorectal cancer cells. Thus, LL-37 as a tumor microenvironment factor plays a critical role in tumorigenesis. Similarly, LL-37 augments the promoting tumorigenesis properties of MSCs by recruiting them to ovarian tumors and enhancing their proliferation and migration, facilitating tumor progression through secreting proangiogenic factors and some cytokines resulted in a significant number of vascular channels and immunosuppressive.

The antitumor activity of LL-37 may be linked to its role to mediated apoptosis and as an immunomodulatory agent. In colon cancer, studies indicated that LL-37 suppressed tumor development through different pathways. Ren et al. suggested that LL-37 inhibited colon cancer by the activation of a GPCR-p53-Bax/Bak/Bcl-2 signaling cascade that triggers AIF/EndoG-mediated apoptosis, rather than caspase-dependent apoptosis [147]. Cheng et al. found that LL-37 inhibited colon cancer development through indirect pathways, which include interference with epithelial-mesenchymal transition of colon cancer cells and suppression of fibroblast-supported colon cancer cell proliferation [148]. As an immunomodulatory agent of LL-37, studies also have shown that this peptide enhanced the sensing of CpG oligodeoxynucleotides by immunocompetent cells (B cells, pDCs, and NK cells), and these CpG oligodeoxynucleotides enhance the antitumor activity through affecting TLR9 [140] and induce IFN-γ expression, proliferation, and activation of NK cells in treated tumors [81]. Furthermore, LL-37 induced an activation and expansion of OVA-antigen-specific $CD8^+$ T cells in draining lymph nodes and the tumor microenvironment [149]. This process was associated with delay in tumor growth, while preclinical studies have also demonstrated that intratumoral injections of LL-37 stimulate the innate immune system by the activation of pDCs [150]. These cells can induct and maintain antitumor immune responses and mediate tumor destruction [151]. These findings suggest that LL-37 could induce antitumor immunity and provide a promising strategy for immunotherapy.

7. Conclusions

Recent work has conclusively demonstrated that human cathelicidin LL-37 represents a chemical defense as an essential component of innate immunity that eliminates invading pathogens and restores homeostasis. In addition to its antimicrobial activities, accumulated evidence reveals pleiotropic functions of LL-37 that influence immune responses (see Figure 1). The immunomodulatory function of LL-37 has

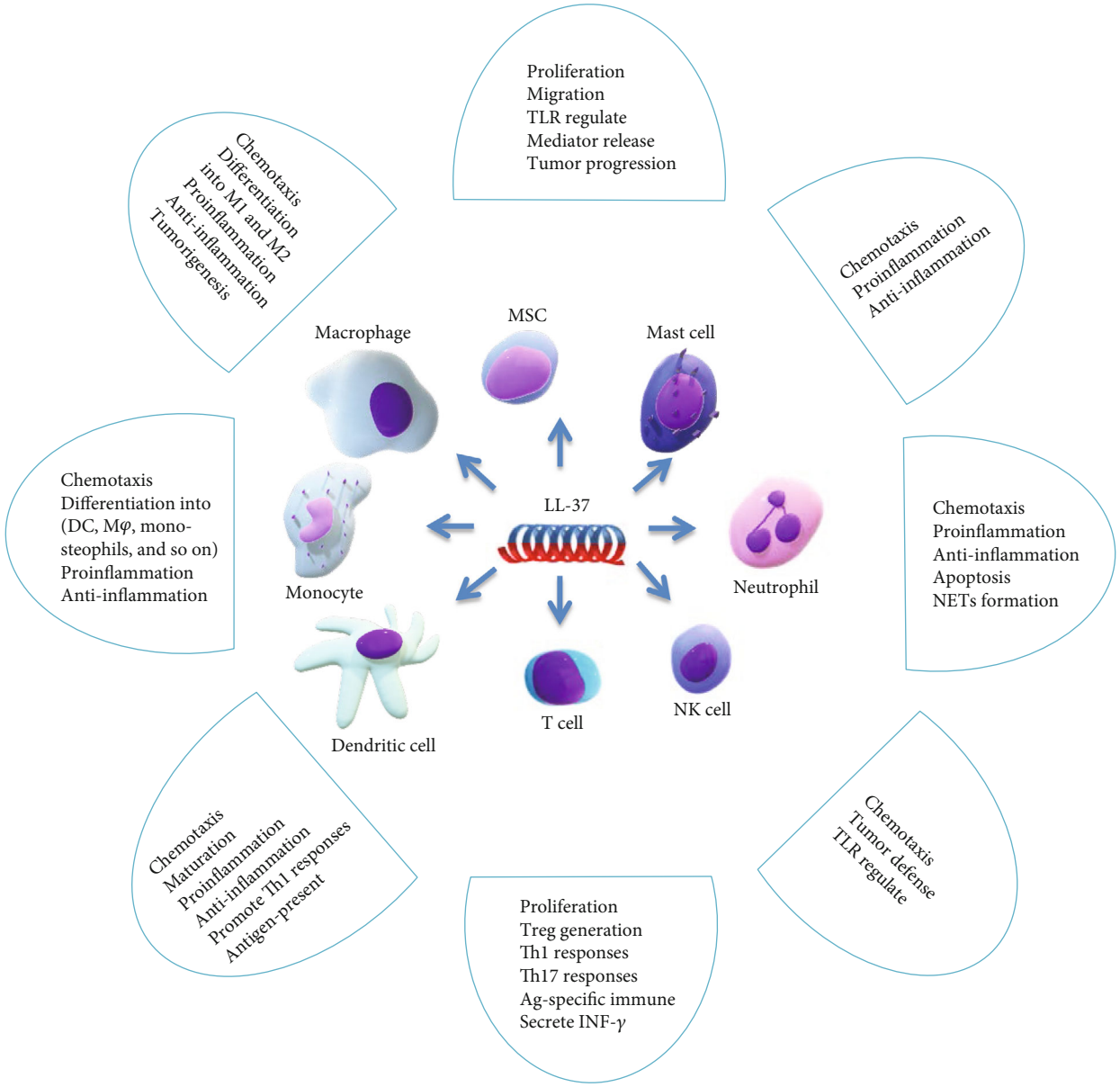

Figure 1: Immunomodulatory function of LL-37.

its two sides. On the one hand, LL-37 can chemotactically activate cells to the infected or abnormal parts of the body and can synergize with other active substances to promote the differentiation of immunocompetent cells into proinflammatory cells that promote immune responses; for example, monocyte can be further differentiated into macrophages or DCs; macrophages can differentiate into M1-type cells, and DCs can further mature to present more antigen; T cells can differentiate into Th1, Th17 type cells, etc.; LL-37 can stimulate immunocompetent cells to secrete proinflammatory cytokines, chemokines, costimulatory factors, cellular receptors, etc., promote immune response, exert its anti-infective and antitumor effects, bridge innate immunity and adaptive immunity, and promote the responses of Th by DCs to start up the secondary immune system. On the other hand, LL-37 also has the ability to inhibit inflammation and promote carcinogenesis. This peptide can augment the release of anti-inflammatory cytokines, neutralize bacterial LPS, inhibit the release of proinflammatory factors, limit the expansion of inflammation, maintain the body's immune balance, and at the same time, recruit MSCs into tumors to enhance the immunosuppressive effect; in the tumor environment, LL-37 facilitate macrophage differentiation to anti-inflammatory M2-type cells, along with MSCs exerting carcinogenesis.

Under normal circumstances, LL-37 can help the body maintain homeostasis, but once this steady state is broken, LL-37 can become a disease-causing factor (see Figure 2). In IBD, psoriasis LL-37 is overexpressed. In the case, LL-37 promotes the progression of inflammation, destroying the body's homeostasis and causing autoimmune disease. In tumors, overexpressed LL-37 can affect MSCs and macrophage to promote tumor cell growth and promote tumorigenesis. Due to the ubiquitous expression in different

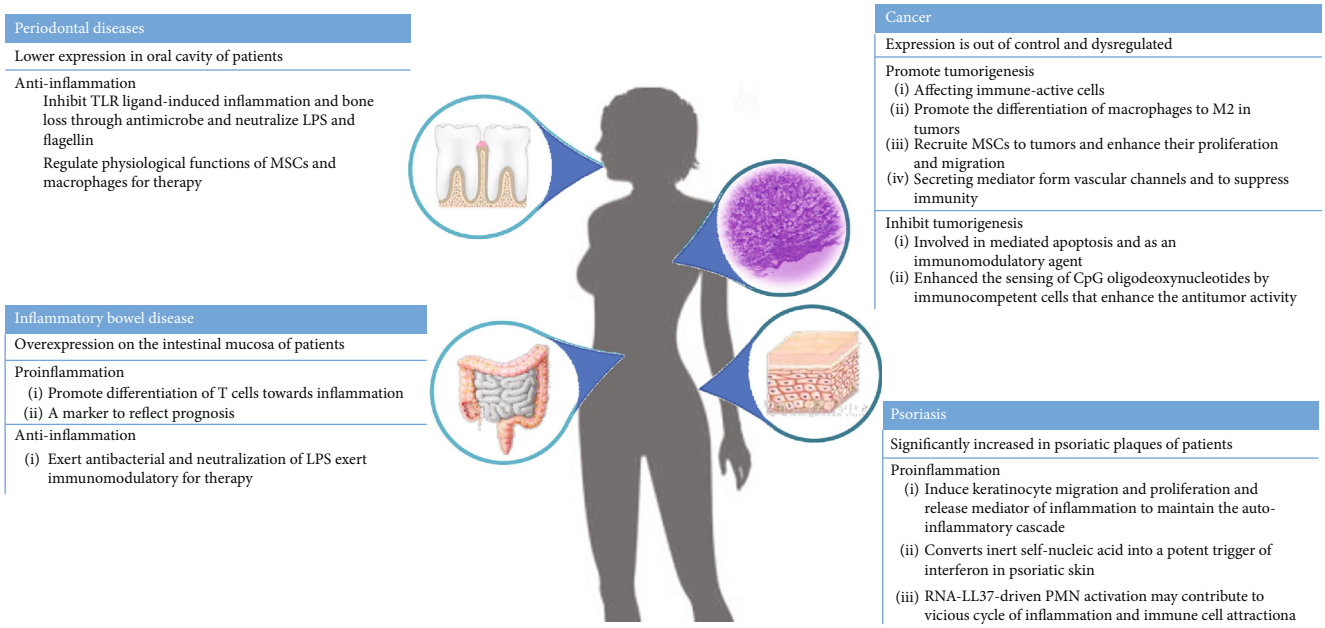

Figure 2: The roles of LL-37 in the diseases.

anatomical sites, the production of LL-37 appears to be regulated in a tissue- or even cell-specific manner. The insufficient expression of LL-37 may increase susceptibility to infections and inflammation, such as periodontal diseases; the patients with severe alveolar bone loss or even periodontal ligament infection always accompany with low levels of LL-37 in serum and saliva. Further advances in understanding the biological activity of LL-37 will create an attractive target for therapeutic intervention in infectious and inflammatory diseases.

Conflicts of Interest

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

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Research Article

A 5-Gene Signature Is Closely Related to Tumor Immune Microenvironment and Predicts the Prognosis of Patients with Non-Small Cell Lung Cancer

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Purpose. Establishing prognostic gene signature to predict clinical outcomes and guide individualized adjuvant therapy is necessary. Here, we aim to establish the prognostic efficacy of a gene signature that is closely related to tumor immune microenvironment (TIME). *Methods and Results*. There are 13,035 gene expression profiles from 130 tumor samples of the non-small cell lung cancer (NSCLC) in the data set GSE103584. A 5-gene signature was identified by using univariate survival analysis and Least Absolute Shrinkage and Selection Operator (LASSO) to build risk models. Then, we used the CIBERSORT method to quantify the relative levels of different immune cell types in complex gene expression mixtures. It was found that the ratio of dendritic cells (DCs) activated and mast cells (MCs) resting in the low-risk group was higher than that in the high-risk group, and the difference was statistically significant ($P < 0.001$ and $P = 0.03$). Pathway enrichment results which were obtained by performing Gene Set Variation Analysis (GSVA) showed that the high-risk group identified by the 5-gene signature had metastatic-related gene expression, resulting in lower survival rates. Kaplan–Meier survival results showed that patients of the high-risk group had shorter disease-free survival (DFS) and overall survival (OS) than those of the low-risk group in the training set ($P = 0.0012$ and $P < 0.001$). The sensitivity and specificity of the gene signature were better and more sensitive to prognosis than TNM (tumor/lymph node/metastasis) staging, in spite of being not statistically significant ($P = 0.154$). Furthermore, Kaplan–Meier survival showed that patients of the high-risk group had shorter OS and PFS than those of the low-risk group ($P = 0.0035$, $P < 0.001$, and $P < 0.001$) in the validating set (GSE31210, GSE41271, and TCGA). At last, univariate and multivariate Cox proportional hazard regression analyses were used to evaluate independent prognostic factors associated with survival, and the gene signature, lymphovascular invasion, pleural invasion, chemotherapy, and radiation were employed as covariates. The 5-gene signature was identified as an independent predictor of patient survival in the presence of clinical parameters in univariate and multivariate analyses ($P < 0.001$) (hazard ratio (HR): 3.93, 95% confidence interval CI (2.17–7.1), $P = 0.001$, (HR) 5.18, 95% CI (2.6995–9.945), $P < 0.001$), respectively. Our 5-gene signature was also related to EGFR mutations ($P = 0.0111$), and EGFR mutations were mainly enriched in low-risk group, indicating that EGFR mutations affect the survival rate of patients. *Conclusion*. The 5-gene signature is a powerful and independent predictor that could predict the prognosis of NSCLC patients. In addition, our gene signature is correlated with TIME parameters, such as DCs activated and MCs resting. Our findings suggest that the 5-gene signature closely related to TIME could predict the prognosis of NSCLC patients and provide some reference for immunotherapy.

1. Introduction

Lung cancer remains the leading cause of cancer morbidity and mortality, with 2.1 million new lung cancer cases and 1.8 million deaths expected in 2018 [1]. NSCLC accounts for up to 85% of all lung cancers and mainly comprises adenocarcinoma (65%) and squamous cell carcinoma (30%) histologies [2]. In the past few years, although molecular diagnostics and new treatments (targeted therapy, immunotherapy, etc.) have made much progress and the 5-year survival rate of most patients has increased slightly, the overall prospects have not been very large [3, 4].

The current TNM staging system is the best predictor of prognosis and the standard for guiding NSCLC treatment decisions [5]. However, due to the heterogeneity of the tumor itself and the complexity of the pathogenesis, even patients with the same TNM stage and treatment may exhibit various clinical outcomes [6]. Through microarray gene expression profiling to analyze and screen gene expression characteristics and establish a prognostic gene signature, it is better to predict clinical outcomes and guide the adjuvant treatment of individual patients than TNM staging. So far, several studies based on gene expression signatures have been shown to classify various cancer patients into different prognostic groups with different clinical characteristics [7–11]. However, the gene signatures closely related to TIME have not been found in NSCLC.

The type, density, and location of immune cells in the tumor microenvironment play an important role in the development of the disease [12]. Therefore, immunological structures based on the tumor microenvironment should be used as a separate component in the classification system [13]. Incorporating TIME parameters into gene signature will be more conducive to individualized treatment options [14]. However, regardless of the single monoclonal antibody immunohistochemistry technique or the flow cytometry of multiple antibodies, consistent and accurate data on immune cell composition were not obtained [15–20]. Therefore, the exact immune cell content in different tumors of NSCLC remains accurately undetermined. Several reports indicated that the relative levels of distinct immune cell types by the analytical platform CIBERSORT could estimate the immune cell composition in a tumor [21–23].

In this study, we used downloaded gene expression data and identified a 5-gene signature using univariate survival analysis and LASSO to distinguish between two prognostic groups (low and high risk). Then, we used the CIBERSORT method to quantify the relative levels of different immune cell types in complex gene expression mixtures. Furthermore, the validity and reliability of the 5-gene signature were further verified. Our findings suggest that the 5-gene signature closely related to TIME could predict the prognosis of lung cancer patients and provide some reference for immunotherapy.

2. Materials and Methods

2.1. Data Source and Processing. Gene expression profiling data of NSCLC patients were downloaded from Gene Expression Omnibus datasets (GEO; GSE103584, GSE31210, GSE41271) and the Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/). Microarray analysis of 130 NSCLC patients in GSE103584 is based on Cancer SCAN panel [24]. The dataset GSE103584 was used as a training set for model construction, and data in GSE31210 [10], GSE41271 [25], and TCGA were applied to verify the validity of the model.

2.2. Screening for Prognosis-Related Genes and Building Risk Models. The LASSO was a better high-dimensional regression classifier and was used to select the key genes influencing patient outcomes [26]. The LASSO 1000 iterations were performed using the publicly available R package glmnet [27]. Multiple genomes containing the optimal solution were received after multiple dimensionality reduction. At the same time, for the stability and accuracy of the results, a random sampling method of leave-one-out cross validation (LOOCV) was used to select a set of genes to construct a prognostic model [26].

According to the selected genetic model, a risk formula of risk score was constructed to evaluate the high-risk and low-risk groups. The formula for obtaining the score is $\Sigma_i \omega_i \chi_i$, where ω_i and χ_i are the coefficients and expressed value of each gene. The risk score for each sample in the data in the training set was calculated according to the formula, and the best cutoff value was generated using X-tile plots [28]. This threshold was set to classify patients: higher than the best cutoff for the low-risk group and lower than the risk score for the high-risk group.

2.3. Estimating the Composition of Immune Cells. To estimate the immune cell composition in the sample, the analytical platform CIBERSORT (https://cibersort.stanford.edu/) was used to quantify the relative levels of distinct immune cell types within a complex gene expression mixture [29]. The analysis was performed with an arrangement of 100 default statistical parameters. The activation and quiescence state of the same type of immune cells were analyzed as a whole. CIBERSORT's deconvolution of gene expression data provides valuable information about the composition of immune cells in a sample.

2.4. Analyzing Pathways with Differential Enrichment. GSVA, a pathway enrichment method that estimated variation of pathway activity over a sample population, was used to analyze changes in a pathway in each sample. GSVA was an open-source software package for R which forms part of the Bioconductor project and could be downloaded at http://www.bioconductor.org [30].

The prediction of the pathway under different disease states was made by the signal value of the gene and the

pathway in which the gene was located. Firstly, the enriched score value of each sample was predicted by the signal value of the gene, and then the enrichment difference between the two groups was calculated, and the pathway with differential enrichment in the two groups was obtained. The screening standard $P < 0.05$, and the FDR < 0.05.

2.5. Validation of the Validity and Reliability. Univariate survival analysis of the gene signature was assessed by using survival in R language ($P < 0.05$) [31]. Then survival receiver operating characteristic curve (ROC) was used to complete the area under the curve (AUC) of 5-gene signature and TNM classification [32]. External data from GSE31210, GSE41271, and TCGA were applied to verify the reliability of the risk model's impact on the prognosis of the patients.

Fisher exact was used to assess the correlation between different gene mutation types and risk models. The univariate and multivariate Cox proportional hazard regression analyses were used to evaluate independent prognostic factors associated with survival. Risk model, lymphovascular invasion, pleural invasion, chemotherapy, and radiation were employed as covariates.

3. Result

3.1. Screening Genes Associated with Prognosis and Building Risk Models. There are 13,035 gene expression profiles from 130 tumor samples in the data set GSE103584 (Supplementary material 1). First, the data of GSE103584 was processed uniformly, and then the genes detected in more than 50% of the samples were screened out and normalized. We applied the LASSO Cox regression model to predict and analyze the genes most relevant to prognosis in the 130 sample data. A random sampling method of 10-cross validation was used to construct a prognostic model containing five genes (Figure 1(a)). Through calculation and verification, it is found that the model constructed by 5 genes has the lowest error rate (Figure 1(b)). Figure 1(c) shows the specific information and coefficients of the five genes. Characteristics of the patient in the training set (GSE103584) are given in Table 1.

3.2. Estimating the Composition of Immune Cells. We used CIBERSORT to estimate the immune cell composition of 130 samples and quantify the relative levels of different cell types in a mixed cell population. All results were normalized to proportions by cell type (Supplementary material 2). As shown in Figures 2(a) and 2(b), we compared different types of cells in the low-risk group and the high-risk group. It was found that the ratio of dendritic cells activated and mast cells resting in the low-risk group was higher than that in the high-risk group, and the difference was statistically significant ($P < 0.001$ and $P = 0.03$). The results suggested that the immune cells in the low-risk group were better activated.

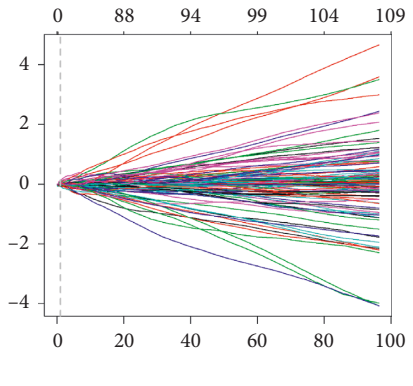
3.3. Analysis of Differential Pathways. By performing GSVA analysis on the differential genes of the low-risk group and the high-risk group, the changes in the relevant pathways in different states were obtained. Figure 3 shows the changes in the pathways of 130 samples in the low-risk and high-risk groups. The result of the enrichment is SHEDDEN_LUNG_CANCER_GOOD_SURVIAL_A4, indicating that the prognostic grouping of the data is consistent with other data. LIAO_METASTASIS is gradually increasing in the low-risk group and the high-risk group, indicating high meta-expression of metastasis-related genes in the high-risk group.

3.4. Validation of the Validity and Reliability. Survival analysis in R language pack was applied to examine the effects of different groups on the prognosis of NSCLC. Kaplan–Meier survival curves for relapse-free survival indicated the probability of recurrence in the high-risk group and the low-risk group. The results showed that patients in the high-risk group had shorter disease progression times than those in the low-risk group (Figure 4(a), $P = 0.0012$). Kaplan–Meier survival curves for overall survival were used to represent the survival probabilities of the high-risk group and the low-risk group. The results showed that patients in the high-risk group had shorter overall survival than patients in the low-risk group (Figure 4(b), $P < 0.001$).

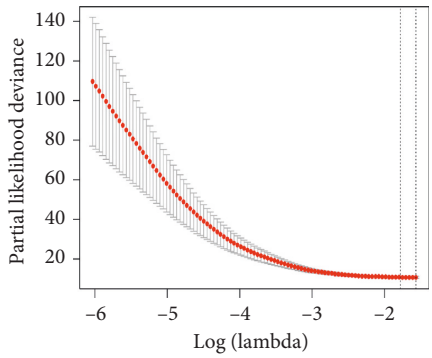
To further validate the accuracy of the risk prediction model, we established a ROC plot of the hazard model and TNM staging. As shown in Figure 4(c), we found that risk prediction models could be more sensitive to prognosis than TNM staging, in spite of being not statistically significant ($P = 0.154$).

Furthermore, external data from GSE31210, GSE41271, and TCGA were applied as a validating set to verify the validity and reliability of the 5-gene signature impact on the prognosis of the patients. Kaplan–Meier survival showed that patients in the high-risk group had shorter overall survival than patients in the low-risk group (Figure 5(a), $P = 0.0035$ and Figure 5(b), $P < 0.001$) and patients in the high-risk group had shorter progression-free survival than those in the low-risk group (Figure 5(c), $P < 0.001$).

3.5. Correlation with Mutant Genes and Clinical Information. By observing the correlation between the predicted risk model and different mutant genes, we found that EGFR mutations were related to the risk model grouping ($P = 0.011$), and EGFR mutations were mainly enriched in low-risk, indicating that EGFR mutations affect the survival rate of patients (Table 2). However, there was no correlation between ALK and KRAS gene mutations and risk models ($P > 0.05$). The univariate and multivariate Cox proportional hazard regression analyses were used to evaluate independent prognostic factors associated with survival. Risk model, lymphovascular invasion, pleural invasion, chemotherapy, and radiation were employed as covariates. It was found that the risk model constructed by the 5-gene signature was an independent risk factor for prognosis (Table 3, $P < 0.001$).



(a)



(b)

Gene	Coefficient
SLC04C1	0.076599572
ELAC1	0.002406076
HLF	0.007256909
ZNF204P	0.036331089
ST3GAL5	0.029739516

(c)

Figure 1: Screening genes associated with prognosis and building risk models. (a) Trend graph of LASSO coefficients. (b) Partial likelihood deviation map. (c) The name and coefficient of the 5-gene signature closely related to the immune system.

Table 1: Clinicopathological characteristics of NSCLC patients in the training set.

Variables	Number	%
Age		
<65	37	28.5
≥65	93	71.5
Sex		
Female	34	26.2
Male	96	73.8
Histology		
Adenocarcinoma	96	73.8
Squamous	31	23.8
Other	3	2.3
T stage		
Tis	5	3.8
T1	53	40.8
T2	49	37.7
T3	16	12.3
T4	7	5.4
N stage		
N0	104	80
N1	12	9.2
N2	14	10.8
Radiation		
Yes	14	10.8
No	116	89.2
Chemotherapy		
Yes	37	28.5
No	93	71.5
EGFR status		
Yes	19	14.6
No	82	63.1
Unknown	29	22.3
ALK status		
Yes	2	1.5
No	97	74.6
Unknown	31	23.8
KRAS status		
Yes	24	18.5
No	77	59.2
Unknown	29	22.3

4. Discussion

Based on gene expression data and survival analysis techniques, we screened a 5-gene signature for predicting the prognosis of NSCLC patients. That is, differential expressions of 5 genes among Solute carrier organic anion transporter family member 4C1(SLCO4C1), ElaC ribonucleaseZ1(ELAC1), Hepatic leukemia factor (HLF), Zinc finger protein 204, pseudogene (ZNF204P), and ST3 beta-galactoside alpha-2,3-sialyltransferase 5 (ST3GAL5) will influence progression-free survival and survival time of NSCLC patients. External data from GSE31210, GSE41271, and TCGA were applied to verify the reliability of the 5-gene signature impact on the prognosis of the patients. To further validate the accuracy of the 5-gene signature, we established a ROC map of the hazard model and TNM staging. The sensitivity and specificity of the gene signature were better and more sensitive to prognosis than TNM staging, in spite of being not statistically significant ($P = 0.154$).

We not only confirmed the stability and accuracy of the 5-gene signature, but also found it closely related to other clinical information. The changes in the relevant pathways in the differential genes of the low-risk group and the high-risk group were obtained by performing GSVA analysis. The results showed that the high-risk group identified by 5-gene signature had metastatic-related gene expression, resulting in lower survival rates. Our 5-gene signature was also related to EGFR mutations ($P = 0.011$), and EGFR mutations were mainly enriched in the low-risk group, indicating that EGFR mutations affect the survival rate of patients. The univariate and multivariate COX regression model analysis was used to analyze the correlation between the 5-gene signature and other clinical factors. The 5-gene signature is an independent risk factor for prognosis ($P < 0.001$). These results suggest that our characteristics may contribute to clinical management.

Infiltrating immune cells are an integral component of the tumor microenvironment and play an important role in

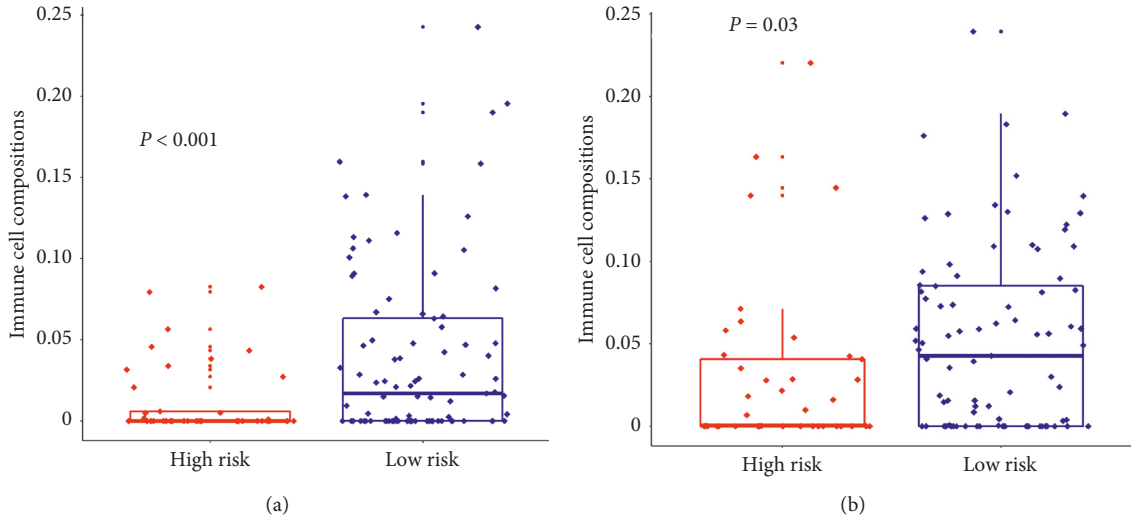


FIGURE 2: Estimating the composition of immune cells. (a) The ratio of dendritic cells activated in the high-risk and low-risk groups. (b) The ratio of mast cells resting in the high-risk and low-risk groups.

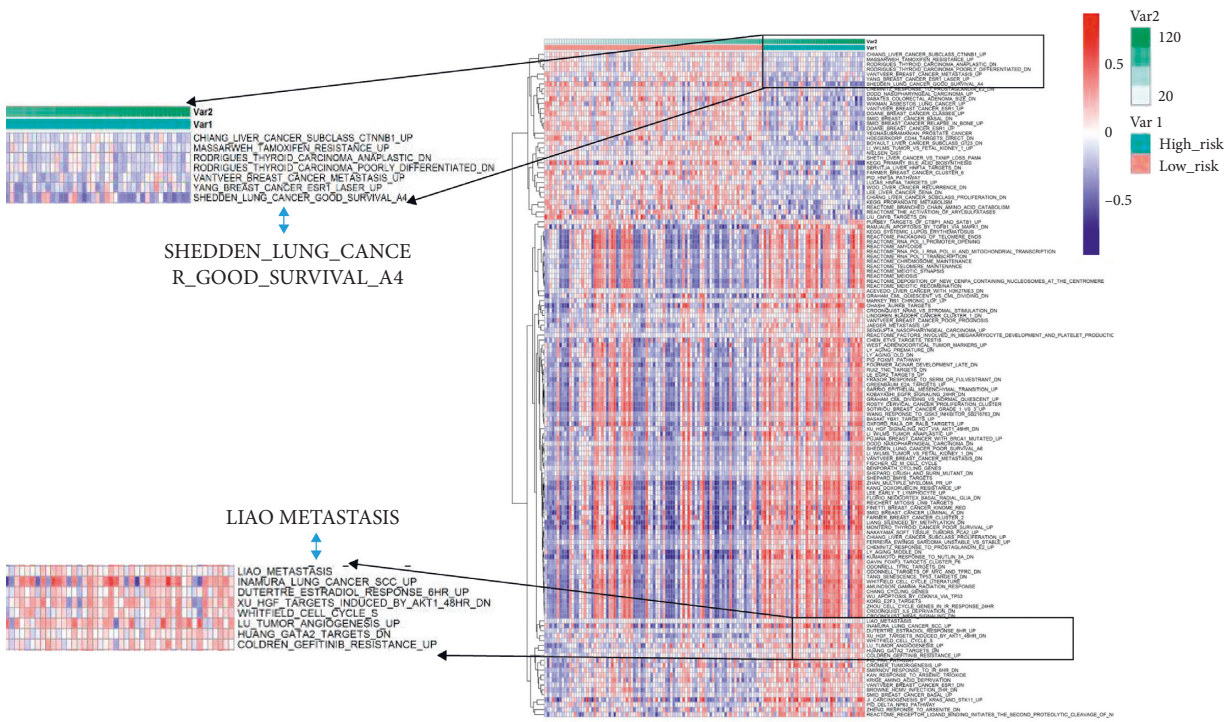


FIGURE 3: The changes in the pathways of 130 samples in the low-risk and high-risk groups.

increasing the effectiveness of immunotherapy [33]. This infiltrating immune cell is usually a heterogeneous mixture of immune cells, including cell types associated with activity and inhibition [34]. Because of the need for different types and subtypes of TIME to be identified in the immunotherapy of tumors, their characteristics and differences are identified. In order to make substantial progress, bioinformatics techniques are used to assess the composition, functional status, and cellular localization of immune cells. Based on the gene signature, a more precise classification of patients based on their TIME will better observe overall survival and response to immunotherapeutic agents.

More importantly, we found that the 5-gene signature is closely related to TIME parameters. The success of cancer immunotherapy has revolutionized cancer treatment and has used TIME parameters (immune cell composition and proportion) as predictive immunotherapy markers [12]. Detailed characterization of immune cell composition in tumors may be the basis for determining the prognostic and predictive biomarkers of immunotherapy. Dendritic cells

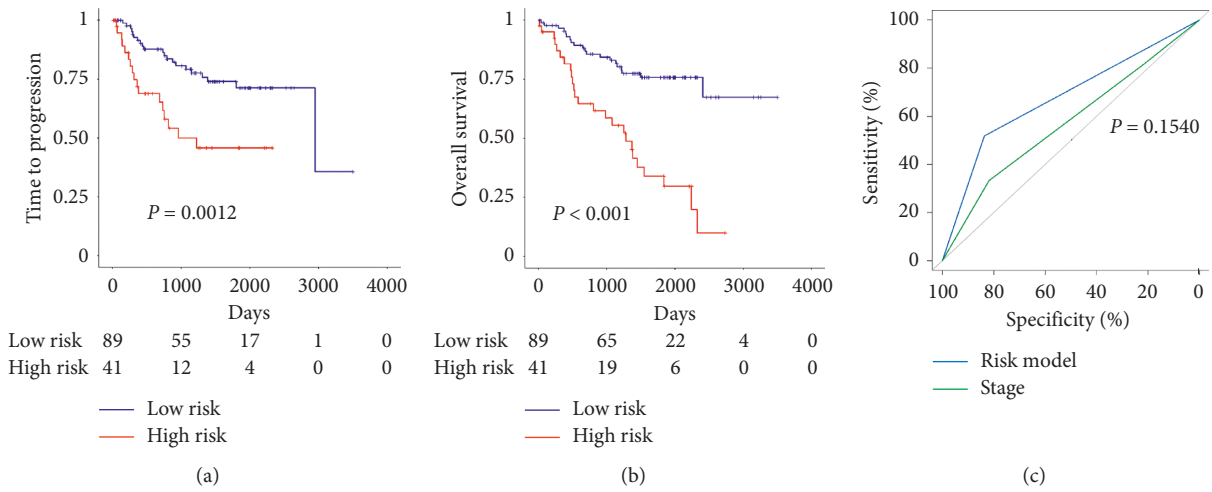


Figure 4: Kaplan–Meier survival curves and ROC curves in the training set. (a) Kaplan–Meier survival curves for relapse-free survival in the training set. (b) Kaplan–Meier survival curves for overall survival in the training set. (c) ROC curves of the risk model and TNM staging in the training set.

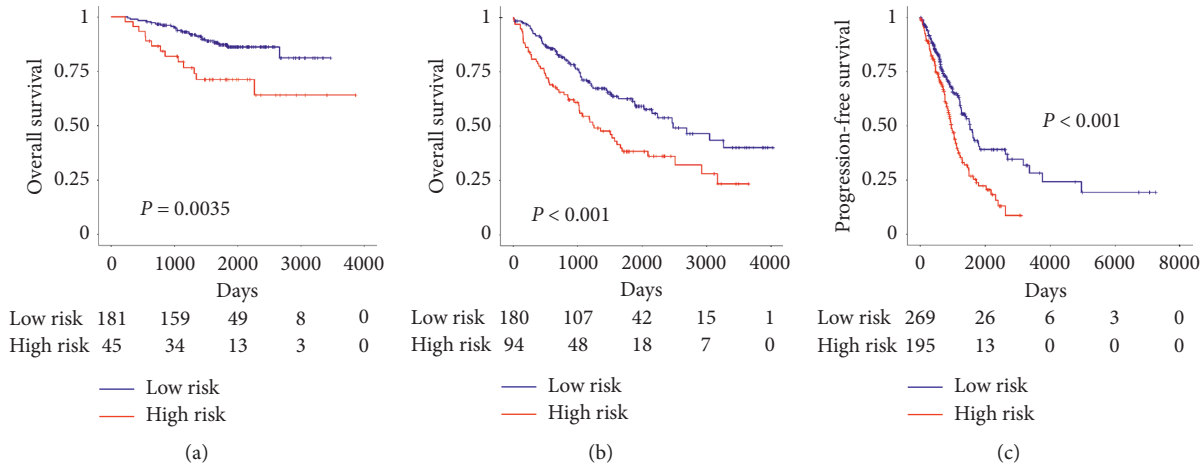


Figure 5: Kaplan–Meier survival curves for overall survival and progression-free survival in the validating set. Kaplan–Meier survival curves for overall survival in the (a) GSE31210 set, (b) GSE41271 set, and (c) TCGA.

Table 2: The correlation between the 5-gene signature and different mutant genes.

Variables	Low risk	High risk	P
EGFR status			0.0112
Yes	18	1	
No	54	28	
ALK status			1
Yes	2	0	
No	57	20	
KRAS status			0.7944
Yes	17	7	
No	68	29	

(DCs) are one of the core components of the immune system responsible for initiating an adaptive immune response that penetrates tumors and processes and presents tumor-derived antigens to naive T cells [35]. DC plays a key role in eliciting antitumor T cell immunity and thus represents the primary therapeutic target for cancer immunotherapy [36, 37]. Mast cells (MC) are thought to be involved in the regulation of innate and adaptive immune responses [38]. Furthermore, it is now recognized that MC is not only used as an effector cell but also induces T cell activation, recruitment, proliferation, and cytokine secretion in an antigen-dependent manner and affects regulatory T cells [39]. At present, it is increasingly found that mast cells play an important role in antitumor immunity [40]. We used CIBERSORT to estimate the immune cell composition of 130 samples to quantify the relative levels of different cell types in a mixed cell population and compared different types of cells in the low-risk group and the high-risk group. It was found that the ratio of dendritic cells activated and mast cells resting in the low-risk group was higher than that in the high-risk group, and the difference was statistically significant ($P < 0.001$ and $P = 0.03$). The results suggested that the presence of immune cells was better activated and the prognosis was better in the low-risk group. In summary,

Table 3: The univariate and multivariate Cox proportional hazard regression analyses between the 5-gene signature and other clinical factors of NSCLC patients.

Variables	Univariable analysis				Multivariable analysis			
	HR	Lower	Higher	*P*	HR	Lower	Higher	*P*
5-gene signature (high vs. low)	3.93	2.17	7.1	0	5.18	2.6995	9.945	<0.001
Lymphovascular invasion (yes vs. no)	1.37	0.58	3.26	0.476	1.03	0.4226	2.514	0.947
Pleural invasion (yes vs. no)	1.15	0.6	2.2	0.679	1.38	0.6977	2.745	0.352
Chemotherapy (yes vs. no)	1.1	0.59	2.05	0.76	0.94	0.4066	2.168	0.883
Radiation (yes vs. no)	1.26	0.56	2.84	0.579	1.42	0.4903	4.107	0.519

the 5-gene signature closely related to TIME parameters could predict the prognosis of lung cancer patients and provide some reference for immunotherapy.

Notably, among the 5-gene signature, only the gene HLF is involved in tumor immunity and the gene ST3GAL5 is involved in tumor invasion, migration, and proliferation. There are two other genes (SLCO4C1, ELAC1) that may have a relationship with the development of tumors, but there is no clear report. The ZNF204P gene has not been reported. SLCO4C1 is a key tumor suppressor gene in head and neck cancer that can be inactivated by "larger promoter" methylation and somatic mutations [41]. Overexpression of the human kidney-specific organic anion transporter SLCO4C1 in rat kidneys reduces hypertension, cardiac hypertrophy, and inflammation in renal failure [42]. Hepatic leukemia factor (HLF) is a critical transcription factor that plays an important regulatory role in many cancers, especially leukemia [43, 44] and may be involved in therapeutically induced immunogenic cell death [45]. HLF is a gene involved in the transformation from E1 to E2, and its inhibition can produce a more immunogenic microenvironment [46]. Overexpression of ST3GAL5 significantly promoted the proliferation and invasion of hepatoma cells. In contrast, knockdown of ST3GAL5 inhibited proliferation and metastasis of hepatoma cells [47]. This indicates that ST3GAL5 is closely related to the invasion and metastasis of liver cancer. In addition, ST3GAL5 has been reported to be positively associated with high risk of childhood acute leukemia and is associated with multidrug resistance in human acute myeloid leukemia, indicating the role of ST3GAL5 in cancer development and progression [48, 49]. ELAC1 appears to correspond to the C-terminal half of 3′tRNase from ELAC2 and it was found that ELAC1 also has 3′-tRNase activity, possibly encoding a candidate prostate cancer susceptibility gene for tRNA 3′ processing endoribonucleases [50]. From the above results, we can see that our gene signature not only identifies new promising biomarkers but also may provide a direction for the study of TIME mechanisms.

Here, we identify that the 5-gene signature is a powerful and independent predictor that could predict the prognosis of lung cancer patients. In addition, our gene signature is correlated with TIME parameters, such as DCs activated and MCs resting. Our findings suggest that the 5-gene signature closely related to TIME could predict the prognosis of lung cancer patients and provide some reference for immunotherapy.

Data Availability

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for noncommercial purposes, without breaching participant confidentiality.

Ethical Approval

This study was approved by the ethics committee of the Shandong Cancer Hospital and Institute affiliated to Shandong University and was consistent with the Helsinki Declaration.

Consent

This study was mainly based on the Gene Expression Omnibus datasets (GEO; GSE103584, GSE31210, GSE41271) and the Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/), and personal privacy information was not involved, so the informed consent was not needed.

Conflicts of Interest

All authors declare that there are no potential conflicts of interest.

Authors' Contributions

Jia Li and Huiyu Wang contributed equally to this study.

Supplementary Materials

Supplemental material 1: 13,035 gene expression profiles from 130 tumor samples in data set GSE103584. We used CBERSORT to estimate the immune cell composition of 130 samples and quantify the relative levels of different cell types in a mixed cell population. Supplementary material 2: the standardized relative proportion of all cell types. (*Supplementary Materials*)

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Review Article

The Effects of Secretory IgA in the Mucosal Immune System

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Immunoglobulin A (IgA) is the most abundant antibody isotype in the mucosal immune system. Structurally, IgA in the mucosal surface is a polymeric structure, while serum IgA is monomeric. Secretory IgA (sIgA) is one of the polymeric IgAs composed of dimeric IgA, J chain, and secretory component (SC). Most of sIgAs were generated by gut and have effects in situ. Besides the function of "immune exclusion," a nonspecific immune role, recent studies found it also played an important role in the specific immunity and immunoregulation. Thanks to the critical role of sIgA during the mucosal immune system homeostasis between commensal microorganisms and pathogens; it has been an important field exploring the relationship between sIgA and commensal microorganisms.

1. Introduction

Mucosal surfaces provide a physical barrier to defend foreign pathogens as well as to involve the tolerance of the commensal microbes or harmless food antigens. The protection of these surfaces is ensured by the mucosal immune system, designated as the mucosa-associated lymphoid tissues (MALT), which consists of mucus layers and epithelium cells, together with lymphoid tissues and immune molecules in the mucosal lamina propria [1, 2]. The immunoglobulin A (IgA) is the predominant antibody isotype in the mucosal immune system, which widely exists in the gastrointestinal tract, respiratory tract, vaginal tract, tears, saliva, and colostrum. Normally, serum IgA shows a monomeric structure, while the mucosal IgA shows polymeric. The function of the former is still unclear [3]. Distinctively, we designated the subtype of IgA composed of two monomeric IgA, secretory component (SC), and J chain as secretory IgA (sIgA) [4], which is the major effective form of mucosal IgA. There are also trimeric sIgA, tetrameric sIgA, and larger polymeric IgA in the upper respiratory tract of healthy humans. Among them, tetrameric IgA has a broad neutralizing function against influenza viruses [5]. Previous studies showed that mucosal immunity is segregated from systemic immune responses [6, 7]. The mucosal system can maintain the balance in the mucosal immunity between the commensal microorganisms and defenses the pathogens on the mucosal surface because of sIgA contribution [8]. Conversely, research showed there was a lack of IgA-secreting B cells in neonates until exposure to bacteria, suggesting that the commensal microorganisms were able to induce sIgA secretion [9, 10]. In humans, sIgA was also a major immunoglobulin in colostrum, which integrates the mucosal immune systems of mother and child for great protective functions [11]. However, selective IgA deficiency, a common primary immunodeficiency, often presents an asymptomatic phenotype or mild consequences, which may question the significance of IgA [12]. In this review, we will discuss the mechanism of sIgA generation and their function during the mucosal immune response.

2. Structure of sIgA

As an immunoglobulin, IgA has two identical heavy chains and two identical light chains. There is a flexible hinge region to separate above chains into two Fab regions-binding the antigens and an Fc region-mediating the effects [13]. In human, IgA has two subsets termed IgA1 And IgA2. The hinge region of IgA1 contains a 13-amino acid longer extension, ranging from three to six, variable O-glycan substitutions but not in IgA2 [4, 14]. Although both IgA1 and IgA2 carry N-linked glycosylation sites at every heavy chain, the latter has two additional N-linked oligosaccharides that may resist to the proteolytic activity of the bacteria in secretions better than the former [12].

Dimeric IgA (dIgA) was made of two monomeric IgAs linked in the penultimate Cys residues of their Fc regions via J (joining) chain and IgA2 is preferred. J chain is a small polypeptide to form pentameric IgM and dimeric IgA, but little is known about the function of J chain due to the technical limitation [15]. When one dIgA is bound to the polymeric immunoglobulin receptor (pIgR) at the basolateral side of the epithelium thereby transported to the luminal side, the dIgA-binding portion of the pIgR is cleaved to form the molecule sIgA [16]. The pIgR fragment of sIgA is called secretory component (SC) to support the stability of sIgA [17].

Although both IgA1 and IgA2 can form sIgA, the variety of subclass proportions will happen in different tissues. For example, there are 80 to 90% IgA1 in nasal and male genital secretions, 60% IgA1 in saliva, and 60% IgA2 in colonic and female genital secretions [4].

3. Induction of sIgA

The mucosal immune system can principally be divided into inductive sites and effector sites [18]. The classical sIgA inductive sites are gut-associated lymphoid tissue (GALT) including Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and mesenteric lymph nodes (MLNs). The GALT contains at least 80% plasma cells (PCs) and 90% sIgA of the body [19]. It has been estimated that approximately 3 g of sIgA is exported into the gut lumen of an adult human every day [20]. Craig and Cebra reported that Peyer's patches (PPs) were the principal precursor source of IgA^+ PCs [21]. In addition, the nasopharynx-associated lymphoid tissues (NALT) and the bronchus-associated lymphoid tissues (BALT) are also mucosal immune inductive sites [22].

3.1. Antigen Presentation of DCs. Peyer's patches were covered with an epithelial monolayer, follicle-associated epithelium (FAE), containing microfold cells (M cells) inside. Beneath the FAE, subepithelial dome (SED) covers the B follicles, while the DCs exists in the SED [23]. Mucosal antigens were captured by the underlying DCs by extending their dendrites [24] or through the transcytosis of M cells [25]. Evidence showed both FAE and small intestine goblet cells (GCs) were involved in the antigen uptake [26, 27].

Upon antigen presentation by DCs, T cells and B cells were activated and IgA class switch recombination (CSR) were mediated in the mucosal B cells, which replaced the immunoglobulin heavy chain C regions ($C\mu$) with the downstream $C\alpha$ gene [28]. There are long repetitive switch (S) regions preceding $C\mu$ and downstream $C\alpha$. Activation-induced cytidine deaminase (AID) converts cytosines in S regions to uracils by the deamination lesions, which instigates the CSR. These uracils lesions are subsequently removed by two DNA repair factors, resulting in DNA double-strand breaks (DSBs) and recombination between upstream S region and downstream S region [29]. Additional, TGF-β has a critical role in this process, which binds to the TGFβR on the surface of B cells, thereby leading to the SMAD3/4 and Runx3 activation and subsequently combining with the TGF-β responsive elements in the Iα promoter of the IgA heavy chain gene [30].

3.2. T-Dependent (TD) Mechanism and T-Independent (TI) Mechanism. In terms of the participation of T cells in this process, the IgA CSR were divided into T-dependent (TD) mechanism and T-independent (TI) mechanism. The former required interaction between CD40 on the surface of B cells and its ligand CD40L derived from T cells, resulting in high-affinity antigen-specific IgA production to neutralize the pathogens [31]. T follicular helper (Tfh), Foxp3 $^+$Treg, and Th17 cells are involved in promoting the IgA response in the intestine by the release of various cytokines, such as IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, and IL-21, to further promote the CSR to IgA [32]. However, evidences have demonstrated that CD40 deficiency in human and mice retain IgA production [33, 34], suggesting that CSR to IgA could occur via TI mechanism, which produced commensal-reactive IgA through innate immune cells such as innate lymphoid cells (ILCs) and plasmacytoid dendritic cells (pDC) [2, 31, 35]. During the TI pathway, BAFF (B-cell activating factor of the TNF family) and APRIL (A proliferation-inducing ligand), two members of TNF family, are responsible for stimulating CSR to IgG or IgA in human [36].

3.3. sIgA Effector Sites. IgA gene sequences of wild type mice present substantial somatic hypermutation (SHM), which reveal the germinal center (GC) origin in the PPs [37]. Due to the food antigens and the microbiota in the gut, GC in the PPs constantly presents and B cells can repeatedly enter into preformed GC in the recirculation, which contributes to the B cell affinity maturation and the formation of long-lived plasma and memory B cells [38]. After terminal differentiation to plasmablasts and plasma cells (PCs), IgA^+ B cells migrate into the bloodstream and prefer to home the mucosal inductive sites preferentially and other secretory effector sites [18]. Migration and interaction happened between different sIgA inductive sites and sIgA effector sites. Lung dendritic cells can also induce IgA CSR and generate protective gastrointestinal immune responses [39]. Lactating mammary glands are also vital sIgA effector sites, and the antigenic stimulation from maternal gut and airways could result in the sIgA specificity for intestinal and respiratory pathogens [40].

4. Functions of sIgA

As a primary antibody class found in various external secretions, sIgA has unique structural and functional features

not observed in other antibody classes. Classically, sIgA eliminates the pathogens with immune exclusion via nonspecific immunity [8]. Apart from that, sIgA plays an indispensable role in specific immunity elicited by pathogens. For example, sIgA can be elicited by mucosal vaccines against influenza virus and colitogenic bacteria in inflammatory bowel disease (IBD) [41, 42]. One of the hallmark characters in the mucosal immune system is the microbe colonization [43]. A study has confirmed that both TI and TD immune responses are involved in coating different commensal bacteria with sIgA [44]. In conclusion, response to the pathogens and induction of tolerance under normal conditions such as innocuous food antigens or commensal bacteria are dual functions of sIgA to maintain the homeostasis in mucosal sites.

4.1. Immune Exclusion. Traditionally, IgA is thought as a noninflammatory antibody at mucosal sites. Due to its polymeric structure and the oligosaccharide side chains of SC [45], sIgA is concentrated in the mucus out layer [46], noncovalently cross-linking microorganisms, promoting microorganisms clump together in situ. Furthermore, the abundant hydrophilic amino acids of IgA Fc and glycosylation of IgA and SC result in the hydrophily of sIgA, to entrap microorganisms [4], and then peristaltic bowel movements help remove the bacteria clumps. The process of agglutination, entrapment, and clearance processes are called immune exclusion [47].

4.2. Multiple Neutralizing Properties. Immune exclusion presents the nonspecific immunity function of sIgA. sIgAs have more extensive protective functions. Firstly, sIgA coating and the steric hindrance help block microbial adhesins to interact with the epithelium, sIgA can also inhibit specifically pathogens by direct recognition of receptor-binding domains such as reovirus type 1 Lang (T1L) [48]. The advanced glycosylated IgA heavy chain and SC serve as competitive inhibitors of the pathogen adhesion process [47]. Blocking pathogens from interacting with epithelial cells is not the exclusive mechanism by which sIgA exerts its protective function. In addition, sIgA may have direct effects on impacting the bacterial viability or changing pathogenicity. For example, sIgA can interact with flagella to inhibit the *Salmonella* bacterial motility [49], as well as protect from cholera toxin-induced fluid accumulation in a ligated intestinal loop model [36]. SC is proved to interact with a surface protein of *Streptococcus pneumoniae*, choline binding protein A (CbpA) [8]. And the galactose residues of free SC could also neutralize *Clostridium difficile* toxin A and enteropathogenic *E. coli* intimin [50].

4.3. sIgA and Receptor. FcαRI is the most important IgA host receptor, widely expressed in cell types including neutrophils, eosinophils, monocytes, and macrophages. [51], to mediate biological effects such as antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, antigen presentation and release of cytokines, superoxide generation, calcium mobilization, and degranulation [52]. Because of the similar IgA binding site for FcαRI and pIgR, sIgA-FcαRI binding is partly hampered by the steric hindrance of SC. Although sIgA is not able to activate phagocytosis by neutrophils or Kupffer cells, sIgA can initiate respiratory burst activity by neutrophils [53]. This process is dependent on the expression of Mac-1 (CD11b/CD18), suggesting that sIgA needs this integrin coreceptor to bind or activate FcαRI [54]. Besides FcαRI, sIgA has also been described to interact with pIgR, transferrin receptor (Tfr/CD71), asialoglycoprotein receptor (ASGPR), Fcα/μR, FcRL4, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [55]. SIgA immune complexes can reverse transport back into the lamina propria via the Tfr on epithelial cells [56] or via interaction with dectin-1 through microfold cells (M cells) [57]. Besides, sIgA immune complexes of the lamina propria were recognized by DC-SIGN and taken up by subepithelial DCs [58]. The SIGNR1, the mouse homolog of DC-SIGN, can also interact with sIgA and induce the tolerogenic DCs. The sIgA-DCs generate the expression of regulatory T cell, which indicates the potential immunoregulation function of sIgA [59]. Furthermore, polymeric sIgA of the lamina propria also binds and excretes antigens back to the lumen using polymeric Ig receptor-mediated transcytosis across the epithelial cells [60].

4.4. sIgA and Commensal Organisms. As is well-known, some patients with selective IgA deficiency show clinically asymptomatic or mild infections but have a higher risk of allergy and autoimmunity [61]. Researches show that both secretory IgM and systemic IgG can replace part of sIgA and establish the second defense line [62, 63]. Is sIgA a redundant component in the immune system? Emphasizing the effects of sIgA on the commensal microorganisms may explain its significance. The modification of microbiota is one of the most features in the selective IgA deficiency patients, and pIgR deficiency mice could inhibit different microbiota [62, 64]. As mentioned above, TD and TI mechanisms help with different-affinity sIgA. Low-affinity antibodies are specific for diverse commensal microorganisms, inclining to host-commensal mutualism, while high-affinity antibodies defend the pathogens [31]. In addition, the TD and TI mechanisms mediate the different sIgA coatings with bacteria [44], leading to different recognition by epithelial cells and DCs [65], and the level of sIgA coating varies between different members of the microbiota [10]. In neonates, maternal IgA is the sole source of sIgA. Evidence revealed that mice which did not receive sIgA in breast milk had a significantly distinct gut microbiota, and these differences were persistent and magnified in adulthood [66]. In addition, the maternal IgG and IgA were reported to inhibit the mucosal T helper cell responses, which revealed the TI mechanisms maintain the host-commensal mutualism in early life [67]. In the programmed cell death 1- (PD-1-) deficient mice, sIgAs were dysregulated and led to the change of microbial communities [68]. Therefore, we propose that TD and TI mechanisms might have synergetic roles in microorganism diversity and commensal homeostasis. However, the precise mechanisms of regulation on the

maintain the homeostasis and the memory B cells diversity and long-lived gut plasma cells are ready to clear [69].

Of note, the relationship between intestinal microbes and IgA is bilateral. The microbiota also modulates the sIgA distribution. A classic example is segmented filamentous bacterium (SFB), a commensal bacterium, remarkably inducing and stimulating multiple types of intestinal lymphoid tissues that generate sIgA [70]. Furthermore, Proietti et al. also proved the microbiota can release ATP to limit the generation of sIgA [71]. However, the effects of the microbe on the distribution of high-affinity and low-affinity sIgA are uncertain. Pabst suggested the model that all microorganisms could induce the high-affinity antibodies albeit vary different immunostimulatory activities and kinetics [31].

5. Conclusion

The mucosal system is the first line of immune defense while the sIgA is the first line of mucosal immunity. In this review, we have described the significant dual function of sIgA for maintaining immune homeostasis in mucosal compartments and the complexity of the sIgA action modes. sIgA presents a great latent capacity in shaping both the infant mucosal immunity and commensal microbial environments. Since breast milk is the main source of sIgA as well as a fundamental immune component for neonates, it offers a potential therapy in the clinics [66].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Retraction

Retracted: Identification of a Novel *ACTN4* Gene Mutation Which Is Resistant to Primary Nephrotic Syndrome Therapy

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] L. Meng, S. Cao, N. Lin et al., "Identification of a Novel *ACTN4* Gene Mutation Which Is Resistant to Primary Nephrotic Syndrome Therapy," *BioMed Research International*, vol. 2019, Article ID 5949485, 7 pages, 2019.

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Research Article

Identification of a Novel *ACTN4* Gene Mutation Which Is Resistant to Primary Nephrotic Syndrome Therapy

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ACTN4, a gene which codes for the protein α-actinin-4, is critical for the maintenance of the renal filtration barrier. It is well known that *ACTN4* mutations can lead to kidney dysfunction, such as familial focal segmental glomerulosclerosis (FSGS), a common cause of primary nephrotic syndrome (PNS). To elucidate whether other mutations of *ACTN4* exist in PNS patients, we sequenced the *ACTN4* gene in biopsies collected from 155 young PNS patients (≤16 years old). The patients were classified into five groups: FSGS, minimal change nephropathy, IgA nephropathy, membranous nephropathy, and those without renal puncture. Ninety-eight healthy people served as controls. Samples were subjected to Illumina's next generation sequencing protocols using FastTarget target gene capture method. We identified 5 *ACTN4* mutations which occurred only in PNS patients: c.1516G > A (p.G506S) on exon 13 identified in two PNS patients, one with minimal change nephropathy and another without renal puncture; c.1442 + 10G > A at the splice site in a minimal change nephropathy patient; c.2191-4G > A at the cleavage site, identified from two FSGS patients; and c.1649A > G (p.D550G) on exon 14 together with c.2191-4G > A at the cleavage sites, identified from two FSGS patients. Among these, c.1649A > G (p.D550G) is a novel *ACTN4* mutation. Patients bearing the last two mutations exhibited resistance to clinical therapies.

1. Introduction

PNS is a type of immune-mediated glomerular disease, resulting in a series of pathophysiological changes due to increased permeability of the glomerular filtration membranes, thus caused the loss of plasma proteins. Based on clinical characteristics, PNS is usually classified into 5 classes based on clinical features: FSGS, minimal change

nephropathy, IgA nephropathy, membranous nephropathy, and without renal puncture. It is well known that this disease severely affects the growth and development of children [1, 2]. Genetic mutations play a critical role in the etiology of PNS; thus, it is important to clarify the relevant genetic mutations in PNS patients, which could be helpful for developing new diagnostic methods and genetic therapies [3].

α-actinin-4, a protein expressed widely throughout the body, but enriched in the kidney, is encoded by the *ACTN4* gene. Several mutations of this gene have been found, and it usually correlates with abnormal serum levels and the function of α-actinin-4, especially in those patients suffering with FSGS [4]. The presence of the *ACTN4* gene p.Lys255Glu mutation relates to abnormal affinity of the actin-binding domain (ABD), which is mainly due to conformational changes in the molecular structure of α-actinin-4 [5, 6]. An Y265H variant of the *ACTN4* gene has also been detected in an adolescent patient with FSGS [7]. Some patients with the p.Ser262Phe mutation of the *ACTN4* gene have shown full-blown rapidly progressing nephrotic syndrome in early childhood [8]. In addition, the p.G195D and c.465C > T mutations have also been discovered in FSGS patients [9, 10].

However, there is a possibility of the existence of more *ACTN4* mutations in PNS patients. To clarify this, we used Illumina's next generation sequencing technology, in combination with the FastTarget target gene capture method, to screen and sequence peripheral blood samples collected from 155 young PNS patients (≤16 years of age) and compared these with 98 healthy controls. Based on bioinformatic analysis, we confirmed that *ACTN4* mutations exist not only in FSGS patients but also in patients with minimal change nephropathy and those without renal puncture. More importantly, we discovered a new *ACTN4* mutation, which could confer resistance to certain clinical therapies.

2. Results

2.1. Comparison of Serum α-Actinin-4. The serum levels of α-actinin-4 were 544.7 ± 108.11 and 241.20 ± 153.11 ng/mL in healthy controls and PNS patients, respectively. It is significantly different ($p < 0.001$).

2.2. Sequencing Depth and Coverage. The average sequencing depth in the target regions was 216.965–1118.708, 86.50–98.20% target area > 2 × coverage; 82.50–97.10% target area > 10 × coverage; 78.40–95.30% target area > 20 × coverage; and 76.00–94.70% target area > 30 × coverage.

2.3. ACTN4 Gene Mutations Identified from PNS Patients. Analysis of sequencing results revealed 5 *ACTN4* mutations that only occurred in PNS patients (Table 1).

2.4. Exon 13: c.1516G > A (p.G506S) Mutation. This mutation was detected in 2 patients. One was detected in a 5-year-old girl with no family history of nephrotic syndrome but was diagnosed with the disease just over 7 months before being admitted to hospital. Administration of prednisone acetate was not effective, and renal pathological examination showed the characteristics of minimal change nephropathy (Figure 1).

A 13-year-old girl with no family history of nephrotic syndrome was also diagnosed with PNS (without renal puncture) 9 months prior to admission. She was treated with prednisone acetate combined with cyclophosphamide for more than 3 months, and the urinary protein changed from negative to positive during the first month. However, the disease returned when the drug was reduced or when she had an infection. Pathological examination showed minimal renal puncture in this patient and she also had the exon 13: c.1516G > A (p.G506S) mutation.

2.5. Exon 12 at the Splice Site: c.1442 + 10G > A Mutation. This mutation was detected in a 15-year-old boy with no family history of nephropathy syndrome and 30 months after diagnosis. His symptoms were alleviated after receiving prednisone acetate and cyclophosphamide over a 16-month period. The renal pathological examination was similar to Figure 1 and consistent with minimal change in nephropathy.

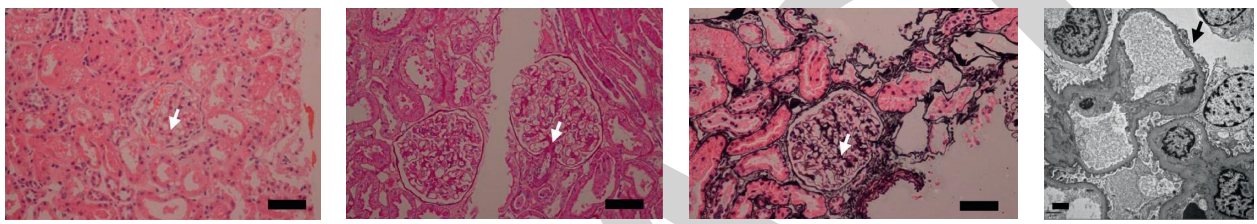
2.6. c.2191-4G > A Mutation. This mutation was detected in two boys: one was 9 and another was 13 years old. Neither boys had a family history of renal diseases. The first one received irregular treatment with prednisone acetate due to repeated renal pathology. Under pathological examination, marked visible glomerular sclerosis was detected, which was consistent with focal stage glomerulosclerosis with acute tubular injury (Figure 2). The latter one showed alleviated symptoms after receiving hormone treatment combined with the immunosuppressant–cyclophosphamide. The renal pathological examination was similar in both patients.

2.7. Exon 14: c.1649A > G (p.D550G) and Exon 18: c.2315C > T (p.A772V) Mutations. The presence of these two mutations was detected in of two patients. One was an 8-year-old boy and another was a 11-year-old girl. Both were diagnosed with nephropathy syndrome. There was no prior family history of renal disease. Renal pathological examination showed mild glomerular lesions with characteristic mesangial cell and stromal cell proliferation, but no obvious change of the thickness of the basement of membrane. Photomicrographs were similar to that observed in Figure 2. However, both patients showed no alleviation of symptoms after receiving standardized treatment for PNS.

Importantly, c.1649A > G (p.D550G), a mutation of *ACTN4* gene, had not been reported previously in any of the major databases including dbSNP, 1000Genomes, ESP6500, ExAC03, ExAC03_EAS, gnomAD, Hrcr1, Kaviar_20150923, and GENESKYDB_Freq (Table 2). There was a suggestion that the presence of this mutation could possibly result in the patient being resistant to clinical therapies.

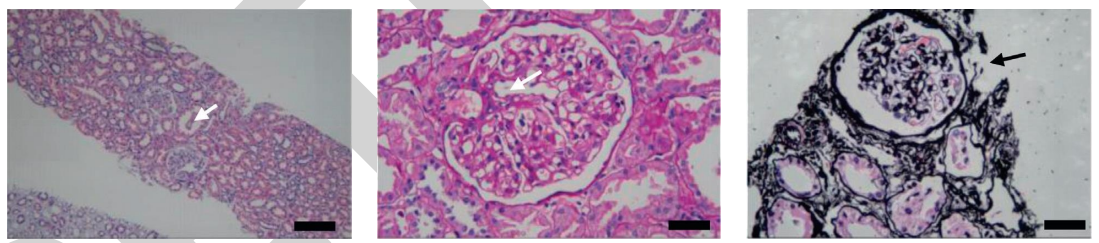
Table 1: *ACTN4* mutations detected from PNS patients. Five types of *ACTN4* mutations were detected in samples obtained from 155 PNS patients which were then classified into groups: FSGS, minimal change nephropathy, IgA nephropathy, membranous nephropathy, and without rental puncture. Mutation areas and related functions are listed and mutated patient's numbers are documented. * represents a mutation detected from the same patients.

Gene region	Function	Predicted protein variants: NM_001322033 NM_004924	SNP ID	FSGS	Minimal change nephropathy	IgA nephropathy	Membranous nephropathy	Without renal puncture
Exonic	Nonsynonymous SNV	Exon13:c.1516 G > A (p.G506S)	rs753348354		1			1
Splicing		Exon12:c.1442 + 10G > A	rs772524653		1			
Splicing		Exon18:c.2191-4G > A	rs371779934	2				
Exonic	Nonsynonymous SNV	Exon14:c.1649 A > G (p.D550 G)		2*				
Exonic	Nonsynonymous SNV	Exon18:c.2315 C > T (p.A772 V)	rs760946329	2*				



(a) (b) (c) (d)

Figure 1: Minimal change of nephropathy was noted under renal pathological examination. (a) HE staining showed mild lesions of the glomeruli, pointed by the white arrow. Scale bar indicates 40 μm. (b) PAS staining showed obvious proliferation of mesangial cells and matrix, pointed by the white arrow. Scale bar indicates 25 μm. (c) PASM staining showed swollen epithelium, vascular degermation, diffused foot processes, and mesangial cell and stromal segmental hyperplasia, pointed by a white arrow. Scale bar indicates 25 μm. (d) Transmission electron microscope imaging showed podocytes diffuse fusion, pointed by a black arrow. Scale bar indicates 2 μm.



(a) (b) (c)

Figure 2: Familial focal segmental glomerulosclerosis under renal pathological examination. (a) HE staining of Kidney biopsies showed tubular lumen expansion and the disappearance of the brush border, pointed by the white arrow. Scale bar indicates 100 μm. (b) Mesangial cell and stromal cell proliferation, pointed by the white arrow. Scale bar indicates 15 μm. (c) Capillary spasm opening and little or no significant thickening of the basement membrane, pointed by the black arrow. Scale bar indicates 25 μm.

3. Discussion

Previous studies proved that *ACTN4* mutations play an important role in the development of PNS [4, 11]. Detsika et al. found that the abnormal expression of α-actinin-4 and glomerular-associated proteins was related to the pathogenesis of FSGS [12]. Xie et al. found that the expression of α-actinin-4 in renal podocytes was decreased in FSGS and patients with IgA nephropathy [13]. Bartram et al. found that *ACTN4* gene p.g195 d mutation in FSGS sporadic children and uroepithelial cells showed that the expression levels of α-actinin-4 was lower than those in healthy controls [9], while Wagrowska-Danilewicz et al. showed no change in α-actinin-4 expression in patients with minimal nephrotic patients, but lesions were observed in the glomeruli [14].

Luimula et al. found that the expression of α-actinin-4 protein in nephrotic rats did not change significantly, but abnormal distribution and increases of α-actinin-4 mRNA expression were seen. They suggested that the difference between the two experimental results may be related to the different experimental models used (puromycin rat/*in vitro* cultured glomerular podocytes) [15]. Suvanto et al. found that the renal podocytes slit diaphragm protein expression

Table 2: The probability of all low-frequency samples in the database. Five types of mutations were detected, checked, and compared through genome databases and/or other software, including Freq_Alt (1000 g), 1000 g_chbs, Exac03, ExAC03_EAS, esp6500, gnomAD, Hrcr1, Kaviar, GENESKYDBHITS_Freq, and GeneskyGenomeDB. The probability of each mutation is listed.

Polymorphisms: NM_001322033 NM_004924	Freq_Alt (1000 g)	1000 g_chbs	ExAC03	ExAC03_EAS	esp6500	gnomAD	Hrcr1	Kaviar	GENESKYDBHITS_Freq	GeneskyGenomeDB
exon13:c.1516 G > A (p.G506S)			0	0		0		6.5×10^{6}		
exon12:c.1442 + 10G > A			8.446×10^{6}	0.0001		1.233×10^{5}		6.5×10^{6}		
exon18:c.2191-4G > A			1.667×10^{5}	0	0.000077	5.29×10^{5}		1.29×10^{5}		
exon14:c.1649 A > G (p.D550 G) exon18:c.2315 C > T (p.A772 V)			8.284×10^{6}	0.0001		2.844×10^{5}		6.5×10^{6}		

Table 3: A list of gene locus primers and fragment lengths obtained. For the FastTarget Target gene capture method, 34 pairs of primers were used. Each pair of primers was able to achieve a single and clear PCR product.

Target gene	Forward primer	Reverse primer	Product (bps)
ACTN4_1_1	5′-CGCGGCCTTGGTGCCTTTTCT-3′	5′-ACTGGTTCGCCGCGTGGTAGTCC-3′	239
ACTN4_1_2	5′-AGCTGAGGCGGGAGCGGACA-3′	5′-CCCGGGCCCCCTCAGAAAAG-3′	264
ACTN4_2	5′-GCTGCGGTTCTCCTGAGGT-3′	5′-GCTGTGGCAGAGCACCTGT-3′	275
ACTN4_3	5′-TGCTTTTGGAGAACAGAGGAGACT-3′	5′-GTTGTGCTTCAGAGCCTAAAGGTC-3′	290
ACTN4_4	5′-GGAGGAGCCTCACTCTGGTTTTA-3′	5′-GTGAGTGACCCCAAGGAAACAG-3′	261
ACTN4_5	5′-TGGGCTGAGTTCTGAGGGTTTAT-3′	5′-TCTCACAGACCACGACAAAAACA-3′	248
ACTN4_6	5′-CAGACTGCAGTGAATGGGAATTAGT-3′	5′-CGGAGTTAGGGGGTCAGACAG-3′	249
ACTN4_7	5′-GGCTGAGAACTGCCTGAAGAAA-3′	5′-GAAGCACAGTGGTGGCTGAAC-3′	252
ACTN4_8	5′-CCCGTGGATCCCAGTGAGT-3′	5′-CCGTCTGCAAGAGAAATGAGGT-3′	258
ACTN4_9	5′-CCTCCCTGCGTCTTTCACTCT-3′	5′-CAGGGTCAGTCTGTGTGGTGTG-3′	280
ACTN4_10	5′-CTCCTTCCCCTCTGTGAGGAGT-3′	5′-CCTCTGGCTGAGGATAATGAGGT-3′	264
ACTN4_11_1	5′-TAGCAGGAATCGTGGAGAAGTTG	5′-CGGTAGTCGCGGAAGTCCT-3′	262
ACTN4_11_2	5′-GCCCCAAAAGACTATCCAGGAG-3′	5′-AAAGATTACGCTGGCCAAACTG-3′	272
ACTN4_12	5′-CCCTGGGTGCCTCCACTT-3′	5′-ATGCATGCCTGAGAGACAGGAG-3′	274
ACTN4_13	5′-GACAGCCCCTCCAGACTCCT-3′	5′-TGGTGAGAGCCAGGTGATGATA-3′	263
ACTN4_14	5′-GGGTCCAATCCATCTAGCCACT-3′	5′-GGAGCTCACAGGTCTGGACACTA-3′	260
ACTN4_15	5′-CCTCCTGCTCACATACTGACCTG-3′	5′-CACAGAGGCTCTTGGGAAGATG-3′	246
ACTN4_16_1	5′-ATCGTCCATACCATCGAGGAGA-3′	5′-TCCCACTTGGAGTTGATGATTTG-3′	285
ACTN4_16_2	5′-CTCAGCCCATGACCAGTTCAA-3′	5′-CTGCACCTGGCAGAGGAGAC-3′	272
ACTN4_17	5′-CAACTCCAAGTGGGAGAAGGTG-3′	5′-CTTGGAACCTTCTCAGCTCTGTG-3′	287
ACTN4_18_1	5′-CTCCTCCAGGTGGTCAGTGG-3′	5′-GCACCTCCATGGTATAGTTGGTGT-3′	251
ACTN4_18_2	5′-GAGCCACCTGAAGCAGTATGAAC-3′	5′-CCAAAGTGCTGGTCTCTTCAATAA-3′	275
ACTN4_19	5′-TGAACCACGGTGAGGACAGTT-3′	5′-CAGATGCAGAGACGAAGGTGTG-3′	286
ACTN4_20	5′-CTAACTCTGTGTTTCCCTCCCCTAC-3′	5′-GGCGAGGGGAGAAAGAGAGA-3′	282
ACTN4_21	5′-GGCCCCTCTTGCCTACTCTG-3′	5′-CTCGGGCGGAGGAGTGTC-3′	259
ACTN4_22	5′-CCCCTGCCCCACTAAATGTC-3′	5′-ACACACTGGCCCCCTCAG-3′	290
ACTN4_23_1	5′-GCATGGGGGCTGGCGAGAGG-3′	5′-GTCGGGGGTGTTGGGTCAGGTCTC-3′	287
ACTN4_23_2	5′-TGCCCGGTGCCCTCGACTACAA-3′	5′-AGGTTGGGGAGACTTGGGGCCA-3′	279
ACTN4_23_3	5′-CTCTGTATCTATGCAAAGCACTCTCTG-3′	5′-AGGGACCTCAGAGCAAAGGAAGA-3′	286
ACTN4_23_4	5′-GGGATGCCTCACCACACC-3′	5′-GGATGGGGTGCGGTTCAG-3′	287
ACTN4_23_5	5′-CACTTGCCATTGCCAGGAGA-3′	5′-ATCCGTAAGTTAATAAAGTAAA TAGTAATTCTCTGA-3′	288
ACTN4_23_6	5′-TTGTCTGGCCTCACRTGTCT-3′	5′-GGGCAGAGAATCGGCTATGT-3′	257
ACTN4_23_7	5′-TAGCAACRTATCTCTGCCGTCTCTC-3′	5′-CCAGAGGGTGGTTTATCCAGAA-3′	245
ACTN4_23_8	5′-TGATGCTCCTCCGGGTCT-3′	5′-GCTCTGCCCTGGCTCTCCT-3′	281

was reduced in Finnish type congenital nephrotic syndrome, and similar changes were not observed in minimal change nephrotic podocytes. However, *NEPH1 FAT1*, *ACTN4*, and *CD2AP* were found to be expressed normally in proteinuric and nonproteinuric kidneys of minimal change nephrotic patients [16]. Above all, this is a dynamic process which is associated with the course of PNS. Abnormal serum levels of α-actinin-4 could be a consequence of *ACTN4* mutations.

In line with the aforementioned studies, kidney disease is consistent with decreased serum levels of α-actinin-4. In our study, the serum levels of ACTN4 in the healthy group were significantly higher than those of the PNS patients. In addition, we detected only 5 types of *ACTN4* heterozygous mutations from PNS patients. They were c.1516G > A (p.G506S) mutation on exon 13, c.1442 + 10G > A mutation at the splice site, c.2191-4G > A mutation at the splice site, c.1649A > G (p.D550G) on exon 14, and c.2315C > T (p.A772V) on exon 18. Among these, the mutation c.1649A > G (p.D550G) on exon 14 is a newly described mutation, and its presence together with the c.2191-4G > A mutation at the cleavage site in two patients appeared to confer resistance to clinical therapies in their host.

In summary, the *ACTN4* gene is a candidate gene involved in the development of PNS. It is anticipated that, in future its mutations could be helpful for the diagnosis and for the prediction of clinical therapies. Moreover, it could possibly serve as a novel therapeutic target.

4. Materials and Methods

4.1. Study Subjects. Ninety-eight healthy children were recruited from the Physical Examination Center of the Affiliated Hospital of Youjiang Medical College for Nationalities, and all those with nephrotic diseases were excluded after clinical tests. A total of 155 children with PNS (proteinuria ≥50 ng/kg/day and serum albumin <30 g/L, case group) were recruited from the Outpatient Department, Affiliated Hospital of Youjiang Medical College for Nationalities. All the study subjects in the case group were under 16 years of age and were excluded if they had secondary nephrotic syndrome. The 155 patients with PNS were classified into five groups based on pathological tests: FSGS ($n = 47$), minimal change nephropathy ($n = 37$), IgA nephropathy ($n = 36$), membranous nephropathy ($n = 17$), and without renal puncture ($n = 18$).

This research was approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical College for Nationalities. All the parents/guardians of the study subjects understood the research principles and agreed to participate in this study.

4.2. Extraction of DNA. 2 mL of venous blood was collected in EDTA anticoagulant tubes from each subject, and DNA was extracted with a Dneasy Blood & Tissue DNA Extraction Kit (Qiagen, #60606) by following the manufacturer's instructions. The quality of the DNA was validated using an Invitrogen Qbit spectrophotometer.

4.3. FastTarget Target Gene Capture. Primers for sequencing the ACTN4 target region were designed based on a template of the standard human genome and selected/optimized to obtain a clear, single band. Thirty-four pairs of optimized primers (Table 3) were mixed thoroughly into multiplex PCR primer panels according to the protocol, and the standard human genome was used for quality control. Primers with index sequences were used to introduce specific tag sequences compatible with the Illumina platform to the ends of the library by PCR amplification. The reaction used an 11-cycle PCR program to minimize the propensity of products. Amplified products were mixed with equal amounts of buffer and were tapped to obtain the final FastTarget sequencing library. The length of the fragments was determined using an Agilent 2100 Bioanalyzer. After quantification of library molarity, high-throughput sequencing was performed through the Illumina Miseq platform in a 2×150 bp/2×250 bps double-end sequencing mode to obtain FastQ data.

4.4. Bioinformatic Analysis. By using Burrows-Wheeler Aligner (BWA) software (http://bio-bwa.sourceforge.net/) [8], the sequencing data were compared with the UCSC hg19 reference genome, and the GATK standard program was used to validate the preliminary comparison results obtained by the BWA software. The advanced comparison identified SNV/InDel was used to check the accuracy of the sequences. The SNV/InDel of each sample was determined by using the VarScan software and the GATK HaplotypeCaller software, respectively. The SNV/InDel determined by the abovementioned two detection schemes was compared, and all the samples were combined accordingly. SNV/InDel loci were compared with the dbSNP, thousands of human genomes, ESP6500, ExAC03, ExAC03_EAS, genomAD, and Hrcr1Kaviar_20150923 databases by ANNOVAR to evaluate the frequencies, functional characteristics, conservation of these sites, and pathogenicity and to confirm the most significant SNV/InDel sites for the data obtained.

Data Availability

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

Ethical Approval

This study was approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities (Baise, China), in accordance with the Declaration of Helsinki.

Consent

All participants provided written informed consent to participate in this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Lingzhang Meng, Shan Cao, and Na Lin contributed equally to this study. LM, SC, and NL performed the experiments. LM and NL collected blood and performed data analysis, and SC and JZ recruited patients and performed DNA extraction. XC, Y Liang, and KH performed ELISA experiments. ML, DL, and JW performed histology experiments. LY, AW, and GL performed sequencing data analysis and retrieval of genome databases. QL and YG performed the statistical analysis. Y Liu initiated and supervised the project and wrote the manuscript. All the authors approved the final manuscript.

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Review Article

Interactions between Intestinal Microflora/Probiotics and the Immune System

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The digestive tract is home to millions of microorganisms and is the main and most important part of bacterial colonization. On one hand, the abundant bacterial community in intestinal tissues may pose potential health challenges such as inflammation and sepsis in cases of opportunistic invasion. Thus, the immune system has evolved and adapted to maintain the symbiotic relationship between host and microbiota. On the other hand, the intestinal microflora also exerts an immunoregulatory function to maintain host immune homeostasis, which cannot be neglected. In addition, the interaction of either microbiota or probiotics with immune system in regard to therapeutic applications is an area of great interest, and novel therapeutic strategies remain to be investigated. The review will elucidate interactions between intestinal microflora/probiotics and the immune system as well as novel therapeutic strategies.

1. Intestinal Immune System

Gut associated lymphoid tissue (GALT) is composed of the epithelium, lamina propria, and muscular layer [1]. Enterocytes constitute most of the intestinal epithelial cells and are able to absorb sugar, amino acid, and many other nutrients. Some enterocytes express Toll-like receptors (TLRs) and will secrete a series of proinflammatory chemokines (IL-8), cytokines (IL-1, IL-6, IL-7, IL-11, and TNF), and growth factors (SCF and G-CSF) when encountering with pathogens or toxins. These molecules will recruit peripheral neutrophils and mast cells to intestinal subepithelial regions and accelerate activation and differentiation of local lymphocytes. For instance, IL-7 and SCF secreted by intestinal epithelial cells can act synergistically to activate $\gamma\delta$ intestinal intraepithelial lymphocytes (iIELs). Then, activated $\gamma\delta$–iIEL can also secrete cytokines and chemokines to activate $\alpha\beta$–iIEL, thus initiating a more robust adaptive immune response [2–4]. Between intestinal epithelial cells are enteroendocrine cells, paneth cells, and goblet cells. When a pathogen invades the body, paneth cells release certain antibacterial molecules such as defensins into villi in the small intestine lumen while goblet cells secrete mucus to the intestinal surface, which is helpful for maintaining the intestinal barrier [5, 6]. Intraepithelial $\alpha\beta$T and $\gamma\delta$T lymphocytes, NK cells, and NKT cells can also be gathered among intestinal epithelial cells. Intestinal intraepithelial lymphocytes (iIELs) are a unique cluster of cells which reside in intestinal mucosal epithelium and have two different cell sources. Approximately 40 percent of iIELs are thymus-dependent $\alpha\beta$ T cells and their phenotype is similar to peripheral T cells. About 60 percent of iIELs are thymus-independent $\gamma\delta$ T cells. $\gamma\delta$ T cells are innate immune cells with strong cytotoxicity as well as the capacity to secrete

various cytokines. Therefore, iIEL plays a vital role in immunosurveillance and cell-mediated mucosal immunity [7–9].

Lamina propria contains a large number of macrophages and neutrophils as well as a small number of NKT cells, mast cells, and immature dendritic cells. A certain number of mature $\alpha\beta$ T cells and B cells as well as few $\gamma\delta$ T cells also reside in the lamina propria [10, 11]. Lymphocytes in the lamina propria usually congregate together to form intestinal follicle, which contains germinal centers populated by B cells and follicular dendritic cells, topped by immature dendritic cells, macrophages, $CD4^+$T cells, and mature B cells [12, 13]. Located in one side of intestinal follicle that is close to the intestinal luminal are specialized phagocytic cells named M cells, which can transport antigens across the epithelium to the side of basement membrane via transcytosis. Consequently, the antigens interact with the local immune cells and initiate mucosal immune responses where B cells differentiate into IgA secreting plasma cells [14–16]. The elements of intestinal mucosal immunity are summarized in Table 1.

The intestine is a unique organ which is in close contact with microorganisms. Most microbes are destroyed and killed by the harsh gastric acid environment, but a few can still make it through the intestine. The intestinal surface is covered with a large number of finger-like projections called microvilli (also named brush border), whose primary function is the absorption of nutrients. Brush border is wrapped up by a molecule called glycocalyx [17]. Since glycocalyx is a negatively charged and mucoid glycoprotein complex, microvilli could prevent the invasion of pathogenic bacteria. Besides, apical tight junctions of intestinal epithelial cells also ensure that pathogens do not pass through the intestine [18]. A vast population of immune cells reside within these and the underlying structures. As the most crucial intestinal sentinels, Peyer's patches are composed of B-cell follicles, interfollicular regions, macrophages, and dendritic cells [19]. A key function of Peyer's patch is sampling of particulate antigens, mostly bacteria and food through a specialized phagocytic cells called M cells, which can transport material from the lumen to subepithelial dome [20]. Then, local dendritic cells are able to sample antigens and present them to immune effector cells [21]. Nevertheless, intestinal tolerance is mainly mediated by CD4+ Treg cells in the context of uptake of food antigens. These Treg cells secrete IL-10 and TGF-β which exerts suppressive effects on immune cells within the lamina propria. However, a breakdown in the process of immune hemostasis will lead to gut pathology such as food allergy and inflammatory bowel disease [22, 23]. Intestinal barriers including mucin, antimicrobial peptides, and secretory IgA prevent the direct contact between the microorganisms and gut epithelial layer. Barrier destructions can contribute to bacteria influx, activation of epithelium, and inflammatory responses [24]. Proinflammatory antigen-presenting macrophages and dendritic cells are activated and release inflammatory cytokines such as IL-6, IL-12, and IL-23. Th1 and Th17 effector T-cell subsets are polarized and produce inflammatory cytokines such as TNF-α, IFN-γ, and IL-17 [25]. In addition, neutrophils are recruited and undergo dramatic form of cell destruction called NETosis, with the production of neutrophil extracellular traps (NETs) and tissue injuries [26].

2. Intestinal Microflora and Probiotics

There are a large number of microorganisms in the intestine, which are mainly distributed in the colon. It is estimated that over 40 trillion bacteria (including Archaebacteria) inhabit in the colon of adults, with a small proportion of fungus and Protista. In general, each individual carries an average of 600,000 intestinal microbial genes [27, 28]. In terms of bacterial strains, there is a distinct diversity among individuals. Each individual has his unique intestinal microflora, which is determined by host genotype, initial colonization through vertical transmission at birth, and dietary habits [29–32]. In healthy adults, the composition of bacterial flora in feces is stable regardless of time. Bacteroidetes and Firmicutes are two main bacteria in human intestinal ecosystem, accounting for over 90 percent of all microorganisms. The remains are Actinobacteria, Proteobacteria, Verrucomicrobia, and Fusobacteria [33, 34]. Probiotics are microorganisms that may be beneficial to health when consumed in adequate amounts [35]. Lactobacillus and Bifidobacteria are most commonly applied probiotics in clinical practice. Yeast *Saccharomyces boulardii* and *Bacillus* species are also widely used [36, 37]. The function of probiotics is closely related to the species of microorganisms that colonize within the intestine. The interaction between probiotics and host cells as well as intestinal flora is a key factor which influences the host health. Probiotics have an impact on intestinal ecosystem by regulating gut mucosal immunity, by having interactions with commensal microflora or potentially harmful pathogens, by producing metabolites (such as short-chain fatty acids and bile acids), and by acting on host cells through signaling pathways (Table 2). These mechanisms can contribute to the inhibition and elimination of potential pathogens, improvement of intestinal microenvironment, strengthening the intestinal barrier, attenuation of inflammation, and enhancement of antigen-specific immune response [38, 39].

Disturbed intestinal immune niche is a contributory cause for the digestive diseases such as inflammatory bowel disease (IBD), functional dyspepsia, gastroesophageal reflux disease, and nonalcoholic fatty liver disease. IBD patients are characterized by an increase in potentially aggressive gut microbial strains as well as decreased regulatory species [40–42]. Aggressive gut microbial strains activate inflammatory response by inducing Th1 and Th17 effector cells while decreased regulatory species inhibit the generation and function of regulatory cells including regulatory T cells (Treg), B cells (Breg), macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs). This has further resulted in elevated levels of TNF-α and inflammasome and reduced levels of IL-10, TGF-β, and IL-35 [43]. Therefore, dysbiosis of the intestinal flora has contributed to dysfunctional immune system and the chronic inflammation in IBD.

Table 1: Elements of intestinal mucosal immunity.

Structures	Constitution	Effect and mechanism
Lumen	Commensal bacteria	Competitively inhibit pathogenic bacteria Produce antimicrobial substances
	Mucus	Traps pathogens Prevents access to epithelial layer Contains secretory immunoglobulin A
	Glycocalyx	Provides physical barrier
Epithelial layer	Enterocytes	Connected by tight junctions Surface TLRs induce secretion of proinflammatory chemokines, cytokines, and growth factors Capture some antigens
	Goblet cells	Secrete mucus
	Paneth cells	Produce defensins and antibiotic substances
	Enteroendocrine cells	Produce neuroendocrine mediators
	$\gamma\delta$iIELs	Promote $\alpha\beta$iIEL activation through cytokine and chemokine secretion Produce antimicrobial effectors and protect against pathogens Prevent inflammation-induced epithelium damage
	M cells	Capture and transport antigen
Lamina propria	$\alpha\beta$T cells, B cells, DCs, and other APCs	Initiate adaptive immune responses in lymphoid follicles
	Treg cells	Suppress activation and effector function of immune cells

Table 2: Mechanisms of probiotics and host interaction.

Probiotics
Immunologic functions
Stimulate intestinal antigen-presenting cells such as macrophages or dendritic cells and increase immunoglobulin A (IgA) secretion
Regulate lymphocyte polarization and cytokine profiles
Induce tolerance to food antigens
Nonimmunologic functions
Digest food and inhibitory compete with pathogens for nutrition and adhesion
Alter local PH to create an unfavorable microenvironment for pathogens
Generate bacteriocins to inhibit pathogens
Scavenge superoxide radicals
Promote epithelial antimicrobial peptides production and enhance intestinal barrier function

3. Immune Regulation by Microflora and Probiotics

3.1. Promoting the Balance of Th1, Th2, Th17, and Treg Cells. Actually, intestinal microorganism can elicit diverse signals and induce CD4+T-cell differentiation. Invasive bacteria such as ectopic colonization of *Klebsiella* species can induce DCs phagocytosis and release of proinflammatory cytokines (IL-6, IL-12, and TNF), which is closely associated with Th1 polarization. *Bacteroides fragilis* is a kind of symbiotic anaerobic bacteria which colonizes in human lower digestive tract. Polysaccharide A (PSA) in its outer membrane can be recognized by T-cell surface molecule TLR2, which induces differentiation of CD4+T cells into Treg cells. Here, the Treg cells secrete molecules such as IL-10 and TGF-β which exert a suppressive action on immune cells. Actually, it has been demonstrated that administration of PSA or intestinal *Bacteroides fragilis* colonization can prevent intestinal inflammatory diseases in mice models [44–46]. In addition, segmented filamentous bacteria can be presented to T cells by dendritic cells and contribute to the synthesis of Th17 cells in lamina propria of small intestine, thus playing a vital role in antibacterial immune response [47, 48]. Parasites, for instance, *Heligmosomoides polygyrus*, can contribute to a Th2 immune response. The parasite can bind to tuft cells and secret high amounts of IL-25, which then acts upon dendritic cells. Dendritic cells produce IL-4 and TGF-β and induce CD4+ T differentiation into Th2 subset, with upregulated levels of IL-4 and GATA3 transcription factor. The immunomodulatory effects of various probiotics are listed in Table 3.

3.2. Regulation of Intestinal Related Gene Expression. Previous reports have demonstrated that expression of multiple intestinal genes is regulated by probiotics. For instance, *Escherichia coli* and *Lactobacillus rhamnosus* can upregulate mucin expression in intestinal cells to enhance intestinal mucosal barrier. Probiotics can also regulate gene expression of enterocytes and dendritic cells. It has been

Table 3: The immunomodulatory effects of probiotics.

Literature (PMID)	Probiotic strains	Mechanism and immunologic effects
15940144, 11751960	*Lactobacillus reuteri* *Lactobacillus casei*	Promote IL-10 secretion by Treg cells
17521319, 16297146	*Bifidobacterium bifidum*	Promote IL-10 secretion by mature DCs
15585777	*Lactobacillus rhamnosus*	Inhibit T-cell proliferation Decrease IL-2 and IL-4 secretion by mature DCs
15654823	*Bifidobacterium longum*	Promote IL-10 secretion by DCs
21740462	*E. coli* strain, Nissle 1917	Increase FoxP3+ Treg cells
19300508, 18804867	*Lactobacillus casei*, DN-114 001	Increase FoxP3+ Treg cells Promote IL-10 and TGF-β secretion
18670628	*Bifidobacterium infantis* 35, 624	Increase FoxP3+ Treg cells Inhibit TNF-α and IL-6 secretion
19029003	*Lactobacillus reuteri* (ATCC 23272)	Increase FoxP3+ Treg cells
16522473	*Bifidobacterium breve*	Activate TLR2 and promote maturation of DCs Increase IL-10 secretion

demonstrated that probiotic VSL#3 in certain concentrations (10^7 organisms/mL) could alter the DC phenotypes by the upregulation of costimulatory molecule (CD80, CD86, and CD40) expression [49].

3.3. Regulation of Immune Response through Microbial Metabolites. Probiotics can produce a series of metabolites by digesting different foods and impact the immune response within the body.

3.3.1. Short-Chain Fatty Acids. Short-chain fatty acid (SCFA) is fatty acid with carbon chain length of 1–6 carbon atoms. It is produced through fermentation of fibres by probiotics. Intestinal SCFA mainly includes acetate, propionate, and butyrate. SCFA can exert its immunoregulatory function as both extracellular and intracellular signaling molecules [50, 51]. Extracellularly, SCFA can act as ligands for cell surface G protein coupled receptors such as GPR41, GPR43, and GPR109a and regulate immune function indirectly. SCFA can bind to GPR43 in the surface of neutrophils and eosinophils to alleviate intestinal inflammation. GPR109a, which is expressed in colon epithelial cells and innate immune cells, can specifically bind to butyrate and induce differentiation of Treg cells [52, 53]. Intracellularly, SCFA can inhibit histone deacetylases (HDAC) and regulate gene transcription to exert immunoregulatory functions. For example, SCFA can promote acetylation of FoxP3 and synthesis of colon FoxP3+Treg cells to enhance their immunosuppressive function. Butyrate can suppress HDAC activity of macrophages in intestinal lamina propria and inhibit their secretion of inflammatory mediators such as nitric oxide, IL-6, and IL-12 [54, 55]. In addition, SCFA can also promote Tfh-cell production, B-cell differentiation, and antibody synthesis, as evidenced by latest reports [56].

SCFA also plays a crucial role in homing of T cells. Retinol, the main component of vitamin A, can be oxidized into retinaldehyde by retinol dehydrogenase. Retinal can be further oxidized to retinoic acid (RA) in vivo through an enzyme called Aldh1a. SCFA, the metabolites of probiotics, increases the activity of Aldh1a and promotes the conversion of intestine absorbed vitamin A into RA. Dendritic cells in intestinal Peyer's patch (PP) and mesenteric lymph nodes (MLN) express Aldh1a1 and Aldh1a2, respectively, and therefore produce RA locally. When an antigen is presented to T cells by CD103+ dendritic cells in MLD, the local RA induces expression of $\alpha 4$ in T-cell surfaces, which then binds with $\beta 7$ to form $\alpha 4\beta 7$ integrin. The $\alpha 4\beta 7$ integrin can combine with MadCAM-1 molecule of high endothelial vein (HEV) surface. Meanwhile, RA also induces CCR9 expression in T-cell surface, which binds to CCL25 in intestinal epithelial cells [57, 58]. Therefore, probiotics can promote homing of T cells to intestinal mucosa.

3.3.2. Amino Acid Metabolites. Certain essential amino acids are produced as metabolites of probiotics. Particularly, tryptophan (Trp) is closely related to the immune system. Trp can be decomposed into various metabolites by microflora. In the gut, indolic acid derivatives, including indole-3-acetic acid (IAA), indole-3-aldehyde (IAld), indole acryloyl glycine (IAcrGly), indole lactic acid, and indole acrylic acid (IAcrA), originate from Trp catabolism. Specifically, intestinal bacteria, such as *Bacteroides*, Clostridia, and *E. coli*, can decompose Trp to tryptamine and indole pyruvic acid, which are then turned into IAA, indole propionic acid, and indole lactic acid. IAA can combine with glutamine to synthesize indolyl acetyl glutamine in the liver or converted to IAld through aerobic oxidation by peroxidase catalyzation. Indolyl propionic acid can also be further transformed to IAcrA and combine with glycine to produce IAcrGly in the liver or kidney [59]. Indole is the most effective product among various bacterial Trp metabolites. It can also attenuate TNF-α-induced activation of NF-κB and reduce expression of the proinflammatory chemokine IL-8 as well as the adhesive capacity of pathogenic *E. coli* to HCT-8 cells [60]. In addition, both indole and its derivatives (IAld, IAA, and tryptamine) can activate intestinal innate lymphoid cells (ILCs) and regulate local IL-22 synthesis by sensitizing AhR to maintain intestinal mucosal homeostasis [61–63]. Besides, indole has been confirmed to strengthen intestinal epithelial barrier by fortifying tight junctions between cells through the pregnane X receptor (PXR) [64].

Gut commensal *Ruminococcus gnavus* and Firmicutes *C. sporogenes* have the capacity to decarboxylate Trp to tryptamine [65]. Since tryptamine exerts inhibitory effect against IDO1, it is regarded as a potential target in immune escape [66]. Skatole has been reported to inhibit CYP11A1, leading to decreased synthesis of pregnenolone, glucocorticoids, and sex steroids [67]. In the intestine, formation of endogenous steroid hormones, for instance, the anti-inflammatory glucocorticoid cortisol, is essential for the maintenance of intestinal homeostasis [68]. Therefore, skatole has been reported to play a vital role in the pathogenesis of inflammatory bowel disease (IBD).

3.3.3. Bile Acids. Bile acids are mainly converted from cholesterol in hepatocytes and undergo a series of metabolic processes mediated by intestinal microflora in the intestine. With the help of probiotics, primary bile acids, namely, cholic acid and chenodeoxycholic acid, convert to deoxycholic acid and lithocholic acid, respectively [69, 70]. Since intestinal macrophages, dendritic cells, and natural killer T cells express bile acids receptors such as GPBAR1 and FXR, intestinal bile acids can bind to these receptors and suppress NLRP3 mediated inflammatory response to maintain immune homeostasis [71, 72]. In addition, bile acids also regulate chemokine CXCL16 expression on liver sinusoidal endothelial cells (LSECs) and the accumulation of CXCR6+ hepatic NKT cells, which exhibit activated phenotypes and inhibit liver tumor growth [73].

3.3.4. Vitamins. Intestinal microflora has the capacity to synthesize vitamins and is their important source, especially for vitamin B [74]. As is known to all, vitamins play a vital role in regulating the immune system. Vitamin B1 is a key cofactor of tricarboxylic acid cycle. A decrease in vitamin B1 levels results in reduction of naive B cells residing in intestinal Peyer's patch, thus influencing intestinal immune function [75]. As a cofactor of sphingosine-1-phosphate (S1P) lyase, vitamin B6 is involved in the degradation of S1P. Therefore, it plays a fundamental role in maintaining S1P concentration gradient and promoting intestinal lymphocytes migration to periphery [76–80]. Besides, vitamin B also acts as a ligand for immune cells. The interaction is mediated by major histocompatibility complex MHC class I related proteins, which bind to vitamin B2, leading to the activation of mucosal-associated invariant T cells (MAITs) as well as secretion of IL-17 and IFN-γ. From this perspective, vitamin B2 has exerted the function of immune surveillance [81, 82].

At present, the immunoregulatory mechanism of probiotics is still not entirely clear regardless of its great variety and extensive clinical application. It requires further studies to investigate the in vivo process of probiotics through oral administration or enema therapy including the residence time, colonization, and reproduction, impact on original intestinal flora, and microbial interactions. And it is worthwhile to have a focus on the interaction of either microbiota or probiotics with immune system in regard to novel therapeutic applications. Apart from anti-TNF agents and immunomodulators, probiotics, prebiotics, and fecal microbial transplantation have been applied empirically in IBD. In addition, multiple novel strategies have already done in preclinical and clinical trials through targeting certain microbial organisms and altering mucosal immune niches. These strategies include blocking fimH to inhibit AIEC mucosal attachment, introduction of bacteriophages to eliminate pathobionts, and applying CRISPER-CAS editing to generate specific bacteriocins [83–85]. Hopefully, these approaches will be more effective which can be applied in a personalized manner in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Chen-xing Zhang and Hui-yu Wang are co-first authors and contributed equally to the work.

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Review Article

Immunological Aspects of Graves' Ophthalmopathy

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The body's autoimmune process is involved in the development of Graves' disease (GD), which is manifested by an overactive thyroid gland. In some patients, autoreactive inflammatory reactions contribute to the development of symptoms such as thyroid ophthalmopathy, and the subsequent signs and symptoms are derived from the expansion of orbital adipose tissue and edema of extraocular muscles within the orbit. The autoimmune process, production of antibodies against self-antigens such as TSH receptor (TSHR) and IGF-1 receptor (IGF-1R), inflammatory infiltration, and accumulation of glycosaminoglycans (GAG) lead to edematous-infiltrative changes in periocular tissues. As a consequence, edema exophthalmos develops. Orbital fibroblasts seem to play a crucial role in orbital inflammation, tissue expansion, remodeling, and fibrosis because of their proliferative activity as well as their capacity to differentiate into adipocytes and myofibroblasts and production of GAG. In this paper, based on the available medical literature, the immunological mechanism of GO pathogenesis has been summarized. Particular attention was paid to the role of orbital fibroblasts and putative autoantigens. A deeper understanding of the pathomechanism of the disease and the involvement of immunological processes may give rise to the introduction of new, effective, and safe methods of treatment or monitoring of the disease activity.

1. Introduction

Graves' disease (GD) is the most common underlying cause of hyperthyroidism, and the incidence of new cases is estimated at 20 to 50 per 100,000 people per year [1]. It is a multifactorial disease, influenced by genetic, environmental, and endogenous factors. The peak in the disease occurrence is between the ages of 30 and 50 years, but it can occur at any age and affects women more often than men [2]. The cause of hyperthyroidism in GD is circulating autoantibodies directed against the thyrotropin receptor (TSHR), which mimic the action of TSH and excessively activate thyroid follicular cells and consequently stimulate the secretion of thyroid hormones (triiodothyronine and thyroxine), thereby inducing thyroid growth and its vascularization [3]. These processes trigger the development of hyperthyroidism symptoms such as anxiety, fatigue, nervousness, weight loss, moist skin, hair loss, muscle weakness, and palpitations. The extrathyroidal symptoms include localized dermopathy, acropachy, and ophthalmopathy, edematous-infiltrative changes involving orbital soft tissues described as thyroid-associated orbitopathy (TAO), and thyroid eye disease or Graves' ophthalmopathy (GO) since more than 90% are due to GD [4]. GO, defined as an autoimmune inflammatory disorder involving the orbit, is observed in about 2 subjects per 10,000 a year and in 25–50% of patients with GD [5, 6].

Although these patients are predominantly hyperthyroid (90%), patients with GO may also be euthyroid (5%) or hypothyroid (5%) [7]. It is observed that the pathological autoimmune reaction is directed against cross-reactive autoantigens in the thyroid and retrobulbar tissues [6, 8]. Significant involvement of cytokines and immunological mechanisms in the pathogenesis of GO is suggested. Tissue infiltration by cytokine-producing inflammatory cells and extensive remodeling of the eye soft tissues results in a phenotypic picture of the disease (Figure 1). Clinical signs and symptoms include double vision, retracting eyelids, edema, proptosis, and erythema of the conjunctival and periorbital tissues [6]. According to the recommendations of the European Group on Graves' Orbitopathy (EUGOGO), GO is distinguished into three levels of severity: mild, moderate to severe, and sight-threatening [9]. Treatment depends on the GO severity and includes immunosuppressive therapy, orbital irradiation, and surgery (endoscopic orbital decompression). Understanding the role of the immune system in GO may enable the introduction of new therapeutic options in the future.

2. Pathogenesis

Similarly to GD, at the base of GO is the autoimmune response in which the sensitive T cells, as well as autoantibodies against a common autoantigen of the thyroid and retrobulbar tissues, play an important role [10]. This common antigen may be the TSH receptor, as it has been also expressed on fibroblasts and orbital preadipocytes [11]. A correlation between the degree of ocular changes and the level of stimulatory antibodies directed against TSHR (TRAb) has been reported [12]. It has been suggested that another autoantigen may be the insulin-like growth factor-1 receptor (IGF-1R), as immunoglobulins of GD patients may activate the IGF-1R [13, 14]. Autoantibodies directed against this receptor contribute to the activation of orbital fibroblasts in GO, and the increased expression of the IGF-1R has been shown in patients with GD in both the thyroid tissue and the orbital tissues. Varewijck et al. demonstrated a diminished stimulating activity of IGF-1R through the depletion of immunoglobulins of GD patients [15]. Although these antibodies against IGF-1R are potentially implicated in GO development, there are some discrepancies regarding this speculation. Minich et al. have obtained data that do not confirm that the circulation of stimulating antibodies (against IGF-1R) in the patient's blood aggravates GD, nor their usefulness as a diagnostic parameter of the disease [16].

The main processes involved in the pathogenesis of thyroid-associated orbitopathy are cytokine production and inflammation, hyaluronan synthesis, adipogenesis, and myofibrillogenesis. The main sites of ongoing inflammation are the orbital adipose tissue and fibrous tissue of extraocular muscles [17]. The orbital tissues are infiltrated by activated mononuclear cells, such as T cells, and to a lesser extent by plasmocytes, macrophages, and mast cells. Cytokines produced by leukocytes, such as IFN-γ, IL-1α (IL-5), and leukoregulin (lymphokine, produced by activated lymphocytes), lead to the synthesis of glycosaminoglycans (GAG) [18]. The accumulation of GAG leads to extraocular muscle edema [19]. By means of inflammatory mediators (cytokines) or direct cellular interaction, orbital fibroblasts are activated, which exhibit different morphological and functional features as compared to fibroblasts in other localizations. Moreover, the activation of orbital fibroblasts by TRAb indicates the link between GD and GO [20, 21]. Activated orbital fibroblasts proliferate, differentiate into adipocytes and myofibroblasts, and play a key role in the production of the extracellular matrix. Excessive orbital fibroblast activity contributes to expansion, remodeling, and fibrosis of the orbital tissues. In the active phase of orbital changes, as a result of inflammatory cell infiltration and edema, the volume of tissues surrounding the eyes augments, in turn leading to an increase in the intraocular pressure [18]. As a consequence, the eyeball moves beyond the bony edges of the orbit. Moreover, optic nerve compression resulting in optic neuropathy, as well as impaired venous and lymphatic outflow from the orbit, can occur [22]. The final stage (inactive phase) of exophthalmos involves the fibrosis of the eye muscles (Figure 2).

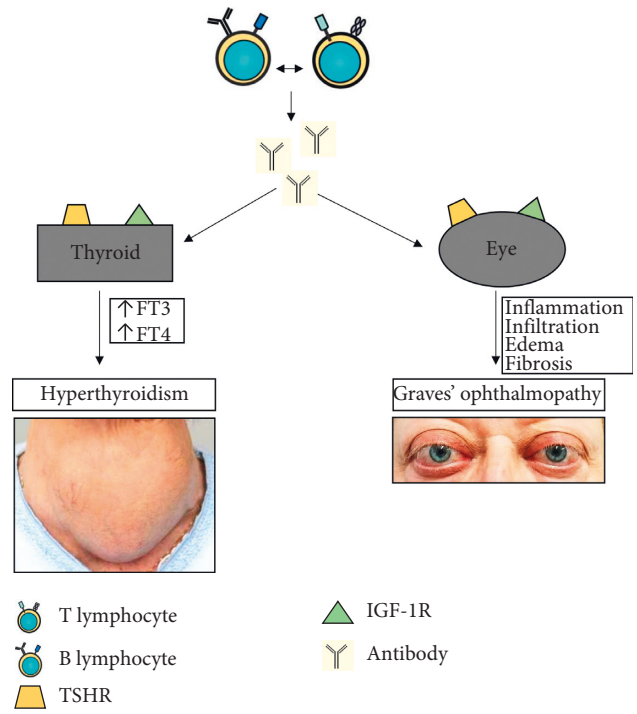

Figure 1: Pathogenesis of Graves' disease (GD) and Graves' ophthalmopathy (GO). GD is an autoimmune disease in which antibodies stimulate the thyroid to produce thyroid hormones leading to hyperthyroidism. One of the most common signs and symptoms is enlargement of the thyroid gland (goiter) while GO is the most frequent extrathyroidal involvement of GD. Inflammation and infiltration extraocular tissues result in edema and fibrosis of these tissues.

3. Cytokine Production and Inflammation

The inflammatory process in orbital tissues leads to migration and infiltration of immune cells, which resembles the process occurring within the thyroid gland. T cells enter the

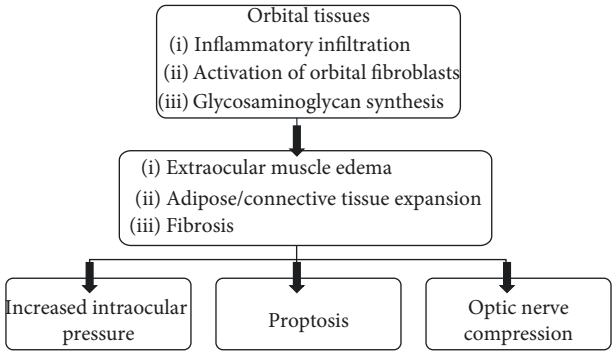

Figure 2: Pathogenesis of edematous-infiltrative changes. Inflammatory infiltration in periocular tissues and activity of orbital fibroblasts lead to expansion and remodeling of tissues. Increased intraocular pressure within the inflexible bony orbit results in proptosis and can contribute to developing optic nerve compression.

soft orbital tissue and release cytokines that contribute to reactivity and tissue remodeling [23]. The initial phase of GO is characterized by increased activity of Th1 lymphocytes, facilitating cell-mediated immunity and producing IL-1β, IL-2, TNF-α, and IFN-γ [24]. These proinflammatory cytokines enhance fibroblast proliferation and hydrophilic GAG production. Furthermore, the inflammatory process leads to the activation of Th2 lymphocytes, which release cytokines, such as IL-4, IL-5, IL-10, and IL-13, activating humoral reactions and the production of IgG [25]. The late phase of GO is characterized by tissue remodeling and fibrosis [26] (Figure 3).

Produced cytokines, chemokines, and growth factors have a huge impact on cells in orbital tissues. IFN-γ induces the production of CXCL9, CXCL10, and CXCL11 by fibroblasts, whereby the migration of lymphocytes to the orbital tissues is promoted [27]. In addition, IFN-γ stimulates the secretion of IL-1β and both (synergistically) stimulate the synthesis of GAG by orbital fibroblasts [28]. However, in contrast to IL-1β, IFN-γ inhibits adipogenesis of fibroblasts [29]. IL-1β has been shown to stimulate the orbital fibroblasts to produce IL-6, IL-8, CCL2, CCL5, and IL-16, which are chemoattractants for T and B cells, monocytes, and neutrophils [30, 31] (Figure 4).

Besides lymphocytes, macrophages, and thyrocytes, orbital fibroblasts also express the costimulatory protein CD40 [32]. The interaction between CD40 ligand (CD154) localized on T cells and the CD40 molecule on the orbital fibroblast surface stimulates the production of various inflammatory mediators (such as IL-1α, IL-6, IL-8, CCL2, and PGE2) by orbital fibroblasts as well as the activity and proliferation of these cells [32]. Prostaglandin E2 (PGE2) participates in B-cell maturation, stimulates the production of IL-6 by orbital fibroblasts, and activates mast cells [33, 34]. The production of PGE2 by orbital fibroblasts is also promoted by leukoregulin, IL-1β (released by macrophages and fibroblasts), and IFN-γ (secreted by activated T cells) [28, 35]. The process of recruitment of autoreactive T lymphocytes is supported by locally produced or circulating adhesion molecules, and the expression of these

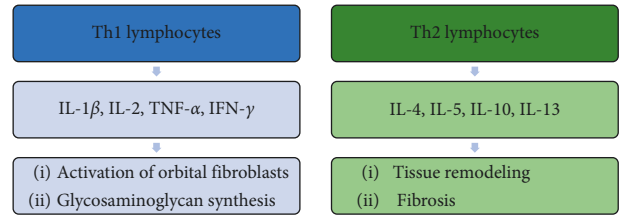

Figure 3: The proportion of T lymphocytes in the pathogenesis of Graves' ophthalmopathy. The initial phase of GO is characterized by increased activity of Th1 lymphocyte-producing cytokines that enhance fibroblast proliferation and GAG production. Th2 lymphocytes involved in the late phase participate in remodeling and fibrosis of periorbital tissues.

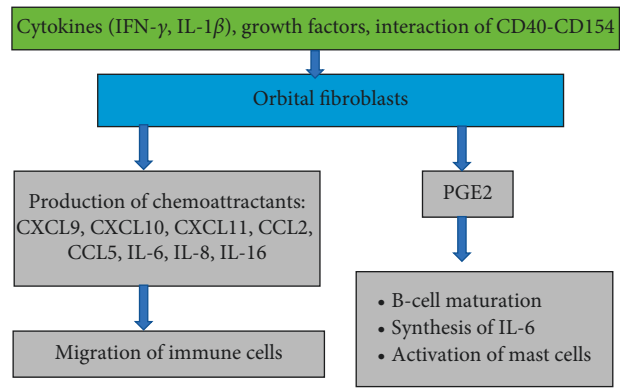

Figure 4: The participation of orbital fibroblasts in orbital inflammation. Cytokines, growth factors, and T cells stimulate orbital fibroblasts to produce chemokines and cytokines. PGE2 produced by orbital fibroblasts activates mast cells and B-cell maturation as well as stimulates the production of IL-6 by orbital fibroblasts.

molecules is induced by cytokines [36]. IL-1α, IL-1β, TNF-α, IFN-γ, and also CD40-CD154 interaction enhance the expression of intercellular adhesion molecule (ICAM-1) on orbital fibroblasts [30, 37, 38]. Adhesive molecules activate T cells and enhance their recruitment, resulting in an increased cell response and development of the active phase of ophthalmopathy. Elevated levels of L-selectin and ICAM-1 have been reported in patients in the active phase of the disease [39].

It is suggested that the cause of the development of GO is a lack of regulatory T lymphocytes (Tregs) control over the inflammatory reaction directed against self-tissues (antigens) [40]. Tregs are responsible for suppressing the immune response by the release of IL-10 and TGF-β [41]. Under physiological conditions, Tregs destroy autoreactive T lymphocytes, directed against thyroid follicular cell antigens [42, 43]. Glick et al. demonstrated an impaired suppressor function of Treg lymphocytes in patients with autoimmune thyroid disease (GD or Hashimoto's disease), who did not receive glucocorticosteroids for a minimum of six months [44]. Klatka et al. reported that patients with GD were characterized by a lower number of Tregs and a higher Th17 lymphocyte count compared to healthy subjects [45]. The significant contribution of Th17 lymphocytes to

inflammatory infiltration is also suggested as their role in autoimmune diseases has been demonstrated [46, 47]. The elevated concentration of Th17 lymphocytes in the peripheral blood of GO patients was reported, but there are no data on the presence of Th17 lymphocytes in the inflammatory infiltration of orbital fat.

4. Hyaluronan Synthesis

An important feature of the processes occurring in retroocular connective tissue, which affects the clinical picture of ophthalmopathy, is the synthesis of large amounts of GAG by orbital fibroblasts [48]. In particular, the accumulation of hyaluronan acid and collagen contributes to the retrobulbar tissue edema. *In vitro* culture of orbital fibroblasts treated with IFN-γ was characterized by higher production of GAG compared to the dermal fibroblasts culture [49]. Similar results were obtained using leukoregulin as a stimulant [50]. The effect of inflammatory mediators, such as IL-1, TNF-α, IFNγ, TGF-β, IGF-1, PDGF (platelet-derived growth factor), and prostaglandins, on the stimulation of orbital fibroblasts for the production of hyaluronan is also indicated [30, 48, 51–53]. Han et al. reported that IL-4 and IFNγ enhance the effect of IL-1β on GAG production by orbital fibroblasts as they augment the induction of hyaluronan synthase-2 (HAS2) expression by IL-1β [28]. Hyaluronan synthases (HASs) expressed on the cell membrane are responsible for the regulation of hyaluronan synthesis [54]. In GO, the major isoform of HAS involved in the synthesis of hyaluronan is HAS2. The balance between synthesis and degradation reflects hyaluronan accumulation. Zhang et al. reported the production of hyaluronidase by orbital fibroblasts [55].

5. Adipogenesis and Myofibrillogenesis

A portion of the orbital fibroblasts is called preadipocytes since they possess the capability to differentiate into mature adipocytes, which distinguishes them from fibroblasts from other locations in the body. This may be due to the high expression of the peroxisome proliferator-activated receptors (PPARγ) [56]. PPARγ belongs to the nuclear receptors of adipocytes, which act as transcription factors and regulate homeostasis of lipids and glucose. Adipogenesis in orbital fibroblasts is enhanced by the activation of PPARγ with rosiglitazone [57]. PPARγ agonists stimulate not only adipogenesis but also the expression of TSHR in cultured orbital preadipocytes. Moreover, they inhibit orbital inflammation and the production of hyaluronan [58]. Microarray studies have shown an upregulation of adipocyte-related genes (genes encoding PPARγ, IL-6, adiponectin, and leptin) in the orbit in GO. The activity of cyclooxygenase-2 (COX2) in activated T cells results in the production of proadipogenic prostaglandins (PPARγ ligands) [59]. COX2 is upregulated in the orbit in patients with GO, and as a result, prostaglandins provoke the process of adipogenesis in orbital fibroblasts [60].

Fibroblast subpopulations Thy1(CD90)+ and Thy1− can be distinguished based on the presence or absence of CD90 glycoprotein expression [61]. Thy1− fibroblasts have a strong ability to differentiate into adipocytes. Studies indicate that IL-1β, IL-6, and PGD2 stimulate fibroblasts towards adipogenesis [30, 52, 62]. It has been shown that this process is inhibited by TNF-α and IFNγ, but not by IL-4. These results agree with the claim that cytokines associated with Th1 lymphocytes are more involved in the early phase of ophthalmopathy rather than in the late phase associated with tissue remodeling and fibrosis. Thy1+ fibroblasts have the potential to differentiate into myofibroblasts, as demonstrated by fibroblasts stimulated by TGF-β, i.e., by a cytokine associated with Th2 lymphocytes [63, 64]. Myofibroblasts play a key role in muscle contraction and the accumulation of collagen in fibrotic tissue. Lehmann et al. have reported that adipocytic differentiation of Thy1− orbital fibroblasts can be inhibited by culture media from Thy1+ orbital fibroblasts, which produce antiadipogenic factors [63]. The involvement of adipose tissue or extraocular muscles in GO patients results from the proportion of Thy1+ and Thy1− orbital fibroblast populations and exposure to TGF-β or another stimulus [6] (Figure 5).

6. Putative Autoantigens and Potential Treatment

6.1. TSH Receptors. Hyperthyroidism associated with GD results from the action of autoantibodies directed against TSHR expressed on the surface of thyrocytes (thyroid epithelium). Studies have demonstrated the presence of the receptor in orbital adipose tissue and also suggested that the shared autoantigen hypothesis can explain the pathogenesis of GO (a common autoantigen of the thyroid and orbital tissues). Orbital adipose tissue of patients with GO (including euthyroid patients) is characterized by greater expression of TSHR than control tissues from people without GD [65, 66]. An elevated level of TSHR has been also noticed in pretibial connective tissue from patients with thyroid-associated dermopathy [67]. Some studies have shown that the level of antibodies against TSHR (TRAb) correlates with the clinical activity and severity of GO [68, 69]. Active GO is associated with a higher expression of TRAb compared to inactive GO. It is suggested that the extrathyroidal and thyroidal TSHR exhibit similar properties [70]. The response of orbital fibroblasts to TRAb is augmented by PDGF-AB and PDGF-BB, whereas TGF-β reduces TSHR expression [51, 71]. TSH, TRAb, and GD-IgG activate orbital fibroblasts and initiate cAMP and PI3K (phosphoinositide 3-kinase) signaling and the production of hyaluronan, ICAM-1, and cytokines, e.g., IL-6, IL-8, CCL2, and CCL5 [72]. In addition, the activation of TSHR induces adipogenesis in orbital fibroblasts [73].

Studies indicate that enhanced *de novo* adipogenesis in the orbit of GO patients increases TSHR expression in this tissue. Cultured orbital fibroblasts under adipogenic conditions have shown higher TSHR expression in mature fat cells than in preadipocyte fibroblasts [74]. Furthermore, PPARγ agonist rosiglitazone and adipogenic conditions trigger the enhanced expression of TSHR and adipocyte-associated genes (adiponectin, leptin, and PPARγ) [57, 65]. Similar findings have been obtained in orbital adipose tissue. In addition, monoclonal TRAbs stimulate adipogenesis in

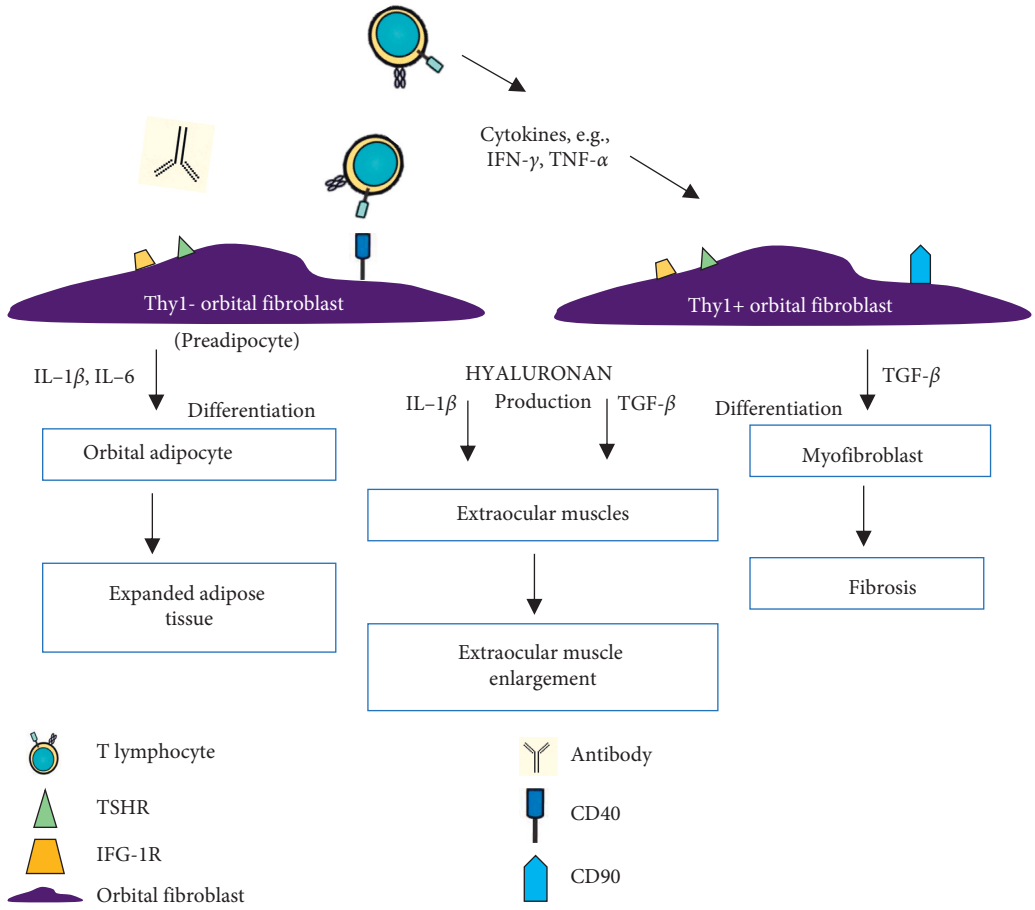

Figure 5: The participation of orbital fibroblasts in orbital tissue remodeling. Orbital fibroblasts express TSHR, IGF-1R, and CD40. Infiltrated immune cells, antibodies, secreted cytokines, chemokines, growth factors, and also CD40-CD154 interactions activate orbital fibroblasts. Inflammatory mediators (Il-1β and IL-6) that enhance adipogenesis activate Thy1− orbital fibroblasts to differentiate into adipocytes. And Thy1+ orbital fibroblasts (with CD90 expression), activated by TGF-β, differentiate into myofibroblasts. Proliferative activity of orbital fibroblasts, their differentiation, and capacity to synthesize extracellular matrix contribute to orbital tissue expansion, remodeling, and fibrosis.

orbital preadipocyte fibroblasts, which indicates the involvement of autoantibodies not only in the overproduction of thyroid hormones in GD but also in an orbital adipose tissue volume increase in GO.

Smith and Hoa have discovered that purified immunoglobulins from patients with GD (GD-IgG including TRAb and other IgGs) participate in the production of hyaluronan [75]. They found that GD-IgG enhances hyaluronan synthesis in GO orbital fibroblasts (through IGF-1R) whereas such properties have not been demonstrated for human recombinant TSH (hrTSH). In addition, only orbital fibroblasts that have undergone adipocyte differentiation are induced to hyaluronan production by GD-IgG, but not by hrTSH [72, 76]. On the other hand, Zhang et al. have shown that, in undifferentiated orbital fibroblasts (not in GO fibroblasts), bovine TSH and TRAb stimulate hyaluronan synthesis [54]. They also demonstrated that GO orbital fibroblasts containing the transfected TSHR-activating mutation increase hyaluronan production.

Due to the fact that the TSHR plays an important role in the pathogenesis of GD, it is believed that this receptor may be a therapeutic target for the treatment of GD [77]. Considering the orbital fibroblast activation through TSHR signaling, small-molecule TSHR antagonists can be used to block signal transduction [78]. These molecules have been found to inhibit cAMP production in human thyrocytes induced by TSH and GD-IgG [79]. TSHR-blocking monoclonal antibodies inhibit hyaluronan production and adipogenesis in cultured human orbital fibroblasts [80]. TRAb K1-70 has antagonist activity and can be useful in the inhibition of stimulating TRAb in GD patients [81]. ATX-GD-59 is an apitope that decreases the production of stimulating TRAb and demonstrates potential for the prevention and treatment of GO [82]. Apitopes—antigen processing independent epitopes—mimic naturally processed CD4+ T-cell epitopes. Regulatory-like T cells (type 1) with immunosuppressive features are induced after the administration of apitopes.

6.2. Insulin-Like Growth Factor-1 Receptor (IGF-1R). Another crucial autoantigen potentially involved in the pathogenesis of GO is IGF-1R. This receptor is expressed

Table 1: Potential therapeutic targets in GO [6, 103].

Target	Treatment	Potential benefit	
TSHR	TSHR-blocking antibody; TSHR antagonist	Inhibition of hyaluronan production and adipogenesis	[109]
IGF-1R	Teprotumumab—IGF-1R-blocking antibody	Inhibition of hyaluronan production and adipogenesis	[87, 88]
CD3	Teplizumab and otelixizumab—CD3 monoclonal antibodies	Induction of tolerance	[89]
CTLA4	Abatacept—CTLA4 analogue	Increased T-cell activation	[90]
CD20	Rituximab—CD20 monoclonal antibody	Increased TRAb production	[5, 94, 95]
TNF and TNF receptor	Adalimumab—TNF-blocking monoclonal antibody; Etanercept—soluble TNF receptor	Inhibition of hyaluronan production and inflammation	[99, 110]
TGF-β	TGF-β-blocking monoclonal antibody	Reduction in fibrosis	[111]
IL-6 receptor	Tocilizumab—IL-6 receptor monoclonal antibody	Inhibition of hyaluronan production and inflammation	[101, 112]
IL-1 receptor	Anakinra—IL-1 receptor antagonist	Inhibition of hyaluronan production and inflammation	[113]

in many tissues, particularly in the thyrocytes and orbital adipose tissue in patients with GD and GO. It belongs to the tyrosine kinase receptors and is involved in processes such as cellular metabolism, growth, apoptosis, and immunity [77]. It also plays a role in the activation of T and B cells. Studies show higher IGF-1R expression in GO orbital fibroblasts than in normal cells [83]. Increased expression of IGF-1R has been found not only in the retroorbital tissue of GO patients but also in the thyroid tissue of GD patients [14]. The stimulation of GO orbital fibroblasts by GD-IgG leading to the synthesis of T-cell chemoattractants, i.e., IL-16 and chemokine RANTES is attenuated by autoantibodies blocking IGF-1R or by transfecting fibroblasts with a dominant negative mutant IGF-IR. This draws attention to the vital role of signaling through IGF-1R in this process [84]. The chemoattractant effect contributes to the recruitment of inflammatory cells into the orbital tissues and promotes the autoimmune response. IGF-1R is found to participate in the differentiation of orbital fibroblasts into adipocytes and in the synthesis of hyaluronan through the action of autoantibodies directed against this receptor [70].

Research indicates that IGF-1 and TSH cooperate in the differentiation and metabolism of thyroid cells [85]. Their common location has been demonstrated in the membrane, in cytoplasmic and nuclear thyroid regions, and also in orbital fibroblasts. Tsui et al. have demonstrated that a monoclonal IGF-1R-blocking antibody inhibits kinase signaling induced by TSH. This antibody can also inhibit M22 (monoclonal TRAb) induced hyaluronan production by orbital fibroblasts. It can result from an association (physical and functional) between IGF-1R and TSHR [86]. Studies have shown that blocking IGF-1R through teprotumumab, a monoclonal antibody, inhibits IGF-1 and TSH action in fibrocytes and reduces the expression of IGF-1R and TSHR [87]. Teprotumumab infusions have great potential in reducing proptosis and the clinical activity score (CAS) in GO [88]. In 2016, the Food and Drug Administration described teprotumumab as a "breakthrough therapy." At present, it is being evaluated in phase III RCT.

6.3. Other Potential Targeted Treatments. Antibodies targeting T cells can be used as a potential therapy since the participation of these cells in the pathogenesis of GO is crucial. Antibodies against CD3 (teplizumab and otelixizumab) lead to the depletion of T cells as in the case of type 1 diabetes [89]. Studies have also found that abatacept, a CTLA4 analogue, diminishes the activation of T cells. This approach was reported to be useful in corticosteroid-resistant rheumatoid arthritis [90]. Furthermore, the application of synthetic peptides in the silencing of autoimmune responses and the induction of T-cell tolerance to autoantigens has been used in experimental autoimmune encephalomyelitis in an animal model of multiple sclerosis [91]. However, none of these approaches connected with inhibiting T cells were investigated in autoimmune thyroid disease [92]. Because CD40-CD154 pathway participates in GD pathogenesis, the anti-CD40 antibody may be a promising approach in the treatment of GD. Iscalimab is one such immunomodulating, human, blocking anti-CD40 monoclonal antibody which can successfully treat Graves' hyperthyroidism [93].

Rituximab, a monoclonal antibody directed against CD20 on B cells, is actively investigated as it expresses an immunosuppressive effect. This monoclonal antibody decreases the production of TRAb [94]. Salvi et al. have demonstrated an improvement in GO activity and severity after the application of rituximab [95]. Although studies conducted by these researchers have also shown favorable effects of treatment by rituximab compared with intravenous methylprednisolone, Stan et al. did not confirm this in the prospective trial [5, 96]. However, long disease duration before treatment initiation may have significantly impacted different results of the mentioned researchers. It seems that rituximab may be vital in the case of a poor response to corticosteroids in patients with GO.

Another possible pathway in the treatment of GO is targeting TNF because of the impact of TNF on the production of MCP-1 by preadipocytes, which is crucial in attracting macrophages [97]. Adalimumab, a monoclonal antibody directed against TNF, was found to reduce

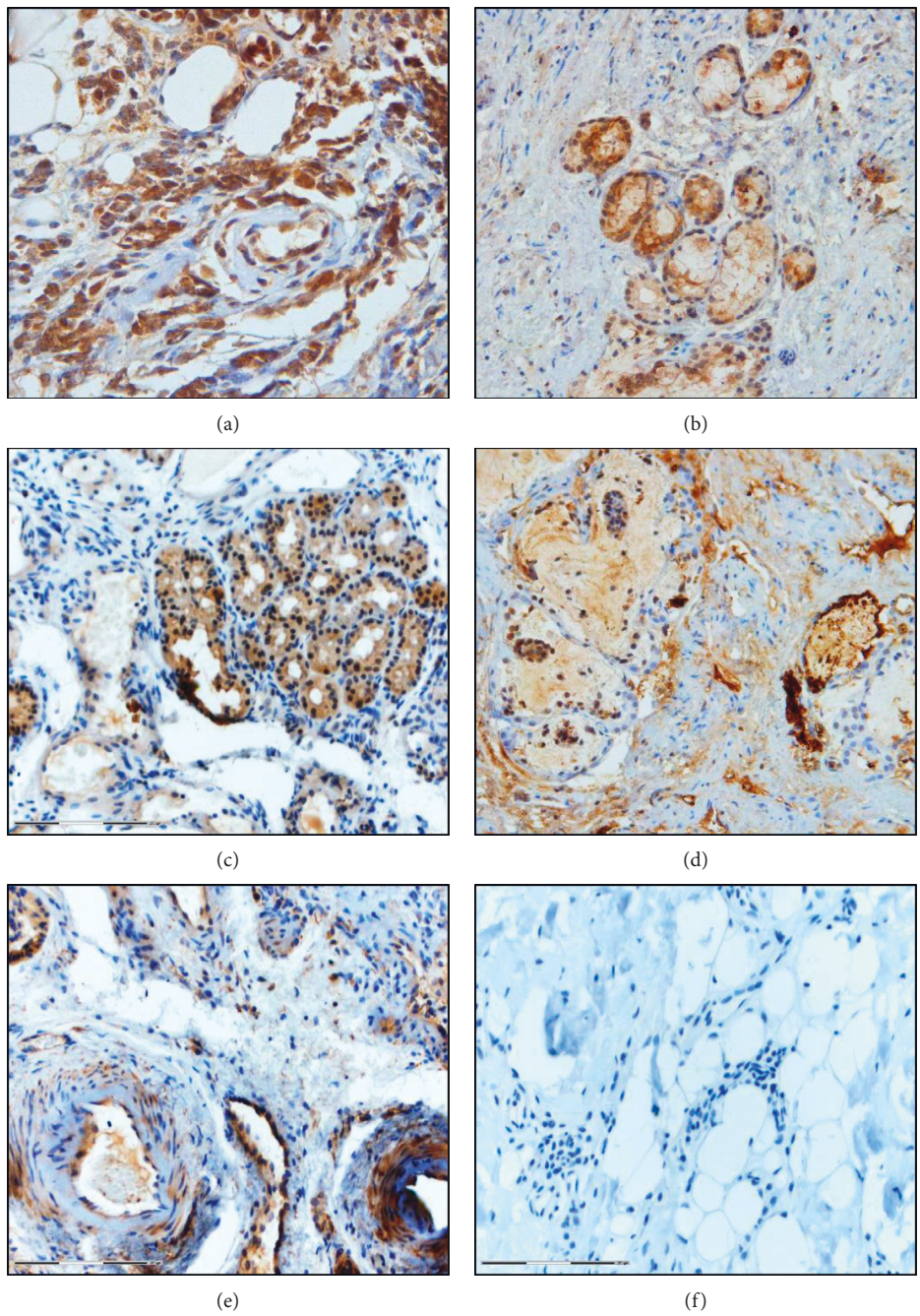

Figure 6: Immunohistochemistry on formalin-fixed paraffin-embedded tissue section of fat tissue of the eye socket obtained from patients who underwent endoscopic orbital decompression due to dysthyroid optic neuropathy: (a) TGF-β (×200); (b) TLR-4 (×100); (c) NF-kappa B (×100); (d) HIF-1α (×100); (e) IL-17 (×100); (f) isotype control (×100). The color reaction was visualized using DAB as a chromogen.

inflammation in active GO and etanercept (soluble TNF receptor) can improve soft tissue changes [98, 99]. As TGF-β demonstrates a profibrotic effect, especially in patients with inactive GO, neutralizing this effect can be beneficial.

Serum concentrations of soluble IL-6 receptor are elevated in patients with active GO and correlate with disease activity [100]. Treatment with an IL-6 monoclonal antibody (tocilizumab) leads to decreased proptosis and improvement in eye muscle motility as well as in severity and activity in corticosteroid-resistant GO [101]. IL-1 is also markedly involved in the pathogenesis of GO. Studies carried out on cultured human orbital fibroblasts have shown that an antagonist of the IL-1 receptor (anakinra) inhibits hyaluronan production and decreases inflammation [102]. Potential therapeutic targets in GO are summarized in Table 1 [6, 103].

7. Conclusions and Future Prospects

GD is an autoimmune disease underlying immune tolerance disorders and reactivity to thyroid autoantigens. One of the

nonthyroid symptoms is GO, in which the autoreactive inflammatory process in the orbital tissues plays the main role. Extraocular muscles and connective tissues are infiltrated by immune cells. This inflammatory infiltration and cytokine production result in the activation of orbital fibroblasts, differentiation, and synthesis of GAG. As a consequence, muscle swelling, adipose tissue expansion, and fibrosis develop. Orbital fibroblasts exhibit particular features as they are a target for TSHR and IGF-1R autoantibodies and also possess the ability to differentiate into adipocytes and myofibroblasts. Our preliminary study indicates that, in the orbital adipose tissue of patients with GO, TGF-β, Toll-like receptor 4 (TLR-4), hypoxia-inducible factor-1α (HIF-1α), nuclear factor kappa B (NF-kappa B), and IL-17 are expressed (unpublished data). It is well known that the expression of these proteins is associated with increased fibrosis, inflammation, hypoxia, and autoimmunity (Figure 6). Toll-like receptors (TLR) are classified as pattern recognition receptors and exhibit expression on monocytes, macrophages, dendritic cells, B cells, and T cells. The signaling pathway activates NF-kappa B, leading to cytokine production. TLRs participate in the development of autoimmune and inflammatory diseases [104]. Liao et al. have reported that TLR-9 gene polymorphisms were associated with an increased risk of GO in male GD patients [105]. HIF-1α is activated in response to cellular hypoxia, which results in tissue remodeling in GO through activation of HIF-1α-dependent pathways in orbital fibroblasts. HIF-1α levels in these cells correlate with the clinical activity score of GO patients [106]. Due to insufficient knowledge regarding the pathomechanism of GO, there is no effective and safe method of treating this disease. The current treatment with the use of methylprednisolone pulses is effective in active moderate to severe GO in about 50% of cases and it carries the risk of complications, including fatalities (thromboembolic complications, sudden cardiac deaths, and severe liver damage) [107, 108]. An in-depth understanding of the function of immune cells as well as fibroblasts, adipocytes, and cytokines in GD patients may, in the future, help to define new treatment modalities or improve monitoring of the disease activity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Delayed Rectifier K^+-Channel Is a Novel Therapeutic Target for Interstitial Renal Fibrosis in Rats with Unilateral Ureteral Obstruction

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Background. Delayed rectifier K^+-channel, Kv1.3, is most predominantly expressed in T-lymphocytes and macrophages. In such leukocytes, Kv1.3-channels play pivotal roles in the activation and proliferation of cells, promoting cellular immunity. Since leukocyte-derived cytokines stimulate fibroblasts to produce collagen fibers in inflamed kidneys, Kv1.3-channels expressed in leukocytes would contribute to the progression of tubulointerstitial renal fibrosis. *Methods*. Male Sprague-Dawley rats that underwent unilateral ureteral obstruction (UUO) were used at 1, 2, or 3 weeks after the operation. We examined the histological features of the kidneys and the leukocyte expression of Kv1.3-channels. We also examined the therapeutic effects of a selective channel inhibitor, margatoxin, on the progression of renal fibrosis and the proliferation of leukocytes within the cortical interstitium. *Results*. In rat kidneys with UUO, progression of renal fibrosis and the infiltration of leukocytes became most prominent at 3 weeks after the operation, when Kv1.3-channels were overexpressed in proliferating leukocytes. In the cortical interstitium of margatoxin-treated UUO rat kidneys, immunohistochemistry revealed reduced expression of fibrosis markers. Additionally, margatoxin significantly decreased the numbers of leukocytes and suppressed their proliferation. *Conclusions*. This study clearly demonstrated that the numbers of T-lymphocytes and macrophages were markedly increased in UUO rat kidneys with longer postobstructive days. The overexpression of Kv1.3-channels in leukocytes was thought to be responsible for the proliferation of these cells and the progression of renal fibrosis. This study strongly suggested the therapeutic usefulness of targeting lymphocyte Kv1.3-channels in the treatment of renal fibrosis.

1. Introduction

Chronic tubulointerstitial nephritis (TIN) is an entity of renal disease characterized by a progressive scarring of tubulointerstitium [1], sometimes deteriorating into end-stage renal disease [2, 3]. The lesion includes tubular atrophy, leukocyte infiltration, and interstitial fibrosis. In addition to drugs and toxins, such as analgesics, antibiotics, Chinese herbs, and heavy metals [4–6], chronic ureteral obstruction and repetitive infection are also the leading causes of chronic TIN, especially in infants [7, 8]. To reproduce the lesion characteristic to renal fibrosis, the animal model of unilateral ureteral obstruction (UUO) was developed in 1970s, which primarily represented the pathology of obstructive nephropathy [9]. In rodent models of UUO, leukocytes, such as lymphocytes, macrophages, neutrophils, and mast cells, are known to infiltrate into the renal interstitium [9–11]. Among them, many studies have focused on the involvement of mast cells in the development of renal fibrosis [12–14], in which mast cells were demonstrated to produce fibroblast-activating factors in addition to chemical mediators [15, 16]. However, we know little about the pathological roles of T-lymphocytes or macrophages in the progression of renal fibrosis, despite their predominance

in the renal interstitium [9–11]. These leukocytes principally express delayed rectifier K^+-channels (Kv1.3) in their plasma membranes, and the channels play critical roles in the activation and proliferation of the cells [17, 18]. Since the cytokines produced by the inflammatory leukocytes directly stimulate the collagen synthesis from interstitial fibroblasts [19], the channels expressed in the leukocytes would contribute to the progression of renal fibrosis in UUO. To clarify this, using a rat model of UUO, we examined the Kv1.3-channel expression in the kidneys and the therapeutic effects of a selective channel inhibitor, margatoxin, on the progression of renal fibrosis and the proliferation/activation of leukocytes there. Here, we clearly show that the numbers of T-lymphocytes and macrophages were markedly increased in UUO rat kidneys at 3 weeks after the operation. We also show that the overexpression of Kv1.3-channels in leukocytes was responsible for the proliferation of these cells and the progression of renal fibrosis. This study strongly suggests the therapeutic usefulness of targeting lymphocyte Kv1.3-channels in the treatment of renal fibrosis.

2. Materials and Methods

2.1. Animal Preparation and UUO Induction. Male Sprague-Dawley rats weighing 150–180 g (Japan SLC Inc., Shizuoka, Japan) underwent unilateral ureteral ligation, as described in previous studies [9–11]. Briefly, after the rats were deeply anesthetized with isoflurane, the left ureter was exposed through a lateral flank incision. Then the ureter was ligated with 3-0 silk at two points under sterile conditions. During the subsequent 1 to 3 weeks, rats had free access to standard rat chow and water ad libitum and were maintained in a humidity- and temperature-controlled room on a 12-hour light-dark cycle. One, two, or three weeks after the operation, the rats were deeply anesthetized and then killed by cervical dislocation. The left kidneys were removed for histological examination and RNA extraction. The contralateral kidneys at 3 weeks after the operation were used as controls. Trunk blood was withdrawn for the measurements of serum creatinine and urea nitrogen levels. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Tohoku University.

2.2. Margatoxin Treatment. For the treatment with Kv1.3-channel inhibitor, margatoxin (Peptide Institute, Osaka, Japan) was dissolved in normal saline to prepare a concentration of 200 nM. After inducing unilateral ureteral obstruction, the rats were intraperitoneally injected with 200 nM/ml margatoxin daily for 3 weeks (margatoxin-treated group). In our previous study, using rat models with advanced chronic renal failure (CRF), 100 nM/ml margatoxin actually ameliorated the progression of renal fibrosis without causing any adverse events [20]. In our preliminary study, since 100 nM margatoxin did not ameliorate the progression of renal fibrosis in UUO rat kidneys, we selected higher dose in the present study. At the end of the observation period, the left kidneys were removed for histological examination and RNA extraction.

2.3. Histological Analyses. Renal cross sections were fixed in 4% paraformaldehyde, embedded in paraffin, and deparaffinized in xylene, and then 3 μm sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome. For fibrosis analysis, Masson's trichrome deposition, expressed as percentages of Masson's trichrome-positive areas relative to the total area, was quantified in each field and averaged, as described in our previous studies [16, 21–23].

2.4. Immunohistochemistry. The 3 μm paraffin sections of 4% paraformaldehyde-fixed kidneys were placed in citrate-buffered solution (pH 6.0) and then boiled for 30 min for antigen retrieval. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and nonspecific binding was blocked with 10% BSA. Primary antibodies were as follows: Mouse anti-collagen type III (1 : 100; Abnova, Taipei City, Taiwan), anti-α-smooth muscle actin (α-SMA) (1 : 100; Thermo Fisher Scientific, Cheshire, UK), anti-CD3 (1 : 50; Thermo Fisher Scientific), anti-ED-1 (1 : 50; AbD Serotec, Oxfordshire, UK), anti-myeloperoxidase (MPO; 1 : 100; Novus Biologicals, Littleton, CO, USA), rabbit anti-Ki-67 (1 : 100; Lab Vision Co., Fremont, CA, USA), and anti-Kv1.3 (1 : 100; Bioss Inc., Woburn, MA, USA). Diaminobenzidine substrate (Sigma Chemical Co., St. Louis, MO, USA) was used for the color reaction. At the end of the staining, the sections were counterstained with hematoxylin. The secondary antibody alone was consistently negative on all sections. Toluidine blue staining was performed by immersion of the sections in 0.1% toluidine blue (Muto Pure Chemical Co., Tokyo, Japan) for 30 min at room temperature. Mast cells were identified by their characteristic metachromasia. For quantitative analysis, the numbers of CD3-, ED-1-, toluidine blue-, MPO-, Ki-67-, and α-SMA-positive cells were counted in high-power fields of the cortical interstitium as described in our previous studies [16, 21–23].

2.5. Real-Time RT-PCR. Total RNAs from freshly isolated renal cortex were extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). First-stand cDNA was synthesized from 2 μg of total RNA of each tissue in 20 μl of reaction mixture using the SuperScript VILO first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). The quantitative RT-PCR was carried out using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies Inc, Gaithersburg, MD, USA) with SYBR Premix Ex Taq II (Takara Bio, Kyoto, Japan). The sequences of the primers used were as follows: KCNA3, forward 5′-GCTCTCC CGCCATTCTAAG-3′, reverse 5′-TCGTCTGCCTCAG-CAAAGT-3′; GAPDH, forward 5′-GGCACAGTCAAGG CTGAGAATG-3′, reverse 5′-ATGGTGGTGAAGACGCC AGTA-3′. The quantity of RNA samples was normalized by the expression level of GAPDH.

2.6. Other Measurements and Statistical Analyses. Serum electrolytes, creatinine, and blood urea nitrogen levels were measured using a chemical autoanalyzer (DRI-CHEM 3500V; Fuji, Tokyo, Japan). Data were analyzed using

Microsoft Excel (Microsoft Co., Redmond, WA, USA) and reported as means ± SEM. Statistical significance was assessed by two-way ANOVA followed by Dunnett's or Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Progression of Renal Fibrosis and Leukocyte Proliferation in UUO Rat Kidneys. Serum creatinine and blood urea nitrogen levels in rats 3 weeks after UUO were compatible with those in normal rats (serum creatinine, 0.43 ± 0.06 mg/dl; blood urea nitrogen, 17.7 ± 0.60 mg/dl; $n = 4$), indicating that renal function was well preserved in UUO rats. However, in these rat kidneys, Masson's trichrome staining and the immunohistochemistry for collagen III, a marker of fibrosis, demonstrated a wide range of staining in the cortical interstitium (Figure 1(a) (A versus B–D), (E versus F–H)), which expanded progressively with the increasing number of postobstructive days. Immunohistochemistry for α-SMA, a marker of myofibroblasts, also demonstrated increasing numbers of positively stained cells within the interstitium (Figure 1(a) (I versus J–L)). These results indicated the progression of renal fibrosis in rat kidneys with UUO, which confirmed the propriety of our rat model [9–11]. In the present study, we did not perform urine examination, since the unobstructed contralateral kidneys in UUO models usually offset the loss of renal function [24]. Additionally, previous studies indicated the lack of proteinuria in UUO models because the injured kidneys were completely obstructed and had no urine output [10]. However, by directly collecting the urine from the injured kidneys, recent studies have revealed the presence of several urinary proteins in UUO rat models, which may serve as candidate biomarkers of renal tubular injury and interstitial fibrosis [9].

In UUO rat kidneys, in addition to the progressive tubular dilatation and atrophy (Figure 1(b) (A versus B–D)), there were an increasing number of small round cells in the cortical interstitium, suggesting the increase in the inflammatory leukocytes. Since these small round cells were positive for Ki-67, a marker for cellular proliferation (Figure 1(b) (E–H)), these leukocytes were considered to proliferate *in situ* within the cortical interstitium.

3.2. T-Lymphocytes and Macrophages Are Prominently Increased in UUO Rat Kidneys. Previously, in our rat models with advanced CRF, the increased numbers of T-lymphocytes or macrophages in the cortical interstitium primarily contributed to the progression of renal fibrosis, since the cytokines produced by the leukocytes stimulated the fibroblasts' activity to produce collagen [20, 22]. Therefore, we examined the distribution of these leukocytes within the interstitium of the UUO rat kidneys (Figures 2(a) and 2(b)). One or two weeks after the induction of UUO, immunohistochemistry demonstrated the infiltration of some CD3 or ED-1-positive cells within the interstitium (Figures 2(a) (A versus B, C) and 2(b) (A versus B, C)). Then by 3 weeks, most of the interstitial leukocytes were positive for either CD3 or ED-1 (Figures 2(a) (D) and 2(b) (D)), indicating that the increased leukocytes were mainly T-lymphocytes or macrophages. In contrast to these leukocytes, there were only a few toluidine blue-positive mast cells in the renal subcapsular interstitial space (Figure 2(c), arrow heads), which did not increase despite the increasing numbers of postobstructive days (Figure 2(c) (A versus B–D)). Immunohistochemistry for myeloperoxidase demonstrated the presence of only a few positive cells through the observation period (Figure 2(d), arrow heads), indicating few infiltration of neutrophils. Figure 2(e) depicts the postobstructive changes in the numbers of CD3-, ED-1-, toluidine blue-, and myeloperoxidase-positive cells, which were counted in high-power views of the cortical interstitium. The differences between the numbers of T-lymphocytes or macrophages and those of mast cells or neutrophils became most prominent at 3 weeks after UUO (Figure 2(e)).

3.3. Leukocytes Overexpressed Kv1.3-Channels in UUO Rat Kidneys. In addition to megakaryocytes or platelets [25, 26], leukocyte, such as T-lymphocytes, or macrophages, predominantly express Kv1.3-channels in their plasma membranes [27]. These channels play pivotal roles in cellular immunity by facilitating calcium influx required for cellular proliferation and activation [18]. Therefore, we examined the leukocyte expression of Kv1.3-channels in UUO rat kidneys (Figure 3). By 2 weeks after UUO, the expression of Kv1.3 mRNA, KCNA3, was significantly increased in the cortex isolated from the rat kidneys (Figure 3(a)). By 3 weeks after UUO, the expression was dramatically increased, which showed similar patterns to the time-dependent progression of renal fibrosis (Figure 1(a)) and the proliferation of leukocytes (Figures 1(b) and 2). In control rat kidneys, as we previously demonstrated [20], immunohistochemistry for Kv1.3 showed weak staining in the cytoplasm of normal proximal tubular cells (Figure 3(b) A). However, at 3 weeks after UUO, Kv1.3 became overexpressed within the cytoplasm of proliferating leukocytes in the cortical interstitium (Figure 3(b) B).

3.4. Therapeutic Effects of a Selective Kv1.3-Channel Inhibitor in UUO Rat Kidneys. In our previous patch-clamp studies, margatoxin, a highly selective Kv1.3-channel inhibitor, almost totally suppressed the channel currents in lymphocytes [28, 29]. In both *in vitro* and *in vivo* studies, this drug was actually demonstrated to repress the proliferation of lymphocytes and their cytokine production [20, 30]. In the present study, to obtain the direct evidence that the overexpression of Kv1.3-channels contributes to the proliferation of leukocytes and to the progression of renal fibrosis, we actually treated the UUO rats with margatoxin and examined the fibrosis or leukocyte marker expression within the kidneys.

3.4.1. Effects of Margatoxin on the Progression of Renal Fibrosis. In margatoxin-treated UUO rat kidneys, Masson's trichrome staining demonstrated much smaller size

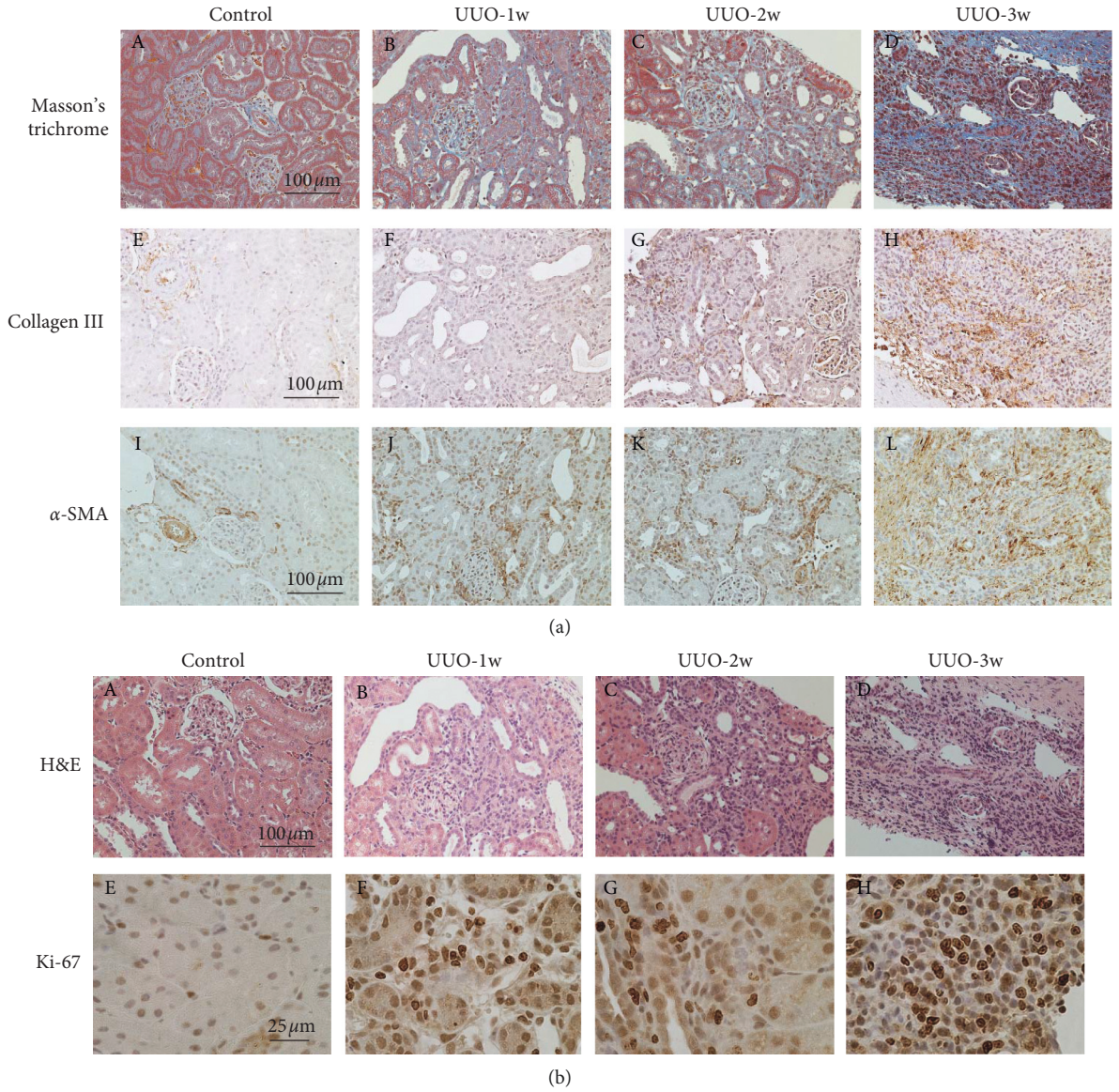

Figure 1: Fibrotic marker expression and leukocyte proliferation in UUO rat kidneys. (a) Masson's trichrome staining and immunohistochemistry using antibodies for collagen III (brown) and α-smooth muscle actin (α-SMA) (brown) in control (A, E, I) and UUO rat kidneys with 1 week (UUO-1w; B, F, J), 2 weeks (UUO-2w; C, G, K), and 3 weeks (UUO-3w; D, H, L) after unilateral ureteral obstruction. Magnification: ×20. (b) Hematoxylin and eosin (H&E) staining and immunohistochemistry for Ki-67 (brown) in control (A, E) and UUO rat kidneys with 1 week (UUO-1w; B, F), 2 weeks (UUO-2w; C, G), and 3 weeks (UUO-3w; D, H) after unilateral ureteral obstruction. (A–D) Magnification: ×20. (E–H) Magnification: ×60.

of the cortical interstitium compared to that in margatoxin-untreated kidneys (Figure 4(a) (B versus A)). There was actually a statistical significance in the percentages of the Masson's trichrome-stained areas relative to the total areas between the margatoxin-untreated and margatoxin-treated UUO rat kidneys (Figure 4(a) C). Additionally, immunohistochemistry α-SMA demonstrated a significant decrease in the number of myofibroblasts in margatoxin-treated UUO rat kidneys (Figure 4(b) B versus A), which was quantitatively confirmed by the decreased number of α-SMA-positive cells in high-power fields (Figure 4(b) C). These results strongly suggested that margatoxin suppressed the number of myofibroblasts and thus halted the progression of renal fibrosis in UUO rat kidneys.

3.4.2. Effects of Margatoxin on Infiltration and Proliferation of Interstitial Leukocytes. In the cortical interstitium of margatoxin-untreated UUO rat kidneys, there were a substantial number of infiltrating leukocytes (Figure 5(a) A), such as CD3-positve T-lymphocytes and ED-1-positive macrophages (Figure 5(a) (C, E)). However, in margatoxin-treated UUO rat kidneys, the numbers of these cells were much smaller in the cortical interstitium (Figure 5(a) (B, D, F)). As shown in Figure 5(a) (G), significant difference was

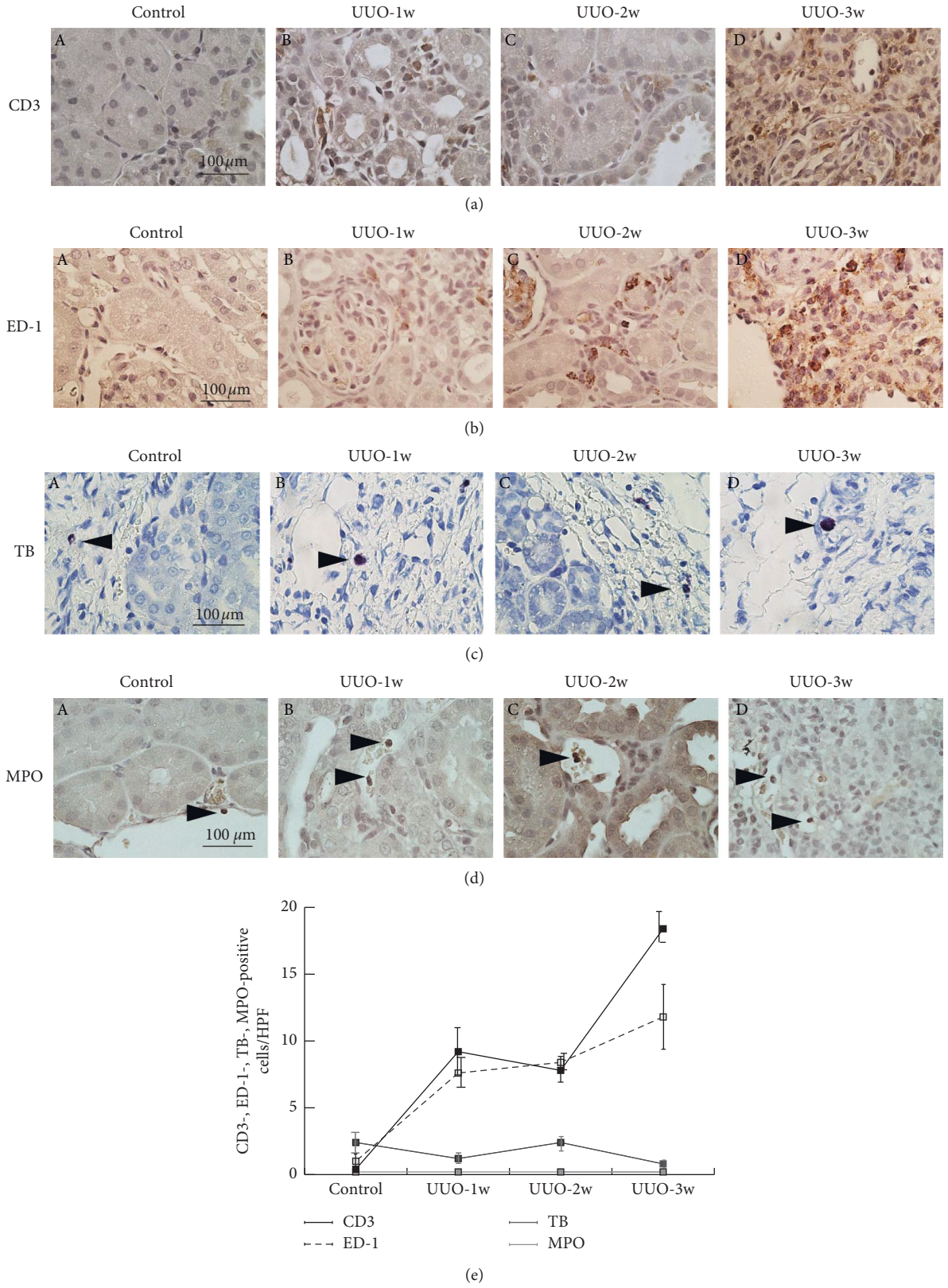

Figure 2: Markers for T-lymphocytes, macrophages, mast cells, and neutrophils expression in the cortical interstitium of UUO rat kidneys. Immunohistochemistry using antibodies for CD3 (a), ED-1 (b) (brown), toluidine blue (TB) staining (c) (blue, arrow heads), and immunohistochemistry for myeloperoxidase (MPO) (d) (brown, arrow heads) in control (A) and UUO rat kidneys with 1 week (UUO-1w, B), 2 weeks (UUO-2w, C), and 3 weeks (UUO-3w, D) after unilateral ureteral obstruction. Magnification: ×60. (e) Numbers of CD3-positive T-lymphocytes, ED-1-positive macrophages, toluidine blue-positive mast cells, and myeloperoxidase-positive neutrophils were counted in high-power views within the cortical interstitium of control, UUO-1w, UUO-2w, and UUO-3w rat kidneys. Values are means ± SEM ($n = 5$).

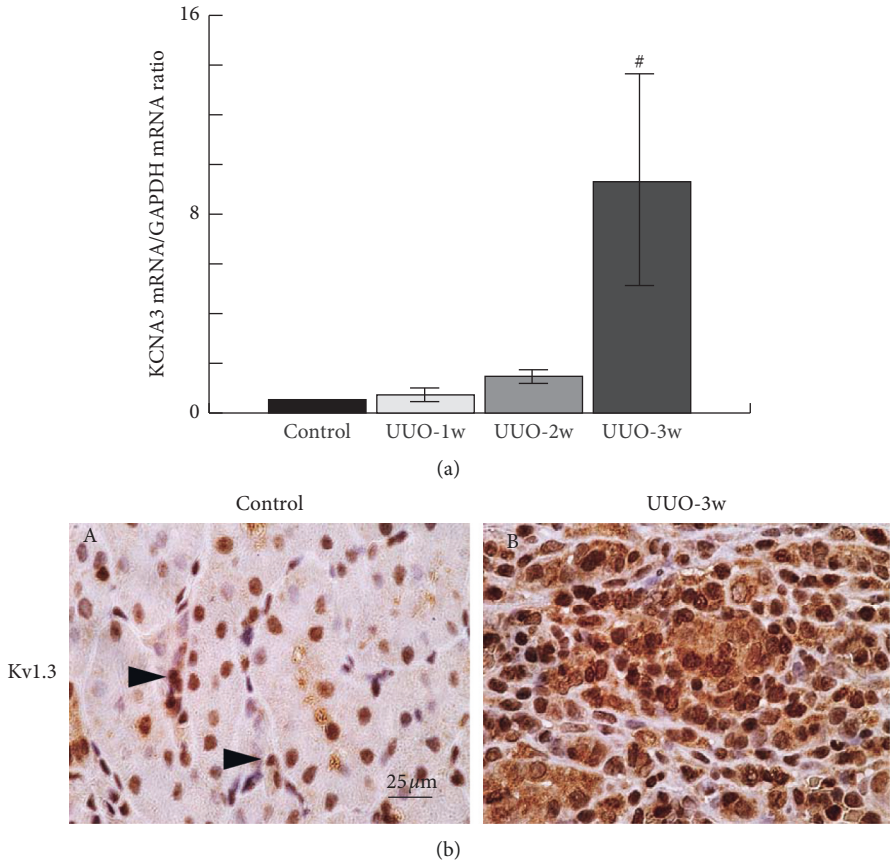

Figure 3: Kv1.3 expression in UUO rat kidneys. (a) KCNA3 mRNA abundance in the renal cortex of control and UUO rat kidneys with 1 week (UUO-1w), 2 weeks (UUO-2w), and 3 weeks (UUO-3w) after unilateral ureteral obstruction. $^{\#}p < 0.05$ versus control rats. Values are means ± SEM ($n = 6$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t-test. (b) Immunohistochemistry using antibody for Kv1.3 (brown, arrow heads) in control (A) and UUO-3w (B) rat kidneys. High-power views of cortical interstitium. Magnification: ×60.

obtained in the number of CD3-positive cells between margatoxin-treated and margatoxin-untreated UUO rat kidneys. These results indicated that margatoxin actually decreased the numbers of inflammatory leukocytes in the renal interstitium of UUO rat kidneys.

Immunohistochemistry for Ki-67 demonstrated a large number of positively stained inflammatory leukocytes within the cortical interstitium of margatoxin-untreated UUO rat kidneys (Figure 5(b) A). However, in margatoxin-treated UUO rat kidneys, there were much less leukocytes positively stained with Ki-67 (Figure 5(b) B). As shown in Figure 5(b) (C), a marked difference was obtained in the numbers of Ki-67-positive cells between margatoxin-treated and margatoxin-untreated UUO rat kidneys. From these results, margatoxin was thought to suppress the *in situ* proliferation of infiltrating leukocytes and thus decreased their numbers within the UUO rat kidneys.

4. Discussion

Using animal models of UUO, previous studies revealed the involvement of mast cells in the development renal fibrosis [12–14] because mast cells release growth factors or cytokines that stimulate the collagen synthesis from fibroblasts [15, 16]. In some studies, tranilast, a mast cell stabilizer, actually ameliorated the progression of renal fibrosis in UUO [31, 32]. However, due to their small occupation in leukocytes that infiltrated into the cortical interstitium, targeting mast cells alone was not enough for the treatment. In the pathogenesis of renal fibrosis, transforming growth factor beta-1 (TGF-β1) plays a major role, since it directly promotes the fibroblast proliferation and stimulates their collagen synthesis [33]. TGF-β1 also activates the downstream Smad signal transduction pathway to generate extracellular matrix [34]. Regarding the mechanism by which tranilast exerted antifibrotic effects [16, 35], this drug was considered to decrease the TGF-β1 expression and repress its activity in the fibrotic kidneys [31, 32, 36, 37], in addition to its mast cell-stabilizing properties [16, 38]. In the present study, we clearly demonstrated the predominance of T-lymphocytes or macrophages over mast cells or neutrophils in UUO rat kidneys, which became most prominent at 3 weeks after inducing UUO (Figure 2). In our previous study using rat models with advanced CRF, proinflammatory cytokines produced by the inflammatory leukocytes actually activated fibroblasts to produce collagen [22]. Additionally, in UUO rat kidneys, the time-dependent increase in T-lymphocytes and macrophages was well

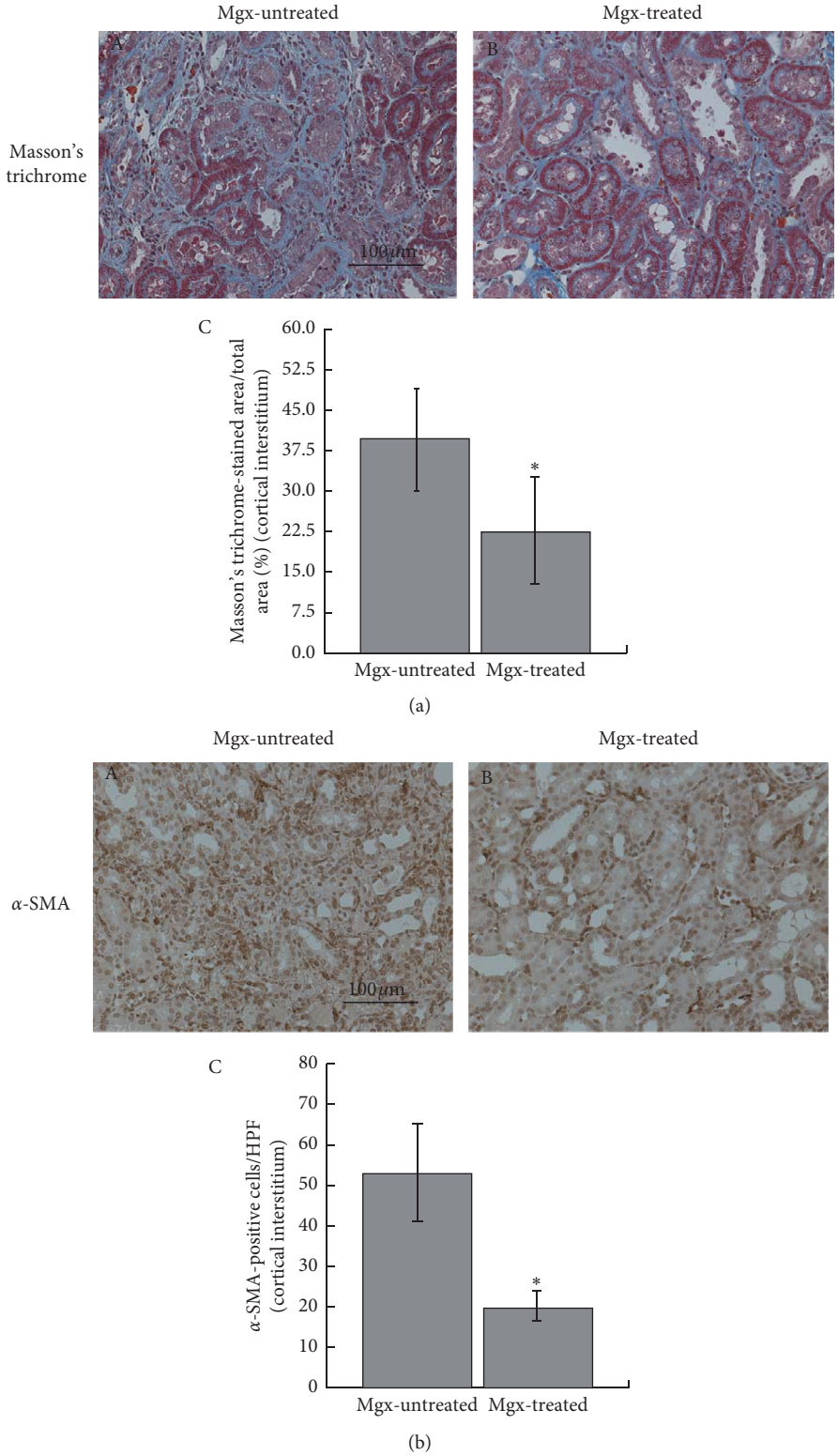

FIGURE 4: Fibrotic marker expression in margatoxin-untreated and margatoxin-treated UUO rat kidneys. (a) Masson's trichrome staining in margatoxin- (Mgx-) untreated (A) and margatoxin-treated (B) UUO rat kidneys. Low-power views of cortex. Magnification: ×20. (C) Masson's trichrome deposition was quantified and expressed as percentages of Masson's trichrome-positive areas relative to the total areas. (b) Immunohistochemistry using an antibody for α-SMA (brown) in margatoxin- (Mgx-) untreated (A) and margatoxin-treated (B) UUO rat kidneys. Magnification: ×20. (C) α-SMA-positive cells were counted in high-power views of the cortical interstitium. $^*P < 0.05$ versus margatoxin-untreated UUO rats. Values are means ± SEM ($n = 5$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

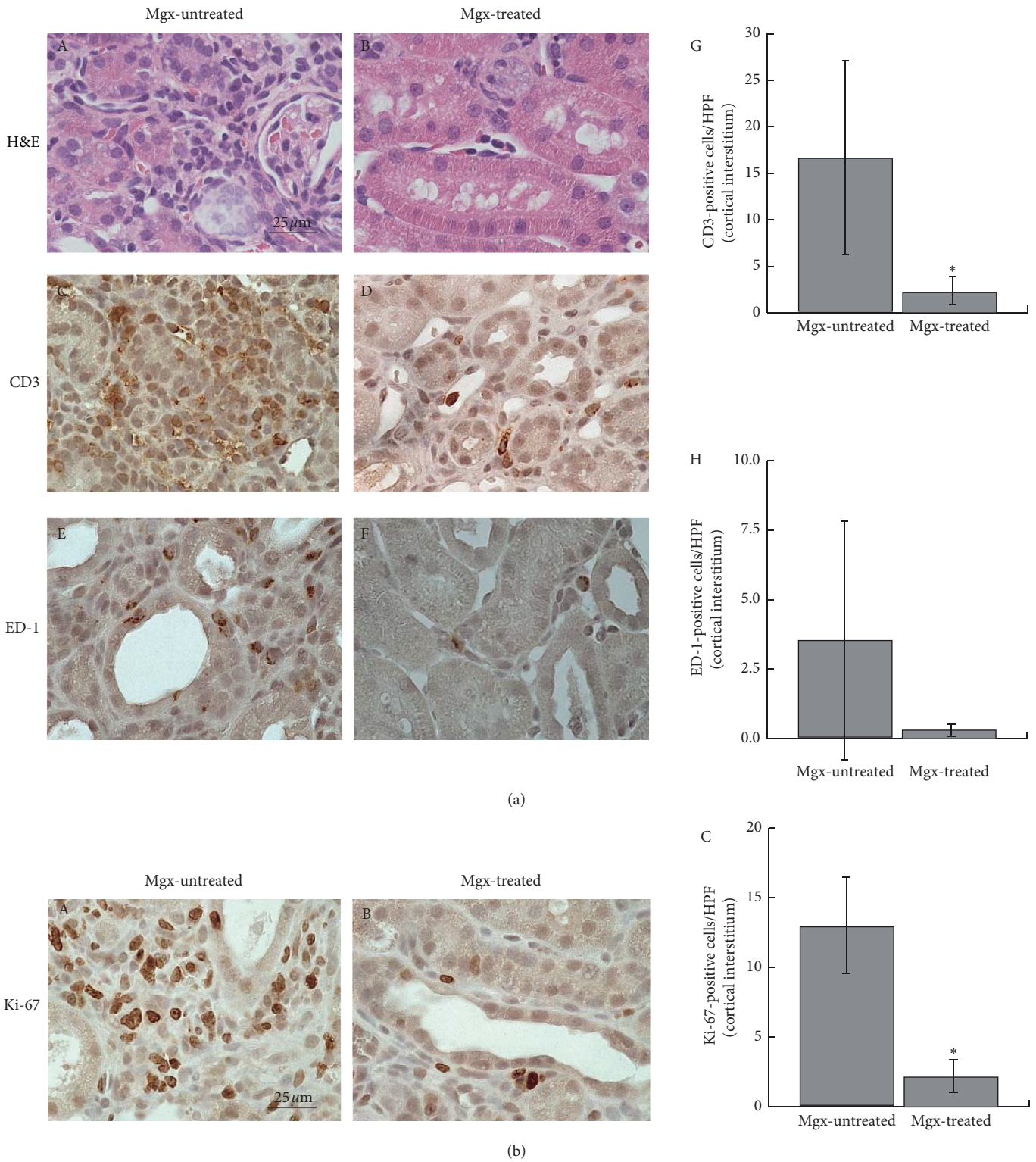

Figure 5: Markers for leukocytes and their proliferation in margatoxin-untreated and margatoxin-treated UUO rat kidneys. (a) Hematoxylin and eosin (H&E) staining and immunohistochemistry using antibodies for CD3 and ED-1 (brown) in margatoxin- (Mgx-) untreated (A, C, E) and margatoxin-treated (B, D, F) UUO rat kidneys. High-power views of cortex. Magnification: ×60. CD3-positive (G) and ED-1-positive (H) cells were counted in high-power views of the cortical interstitium. (b) Immunohistochemistry using antibody for Ki-67 (brown) in margatoxin- (Mgx-) untreated (A) and margatoxin-treated (B) UUO rat kidneys. High-power views of cortical interstitium. Magnification: ×60. (C) Ki-67-positive cells were counted in high-power views of the cortical interstitium. $^{*}P < 0.05$ versus margatoxin-untreated UUO rats. Values are means ± SEM ($n = 6$). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

correlated with such a progression pattern of renal fibrosis (Figures 1(a) and 2). Therefore, these inflammatory leukocytes were thought to be directly responsible for the progression of interstitial renal fibrosis in UUO.

T-lymphocytes and macrophages predominantly express Kv1.3-channels in their plasma membrane [18]. In UUO rat kidneys with longer postobstructive days, these leukocytes overexpressed the Kv1.3-channels within the cortical interstitium of fibrotic kidneys (Figure 3). In previous studies, the overexpression of Kv1.3-channels was noted in isolated cells under certain pathological conditions, such as cancer [39, 40], neuroinflammatory disorder, or ischemic heart disease [41, 42]. In these cells, Kv1.3-channels stimulate calcium signals to facilitate cellular proliferation by generating a driving force for inward calcium flow [17, 43]. In the present study, margatoxin, a selective inhibitor of Kv1.3-channels, suppressed the proliferation of leukocytes (Figure 5) and actually ameliorated the progression of renal fibrosis in UUO rat kidneys (Figure 4). Therefore, as previously shown in cancer cells or neuroinflammatory cells [42, 44], the membrane hyperpolarization bought about by the channels was thought to be responsible for the leukocyte proliferation/activation and the subsequent progression of renal fibrosis. Using a murine model of UUO, Grgic et al. demonstrated a therapeutic usefulness of targeting the intermediate-conductance Ca^{2+}-activated K^{+}-channels ($Kc_a3.1$) in the treatment of renal fibrosis, since these channels were overexpressed in proliferating fibroblasts [45]. From our results, the Kv1.3-channels overexpressed in lymphocytes or macrophages could also be the useful therapeutic target in the treatment of renal fibrosis.

Our recent patch-clamp study revealed that antiallergic drugs, such as cetirizine, fexofenadine, azelastine, and terfenadine, effectively suppressed lymphocyte Kv1.3-channels [46]. These lipophilic drugs were thought to distribute freely into the phospholipid bilayers of cell membrane [46, 47] and thus directly intruded into the composite domains of the channels from inside the membranes. Of note, since azelastine and terfenadine are more lipophilic than the other drugs [47], they would remain within the membranes for a long time, bringing about more continuous inhibitory pattern of the Kv1.3-channels [46]. In previous patch-clamp studies, we also revealed that so-called "commonly used drugs," such as antimicrobials, anti-hypertensives, and anticholesterol drugs, actually exerted inhibitory properties on the Kv1.3-channel currents in lymphocytes [28, 48–51]. Based on such pharmacological characteristics, we could clinically apply these commonly used drugs in the treatment of renal fibrosis. Since these drugs have long been used in daily medical practice, they are more reliable and safer drugs than the selective channel inhibitors that were chemically synthesized originally from venom or scorpion toxins [52–55].

In summary, this study clearly demonstrated that the numbers of T-lymphocytes and macrophages were markedly increased in UUO rat kidneys at 3 weeks after the operation. The overexpression of Kv1.3-channels in leukocytes was thought to be responsible for the proliferation of these cells and the progression of renal fibrosis. This study strongly suggested the therapeutic usefulness of targeting lymphocyte Kv1.3-channels in the treatment of renal fibrosis.

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 no conflicts of interest.

Acknowledgments

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Research Article

Static Low-Angle Squatting Reduces the Intra-Articular Inflammatory Cytokines and Improves the Performance of Patients with Knee Osteoarthritis

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Osteoarthritis (OA) is one of the major diseases leading to disability, and inflammation plays an important role in the pathogenesis of OA. However, inflammation of OA is multifactorial, chronic, and in low intensity, which makes drug-based immunotherapy difficult. Here, we have designed a novel method of exercise—static low angle squat (SLAS), which reduces the intra-articular inflammation of OA knee as well as strengthens the vastus medialis of quadriceps. A two-year follow-up trial of current exercise methods demonstrated long-term, significant improvement in pain relief, range of motion, muscle strength, and knee stability.

1. Introduction

Osteoarthritis (OA) of the knee that is characterized by focal loss of articular cartilage and marginal bone formation, resulting in joint space narrowing and osteophytosis, is one of the top causes of disability among adults [1]. About 12% of Americans above the age of 60 experience symptomatic knee OA [2]. In China, the incidence is 49% of the retired population in the city, and 38% of the people above 60 years in the country side [3].

Although the etiology of OA is incompletely understood, aging, damage of the articular cartilage, and the relevant inflammation process clearly play a major role. After the cartilage is damaged, the degraded metabolites in the joint cavity leads to synovitis. The inflammatory cytokines, proteases, and prostaglandins are therefore secreted into the joint cavity, altering the physicochemical properties of the synovial fluid. The cartilage that rubs abnormally are susceptible to further damage [4]. In recent years, there is growing evidence that inflammation, which is mainly mediated by the innate immune system, plays a key role in the OA pathogenesis. However, there is so far no clinical evidence that patients with knee osteoarthritis could benefit from the immunotherapy, including the corticosteroid injections [5]. This might be due to the fact that the pathogenesis of OA involves multiple inflammatory factors that are often present in a chronic state, relatively low grade.

Researchers showed that patients can benefit from strength training, which has positive effects on pain scores and functional outcomes in knee OA [6]. More importantly, long-term exercise could reduce the inflammatory condition of knee osteoarthritis. Clinical applications show that both failure to remain active and disuse of the affected limb can accelerate impaired joint mechanics and potentially result in articular cartilage softening and matrix dysfunction, leading to more rapid cartilage degeneration [7]. Exercise strengthening the overlying muscles and soft tissue structures of joint could improve gait mechanics, joint movement, fascial tension, and tissue circulation [8]. These

improvement can further upregulate costimulatory factor PGC-1α in skeletal muscle, which negatively regulates NF-κB and inhibits the enhancement of IL-1β, IL-6, and TNFα-induced by NF-κB [9]. These cytokines have been shown to be involved in the regulation of cartilage homeostasis, by promoting cartilage catabolism and inhibiting anabolism.

New evidence has emerged suggesting that moderate physical activity may be beneficial to the people with osteoarthritis [10]. However, there is always discussion about the exercise safety and a more beneficial, long-term, non-pharmacology approach for the rehabilitation of OA is needed. Squat is one of the most frequently used exercises in the field of strength and conditioning, which recruits most of the lower body musculature, including the quadriceps femoris, hip extensors, hip adductors, hip abductors, and triceps surae. In addition, significant isometric activity is conferred to a wide range of supporting muscles to facilitate postural stabilization of the trunk, of which over 200 muscles are activated during squat performance [11]. Nevertheless, squats can be performed at a variety of depths, and deep squat, especially with weight bear, can be harmful to the knee [12]. In China, a static squat that is performed in the low angle, called "horse stance (Ma Bu)," has been put into practice for a long history, which provides a reliable and safe isometric exercise strengthening not only quadriceps but also a set of surrounding muscles. In this study, we designed a novel approach of static low angle squat (SLAS), based on the traditional Chinese exercise. We found that, after one year of SLAS exercise, the levels of proinflammatory factors, TNFα and IL-1β, were significantly reduced in the synovial fluid of OA knee, while the anti-inflammatory cytokine IL-10 increased. Moreover, we performed a large scale of comparative trials to evaluate this method in the OA populations and observed a significant improvement of the HSS scores in the treatment group, including both the functional recovery and pain reduction. Our results suggest that SLAS exercise provides a reliable approach in the rehabilitation of OA, which could be readily accepted by OA patients for long-term benefit.

2. Methods

2.1. Subjects. The inclusion criteria are as follows: the patient was between 54 and 65 years of age, unilaterally or bilaterally involved, with pain in and around the knee joint. Subjects were excluded if they had any deformity of the knee, hip, or back, or had any central or peripheral nervous system involvement, or had received steroids or intra-articular injection within the previous three months or received physiotherapy treatment in the past 6 months.

The study was approved by our Institutional Ethical Committee (IEC), and written consent was obtained from all the participants.

2.2. Methods. Subjects in the experiment underwent 2 years of exercise ($n = 55$). Exercise twice a day for 30 minutes each time. Patients who cannot be completed in one go can perform multiple exercises and adjust the interval accordingly.

SLAS exercise: patients stand with the legs apart; the distance between the knees as well as the feet should be as wide as the shoulders, and then they try to squat down. When bending the knees, patients should try to keep the back straight and adjust the angle of knees from straight down as close to 90 degrees as possible but no less than 90 degrees. However, it should not reach the position that patients feel painful so that any potential damage should be avoided.

Synovial fluids were taken from the OA knee before the exercise and 12 month after. Red blood cells were removed by centrifugation, and the supernatants were frozen at −80°C. Cytokine and BMP-7 (bone morphogenetic proteins) levels were determined with ELISA (R&D) according to the manufacturer's protocol.

The outcome measures for this study were HSS scores, including pain, knee function, ROM, quadriceps strength, deformity, and stability. These variables were measured on both sides of the legs, respectively. All the measurements were taken at baseline (January 2013), 1 year (December 2013), and 2 years (December 2014) after exercise.

2.3. Statistical Analysis. Statistical analyses were performed using the Statistical Package of Social Science (SPSS software version 18.0). The means and standard deviations were computed, and the one sample paired t-test was used to compare pre- and postintervention measures on the pain, knee function, ROM, quadriceps strength, deformity, and stability, respectively. The level of statistical significance was set at $p < 0.05$.

3. Results

To strengthen the quadriceps and the surrounding ligament, we designed a static squat protocol, SLAS, based on the horse stance (Ma Bu) that is the traditional exercise in China. The patients flex their knees (not less than 90 degrees) while exercising and keep their back straight. The knee angle may be reduced after long-term exercise, but the reduction must be the extent to which the patient does not feel pain.

The synovial fluid of OA knee was taken before the exercise and 12 months after the exercise. Samples were analyzed for TNFα, IL-1β, and IL-10 (Table 1). The concentration of proinflammatory cytokine TNFα and IL-1β showed significant decreases after the SLAS exercise compared to before ($p < 0.001$). In contrast, a highly significant increase was found for the anti-inflammatory cytokine IL-10 ($p < 0.001$), suggesting that the SLAS exercise could reduce the inflammation in the OA knee. Correspondingly, the level of BMP-7 in the synovial fluid is significantly reduced in the patients 12 months after SLAS exercise ($p < 0.001$) (Table 1). BMP-7 is an important bone conversion biomarker, which has been shown to correlate with the disease severity of knee OA.

The subjects in the experimental performed the exercise for 24 months. Hospital for Special Surgery (HSS) scores [13] were used to evaluate the outcome for this study. The control and exercise group were chosen randomly, and the patients showed similar HSS scores before the follow-up trial (Table 2).

Table 1: Cytokine levels in the synovial fluid of OA knee.

	TNFα (pg/ml)	IL-1β (pg/ml)	IL-10 (pg/ml)	BMP-7 (pg/ml)
Before	22.43 ± 4.31	80.23 ± 6.54	45.14 ± 5.36	10.50 ± 2.54
After (12 months)	$14.07 \pm 2.89^*$	$43.75 \pm 5.23^*$	$90.45 \pm 4.53^*$	$3.27 \pm 1.38^*$

"*Significant difference."

Table 2: Prescores for HSS.

Group	HSS score	Each item scoring					
		Pain	Function	ROM	Muscle strength	Flexion deformity	Knee stability
Control	61.30 ± 6.40	16.33 ± 2.22	7.95 ± 1.84	12.00 ± 2.31	8.04 ± 1.45	7.76 ± 0.74	9.21 ± 1.28
Exercise	61.44 ± 6.56	16.19 ± 2.43	7.93 ± 1.92	12.00 ± 2.33	7.99 ± 1.62	7.72 ± 0.78	9.17 ± 1.34

Table 3: 12 months postexercise scores for HSS (CI 0.95).

Group	HSS score	Each item scoring					
		Pain	Function	ROM	Muscle strength	Flexion deformity	Knee stability
Control	62.04 ± 6.44	16.19 ± 2.52	7.93 ± 1.80	12.00 ± 2.27	7.99 ± 1.19	7.72 ± 0.72	9.17 ± 1.17
Exercise	$75.35 \pm 9.00^*$	$22.23 \pm 4.85^*$	$9.89 \pm 2.74^*$	$14.40 \pm 2.12^*$	$9.62 \pm 1.16^*$	$9.72 \pm 0.78^*$	9.46 ± 1.09

Table 4: 24 months postexercise scores for HSS (CI 0.95).

Group	HSS score	Each item scoring					
		Pain	Function	ROM	Muscle strength	Flexion deformity	Knee stability
Control	60.94 ± 9.56	16.85 ± 5.04	7.84 ± 2.77	12.36 ± 2.12	7.64 ± 1.15	7.76 ± 0.74	9.46 ± 1.09
Exercise	$85.77 \pm 7.50^*$	$24.01 \pm 4.11^*$	$18.94 \pm 2.71^*$	$14.20 \pm 2.27^*$	$9.43 \pm 1.25^*$	$9.76 \pm 0.74^*$	9.41 ± 1.23

Pain and functional scores were significantly improved after 12 months and 24 months of exercise ($p < 0.001$). After 12 months of regular exercise, there was also a significant difference in ROM and muscle strength scores ($p < 0.001$), with a slight increase in the second year of exercise ($p < 0.05$). The deformity scores were significantly enhanced in the first year ($p < 0.01$) but not altered in the second year. However, there was no significant difference in knee stability between the scores before and after the exercise program ($p = 0.319$) (Tables 3 and 4).

OA often causes joint space narrow that mostly takes place on the inner board of the knee, due to the bias of leg structure and the barycenter of our bodyweight. SLAS exercise could strengthen particularly the vastus medialis muscle of quadriceps and increase the joint space. Consistent to this hypothesis, our follow-up trial demonstrated a significant pain relief and the restoring of functions in the OA patients.

4. Discussion

In this study, we designed a novel exercise approach, SLAS, which has a broad effect on the muscle on the back and surrounding knees. Our study indicated that SLAS exercise reduces the inflammation condition of OA knee. With the large scale of comparative trials, we found that SLAS could in long term reduce the pain and improve the function, mobility, and stability of knee, providing a reliable method for the rehabilitation of OA patients.

The pathogenesis of OA begins with cartilage damage and matrix protein release and is exacerbated by the triggering of DAMP (danger associated molecular patterns) signaling and subsequent chronic inflammation. However, single factor immunotherapy has proven to be useless for OA treatment because of the involvement of multiple inflammatory factors. Furthermore, the low inflammatory intensity in OA does not meet the principles of conventional anti-inflammatory therapies. Therefore, OA treatment requires long-term, reliable treatment for low-grade chronic inflammation. Our data suggest that low-dose long-term exercise such as SLAS can reduce the inflammatory state of the OA knee joint, which may be achieved not only by strengthening the muscles but also by soothing the microenvironment niche of innate immunity in the joint space.

Strength of the quadriceps is one of the intrinsic factors that has been shown to affect the knee joint functions and is closely associated with disability [14]. Many of the resistance-training protocols have focused on strengthening quadriceps, of which straight leg raising (SLR) exercise is often considered. With patient sitting and raising tibias, SLR could largely improve the muscle of quadriceps; however, such exercises may neglect the practice of hamstring that is critical for the stability of knees. It is known that quadriceps strengthening alone is not sufficient to treat the subgroup patients of OA such as tibiofemoral OA [15]. SLAS may be able to motivate the muscle not only on the leg but also in the hip and back, providing more comprehensive improvement of knee stability. Therefore, there is need for studying the

effect of SLAS on the strengthening of hamstring or hip abductor as compared to quadriceps-only programs.

Rehabilitation of OA is usually focused on symptom management, where pain relief, improved joint function, and joint stability are the main goals of treatment. SLAS exercise provides a new rehabilitation program without the risk of causing further damage, which improves knee stability, strengthens the medial femoral muscle of the quadriceps, and expands the joint space. Also, our two-year follow-up trial showed that SLAS exercise could counteract muscle atrophy, reduce pain, and partially restore knee function. In addition, SLAS is designed based on the posture of Chinese traditional exercise, the action and concept of which would be readily accepted by a large population. In practice, SLAS can be performed at home and adjusted accordingly by the patient, which is feasible in the long run.

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 there are no conflicts of interest.

Authors' Contributions

ZZ designed the posture and exercise program. RW, HZ, and KW performed the measurement. LC, YG, and HL performed the statistical analyses. JZ designed and supervised the measurement. All authors commented on the manuscript.

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Research Article

Th1- and Th17-Related Cytokines in Venous and Arterial Blood of Sclerodermic Patients with and without Digital Ulcers

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The earliest clinical manifestation of SSc is usually Raynaud's phenomenon, a small-arteries vasospasm driven by vascular tone dysregulation and microcirculatory abnormalities, resulting in digital ulcers (DU) in up to 50% of patients. Many cytokines as well as growth factors have been shown to play a role in promoting vascular smooth muscle cell proliferation and fibroblast activation, leading to ischemic damage as well as skin fibrosis. We aim to investigate a possible difference in venous and arterial blood levels of many cytokines (Th1- and Th17-related), GM-CSF, and endothelin-1 (ET1) in patients with and without DU. In the same patients, the correlations between capillary damage, evaluated by nailfold videocapillaroscopy (NVC), extension of skin fibrosis, calculated by modified Rodnan skin score (mRSS), and cytokines, ET-1, and GM-CSF levels were also measured. Patients with DU showed venous levels of IL-1β ($p = 0.024$), IL-6 ($p = 0.012$), IL-22($p = 0.006$), and TGF-β ($p = 0.046$) significantly higher compared to arterial levels and arterial levels of GM-CSF and TNF-alpha significantly higher compared to venous levels ($p < 0.001$). NVC abnormalities were correlated with arterial TNFa and venous IL22, IL23, and IL17 levels and negatively correlated with venous ET-1 levels, whereas mRSS showed a negative correlation with IL-21($\rho = -0.427$, $p = 0.050$). The increased Th17-cytokine levels in venous compared to arterial blood of patients with DU suggest local cytokine production on ulcer site. The higher TNFa and GM-CSF levels in arterial blood of DU patients support the attempt to mitigate the hypoxic damage, and the correlation between Th17-cytokines, mRSS, NVC, and ET1 agrees with the potent profibrotic stimulus at the onset of the disease, which decreases as the SSc progresses.

1. Introduction

Systemic sclerosis (SSc) is an autoimmune chronic connective tissue disease, affecting primarily the skin, characterized by a fibrotic involvement of many organs, such as the vascular and gastrointestinal system, lungs, and heart. Based on cutaneous involvement, SSc can be differentiated into two phenotypes, the diffuse SSc (dcSSc) and the limited one (lcSSc). The two phenotypes are also distinguishable on the basis of visceral involvement and autoantibodies pattern.

The earliest clinical manifestation of SSc is usually Raynaud's phenomenon (RP), a small-arteries vasospasm driven by vascular tone dysregulation and microcirculatory abnormalities, resulting in digital ulcers (DU) in up to 50% of patients [1–4].

The vascular damage is usually rapidly progressive, characterized by a recurrent activation and apoptosis of endothelial cells, with consequent intimal thickening, lumen stenosis, and vessel obliteration, leading to tissue hypoxia and to repeated ischemia-reperfusion cycles which induce progressive skin fibrosis.

It is well known that, as a response to vascular damage, endothelial cells release proinflammatory cytokines such as IL-6, IL-1β, and TNF-α and growth factors (GFs), among which are the vascular endothelial growth factor (VEGF) [5] and transforming growth factor β (TGF-β). These inflammatory mediators, in association with the profibrotic stimulus of the endothelin-1 (ET-1), promote leucocyte adhesion to the endothelium, vascular smooth muscle cell

proliferation, and fibroblast activation. This cytokine milieu is observed particularly at sites of digital ulcers [6–8]. In fact, other cytokines related to Th-2, Th-17, and Th-22 have been shown to play a role in the development of capillary damage and fibrotic skin in patients with scleroderma [9].

Until now, no studies focused on sclerodermic digital ulcers have analyzed the different inflammatory patterns mirrored by cytokines measured in arterial and venous patients' serum.

The aim of our study was to evaluate proinflammatory TNF-α, Th-1- (IL-2) and Th-17- (IL1-β, IL-6, IL-9, IL-17, IL-21, IL-22, and IL-23) related cytokines, TGF-β, GM-CSF, and endothelin-1 (ET-1) in arterial and venous blood of patients affected by systemic sclerosis with and without DU and to analyze the correlations between cytokine levels and clinical scores of skin fibrosis and vascular involvement.

2. Methods

2.1. Patients. All consecutive outpatients attending the Clinic of Rheumatic Disease between May 2014 and March 2015, who received the diagnosis of SSc (according to the ACR criteria), were enrolled in the study; the exclusion criteria were smoking, asthma, COPD [10], history of cancer [11] or other autoimmune disease, current corticosteroid or immunosuppressive therapy, and current or recent (last 8 weeks) systemic or respiratory infection. Arterial and venous blood sampling, nailfold video capillaroscopy, and modified Rodnan skin score [12, 13] were obtained in all the patients. In patients presenting digital ulcers, both arterial and venous blood samples were taken from the same side as the ulcers. Our Institutional Review Board for human studies approved the study protocol (no. 0039653), and the study respected the Helsinki Declaration.

Venous blood samples were obtained in twenty healthy nonsmoking subjects, who served as controls.

2.2. Cytokine Assays. Arterial and venous blood cytokines were analyzed by multiplex immunoassay (Bio-Rad Laboratories Inc., Hercules, CA, USA), with Bioplex 100 xMAP technology (Luminex Corp, Austin, TX, USA), and Bioplex Manager 4.1 software (Bio-Rad Laboratories, Segrate, Italy) was used for the data analysis. Every 96-well plate included an 8-point standard curve, and the same assay plate was used for patient and control samples. Table 1 reports the percentage of samples above the detection threshold. Low cytokine concentrations measured not on the linear part of the standard curves were considered below the limit of detection [14, 15]. Further analysis was performed on data set which included parameters (cytokines and growth factors) measured at concentrations higher than the detection limits in over 50% of samples.

2.3. Nailfold Videocapillaroscopy (NVC). Nailfold videocapillaroscopic examination was performed with a epiluminescence video-bio-microscope for immersion microscopy and polarized light microscopy (Videocap, DS Medica, Milan, Italy). Each test was evaluated by two different rheumatologists (CP and EF) using a qualitative score based on the morphology of nailfold capillaries. The patterns identified within the "scleroderma pattern" included (1) "early" NVC pattern: few enlarged/giant capillaries, few capillary hemorrhages, mostly well-preserved capillary distribution, and no evident loss of capillaries; (2) "active" NVC pattern: frequent giant capillaries, frequent capillary hemorrhages, moderate loss of capillaries, mild disorganization of the capillary architecture, and absent or mild ramified capillaries; (3) "late" NVC pattern: irregular enlargement of the capillaries, few or absent giant capillaries and hemorrhages, severe loss of capillaries with extensive avascular areas, disorganization of the normal capillary array, and ramified/bushy capillaries [16].

2.4. Modified Rodnan Skin Score (mRSS). Rodnan skin score, a semiquantitative score based on the fibrotic involvement of the skin, according to Khanna et al. [13] was obtained from all of the patients.

The score ranges from 0 to 51, being analyzed in 17 different areas of the body surface, some of which considered as double. Each area received a score ranging from 0 to 3, on the basis of the following criteria: mRSS = 0 was "normal skin" with fine wrinkles but no skin thickness; mRSS = 1 indicated "mild" skin thickness with possible folding between 2 fingers; mRSS = 2 corresponded to "moderate" skin thickness with difficulty in making skin folds and no wrinkles. mRSS = 3 indicated "severe" skin thickness with inability to make skin folds between 2 examining fingers.

2.5. Statistical Analysis. The statistical analysis was performed using a commercially available statistical package (STATA 10s), and only p values <0.05 were considered statistically significant.

Based on normality distribution tests (Kolmogorov–Smirnov, Shapiro–Wilk, and D'Agostino's K-squared), most cytokine concentrations were not normally distributed in both patients and healthy controls.

A comparison between arterial and venous blood was performed using the nonparametric test for independent or paired samples, based on the cohort of patients (healthy controls or patients' group).

The correlations between arterial or venous cytokines and clinical (mRSS) and instrumental (NVC) parameters were analyzed by regression analysis. Spearman's rank correlation coefficient was calculated according to the nonnormal distribution of data.

3. Results

Twenty-nine patients affected by SSc (28 females and 1 male) were enrolled in the study, as well as 20 healthy controls (12 females and 8 males).

The mean age was 64.5 years (range 28–80) in patients and 59 years (range 47–65) in healthy subjects (n.s.). Patients were then divided into two groups, based on the presence of digital ulcers (20 patients) or not (9 patients). All cytokines

Table 1: Cytokine concentrations (pg/ml) in venous blood of patients affected by SSc and in healthy controls.

	Patients median [CI 95%]	Healthy controls median [CI 95%]	p
TNF-α	2.07 [0.21–8.72]	1.22 [0.87–1.75]	0.008
IL-2	14.57 [3.52–18.42]	6.33 [1.62–22.73]	<0.001
IL-5	0.30 [0.05–10.76]	0.08 [0.00–3.38]	<0.001
IL-9	0.43 [0.11–11.89]	0.90 [0.10–34.25]	n.s.
IL-13	2.92 [0.42–76.85]	2.00 [0.11–9.50]	n.s.
GM-CSF	140.96 [138.65–141.42]	22.28 [20.72–22.56]	<0.001
IL-23	8.84 [0.60–14.79]	1.62 [1.58–1.72]	<0.001
IL-1b	0.18 [0.03–1.22]	0.32 [0.20–0.97]	<0.001
IL-6	3.82 [0.36–30.02]	1.23 [0.12–4.68]	0.011
IL-17	1.08 [0.41–1.65]	0.54 [0.04–0.85]	0.041
IL-21	23.38 [3.73–38.33]	3.60 [3.51–3.87]	<0.001
IL-22	3.75 [0.68–6.48]	1.95 [0.59–6.37]	<0.001
ET-1	15.67 [3.41–48.68]	5.42 [3.32–6.63]	<0.001
TGF-β	6.62 [5.03–26.24]	4.45 [3.82–14.64]	<0.001

Table 2: Comparison between venous and arterial blood cytokine concentrations in patients with and without digital ulcers.

Cytokines	Patients with digital ulcers			Patients without digital ulcers		
	Venous concentration median (pg/ml) [CI 95%]	Arterial concentration median (pg/ml) [CI 95%]	p	Venous concentration median (pg/ml) [CI 95%]	Arterial concentration median (pg/ml) [CI 95%]	p
TNF-α	8.51 [0.21–8.72]	28.81 [0.06–28.87]	<0.001	3.78 [2.06–7.08]	1.64 [0.37–4.03]	n.s.
IL-2	14.47 [9.95–15.03]	15.30 [11.77–15.86]	n.s.	15.24 [12.81–16.70]	16.02 [7.43–17.20]	n.s.
IL-5	0.33 [0.09–1.63]	0.19 [0.05–1.19]	n.s.	0.30 [0.13–4.20]	0.22 [0.02–4.53]	n.s.
IL-9	0.65 [0.11–3.31]	0.36 [0.13–2.47]	n.s.	0.42 [0.11–1.32]	0.37 [0.17–0.62]	n.s.
GM-CSF	140.88 [140.26–140.99]	141.18 [140.81–141.28]	<0.001	140.99 [139.97–141.32]	141.27 [140.84–141.48]	n.s.
IL-23	10.73 [7.14–11.62]	10.73 [10.58–11.68]	n.s.	8.81 [4.80–10.51]	9.60 [8.49–10.74]	n.s.
IL-1β	0.20 [0.17–0.64]	0.14 [0.11–0.16]	0.024	0.18 [0.03–0.67]	0.18 [0.11–0.21]	n.s.
IL-6	3.71 [2.52–11.03]	1.64 [1.22–3.98]	0.012	2.09 [1.23–4.32]	1.95 [0.63–3.01]	n.s.
IL-17	1.08 [0.78–1.18]	1.12 [0.94–1.33]	n.s.	1.17 [0.80–1.25]	0.93 [0.48–1.34]	n.s.
IL-21	23.38 [17.81–27.00]	23.38 [21.42–27.01]	n.s.	23.38 [14.55–27.49]	25.36 [17.04–30.01]	n.s.
IL-22	4.64 [4.13–5.30]	3.62 [3.33–4.60]	0.006	4.52 [3.42–5.23]	5.26 [4.43–5.70]	n.s.
TGF-β	7.10 [6.31–13.10]	7.02 [6.44–7.06]	0.046	6.24 [3.96–11.27]	6.62 [6.17–7.13]	n.s.
ET-1	14.39 [13.51–16.38]	15.88 [11.38–17.40]	n.s.	15.88 [9.36–28.08]	17.37 [10.76–24.55]	n.s.

were detectable both in arterial and venous blood samples in patients and in venous blood samples of controls.

3.1. Comparison between Venous Blood Concentration of Cytokines in Patients and Controls. Serum levels of all the cytokines and ET-1 were significantly higher in the patients compared to healthy controls, except for IL-9 and IL-13 (Table 1).

3.2. Comparison between Arterial and Venous Blood Cytokine Concentration. A significantly higher concentration of IL-1β ($p = 0.024$), IL-6 ($p = 0.012$), IL-22 ($p = 0.006$), and TGF-β ($p = 0.046$) was observed in venous compared to arterial blood samples only in patients with DU, who also showed a significantly higher concentration of GM-CSF and TNF-alpha in arterial compared to venous blood samples ($p < 0.001$) (Table 2).

No significant differences were observed between arterial and venous cytokine concentrations in patients without digital ulcers (Table 2).

3.3. Correlations between Cytokines and Clinical Parameters of Capillary (NVC) and Skin (mRSS) Involvement. Venous IL-22 ($\rho = 0.460$, $p = 0.041$), IL-23 ($\rho = 0.411$, $p = 0.042$), and IL-17 ($\rho = 0.465$, $p = 0.039$) concentrations were positively correlated and ET-1 ($\rho = -0.437$, $p = 0.044$) inversely correlated with NVC pattern in patients with DU, who also showed a significant correlation between NVC and arterial TNF-α concentration ($\rho = 0.460$, $p = 0.045$). A negative correlation between mRSS and venous IL-21 ($\rho = -0.427$, $p = 0.050$) was observed (Table 3).

No correlations were observed between serum cytokines (arterial nor venous) and skin involvement in patients without digital ulcers.

4. Discussion

Th1 and Th17 cytokines, as well ET-1, serum levels were significantly higher in patients compared to controls, indicating a systemic inflammatory status in patients who were not receiving any immunosuppressive drugs.

Looking at the patients with DU, it is interesting that they showed higher concentration of inflammatory (IL-6),

Table 3: Correlations between capillary (NVC) and skin (mRSS) involvement and blood cytokine concentration in patients with digital ulcers.

Cytokines	Nailfold videocapillaroscopy	
Venous blood		
IL-17	$\rho = 0.465$	$p = 0.039$
IL-22	$\rho = 0.460$	$p = 0.041$
IL-23	$\rho = 0.411$	$p = 0.042$
ET-1	$\rho = -0.437$	$p = 0.044$
Arterial blood		
TNF-α	$\rho = 0.460$	$p = 0.045$
	mRSS	
Venous blood		
IL-21	$\rho = -0.427$	$p = 0.050$

TGF-β, and Th17 (IL-1β, IL-22) related cytokines measured in venous compared to arterial blood, while no differences could be appreciated in patients without DU.

This observation suggests a local production of Th1- and Th17-related cytokines, which drive a proinflammatory and profibrotic tissue response.

Actually, Th17-related cytokines, particularly IL-17A, are thought to be involved in the pathogenesis of skin lesions in SSc by inducing adhesion molecules production and promoting collagen synthesis and proliferation [17]. Local skin production of Th1- and Th17-related cytokines has been found by Brembilla et al. [18], who also showed an increase of Th-22 cells in peripheral blood of patients affected by SSc, together with an increase of IL-22 mRNA in skin biopsies, suggesting a potential role of Th-17 and Th-22 in the pathogenesis of tissue fibrosis [18–20]. In our patients, we observed a significant correlation between Th17-related cytokines and capillary damage evaluated by NVC. In the same patients, we showed an inverse correlation between ET-1 levels and NVC scleroderma pattern. This observation may be explained by the well-known profibrotic stimulus played by ET-1 at the onset of disease and its reduction as the SSc progresses.

It has indeed been shown by Chora et al. that high serum ET-1 levels were strongly related to lung and vessel fibrosis only in patients with active SSc and not in the very early or late phases of disease [21].

Our results also found a negative correlation between venous IL-21 and mRSS in patients presenting DU. Zhou and colleagues demonstrated that mRNA of IL-21 was higher in the early phase of SSc, postulating IL-21 could be a biomarker able to identify lesions severity in early SSc [22].

The negative correlation we found between IL-21 and mRSS perfectly fits with the abovementioned finding, due to the elevated inflammation on the ulcer's site at the onset of disease and due to the reduction in cytokine recruitment when skin fibrosis plays a leading role and mRSS is higher.

The higher TNF-α and GM-CSF concentrations we found in the arterial blood samples of patients with DU could mitigate the hypoxic tissue damage, according to the physiological function of these cytokines. It has been demonstrated that the synthesis of TNF-α is increased immediately after ischemic injury, where it plays a role in regulating cells survival or apoptosis and in driving cellular inflammatory responses [5, 23]. Likewise, GM-CSF is a cytokine with pleiotropic functions, ranging from the regulation of proliferation, differentiation, and survival of hematopoietic cells to the mobilization and recruitment of hematopoietic and endothelial stem cells from the bone marrow. It also acts as a differentiation promoter of endothelial cells and fibroblasts and as a stimulatory factor for keratinocyte proliferation. To date, the efficacy of GM-CSF intradermal administration in the treatment of chronic venous leg ulcers is well known [24].

In conclusion, our study provides new information, which might suggest further possible targeted therapeutic approaches for digital ulcers of patients with scleroderma, who are presently lacking in effective approved therapies, except for the antagonists of ET-1.

Data Availability

All data are available if requested.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Comitato etico interaziendale—Città della Salute e della Scienza di Torino—no. 0039653) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

Nicola S., Fornero M., Fusaro E., Peroni C., Priora M., Rolla G., Bucca C. and Brussino L. declare that they have no conflicts of interest.

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Review Article

Interleukin-6 Expression by Hypothalamic Microglia in Multiple Inflammatory Contexts: A Systematic Review

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Interleukin-6 (IL-6) is a unique cytokine that can play both pro- and anti-inflammatory roles depending on the anatomical site and conditions under which it has been induced. Specific neurons of the hypothalamus provide important signals to control food intake and energy expenditure. In individuals with obesity, a microglia-dependent inflammatory response damages the neural circuits responsible for maintaining whole-body energy homeostasis, resulting in a positive energy balance. However, little is known about the role of IL-6 in the regulation of hypothalamic microglia. In this systematic review, we asked what types of conditions and stimuli could modulate microglial IL-6 expression in murine model. We searched the PubMed and Web of Science databases and analyzed 13 articles that evaluated diverse contexts and study models focused on IL-6 expression and microglia activation, including the effects of stress, hypoxia, infection, neonatal overfeeding and nicotine exposure, lipopolysaccharide stimulus, hormones, exercise protocols, and aging. The results presented in this review emphasized the role of "injury-like" stimuli, under which IL-6 acts as a proinflammatory cytokine, concomitant with marked microglial activation, which drive hypothalamic neuroinflammation. Emerging evidence indicates an important correlation of basal IL-6 levels and microglial function with the maintenance of hypothalamic homeostasis. Advances in our understanding of these different contexts will lead to the development of more specific pharmacological approaches for the management of acute and chronic conditions, like obesity and metabolic diseases, without disturbing the homeostatic functions of IL-6 and microglia in the hypothalamus.

1. Introduction

The identification of leptin uncovered the important role of the brain in the regulation of whole-body energy homeostasis [1], which has had a tremendous impact on our understanding of the underlying physiopathology of obesity and a number of related metabolic diseases [2–4]. The hypothalamus, in particular, plays an important role in metabolic regulation, and studies have demonstrated its critical role in the regulation of energy balance by integrating peripheral hormone and neuronal signals of satiety and nutritional status, as well as by directly sensing nutrients [5–8].

The leptin-melanocortin pathway is considered the most robust regulator of whole-body energy homeostasis [9, 10]. This function is provided by two counteracting populations of neurons in the arcuate nucleus of the hypothalamus (ARC), the first of which is proopiomelanocortin (POMC), which has an anorexigenic role, and the second is agouti-related peptide (AgRP)/neuropeptide Y (NPY), which has orexigenic action [3, 11]. These neuronal populations are sensitive to afferent inputs, like leptin and insulin, which regulate both acute and long-term energetic states.

Studies of diet-induced obesity (DIO) and aging have shown that the hypothalamus is targeted by an inflammatory process that leads to defective regulation of energy homeostasis [12–15]. By activating signal transduction through toll-like receptor 4 (TLR4), long-chain saturated fatty acids (SFAs) induce an inflammatory response in the hypothalamus [16]. Signaling through JNK and NF-kB not only increases the mRNA levels of TNF-α, interleukin- (IL-) 1β, IL-6, and IFN-γ

cytokines, but can also lead to endoplasmic reticulum stress, autophagy, and mitochondrial dysfunction [13, 17, 18].

Among the cytokines identified in the hypothalamus, IL-6 has gained considerable attention in studies related to metabolism due to its pleiotropic actions, not only in the pathogenesis of inflammatory disorders, but also in the physiological homeostasis of nervous tissue [19]. IL-6 can stimulate responses in a given target cell in two different ways. The classical signaling pathway corresponds to the binding of IL-6 to its membrane-bound α-receptor, IL-6R, resulting in dimerization of its β-receptor gp130. Alternatively, transsignaling is activated when IL-6 binds to the soluble IL-6R fraction, and this complex can then stimulate distant cells that express gp130 but not surface-bound IL-6R [20, 21]. Both pathways lead to the activation of downstream JAK/STAT signaling, which upregulates the transcription of proinflammatory genes [22, 23]. Although IL-6 levels in the brain are low under physiological conditions, its levels have been reported to be increased in several neurological disorders, predominantly due to neuronal and glial cells [24].

Microglial cells are the resident macrophages of the central nervous system (CNS) and are widely distributed throughout the brain. They originated from primitive macrophages in the yolk sac and form a population that is distinct from bone marrow-derived macrophages (BMDM) [25, 26]. Under normal physiological conditions, they are relatively quiescent; however, after being exposed to injury or infection stimuli, they undergo morphological and functional changes [26]. Many mechanisms by which microglia can be activated have been described [27, 28]. In the mediobasal hypothalamus (MBH), glial cells, which also include astrocytes, have been implicated in initiating and propagating an inflammatory process resulting in gliosis [29, 30]. Gliosis is characterized by an increased number of glial cells, hypertrophy of the cell bodies and processes, and other physiological changes. It occurs, in part, because microglial cells are able to sense changes in the surrounding environment and can quickly become activated to either a proinflammatory (M1) or anti-inflammatory (M2) phenotype. When activated, microglia release cytokines, including IL-6, in addition to other chemokines and growth factors, in order to mitigate or prevent damage to the brain due to the insult.

Although there has been significant progress in studies involving glial cells and cytokines, especially in areas of the brain that are important for metabolic physiologic control or neurodegenerative diseases, the relationship between microglia and IL-6 in the brain remains unclear. In this review, we searched for studies that evaluated IL-6 from a microglial origin in the hypothalamic environment. Data from articles were systematically reviewed to identify the hypothalamic microglia status (whether activated and/or the source of IL-6) and IL-6 expression (whether increased, unaltered, or decreased) in multiple conditions related to inflammatory/pathological processes.

PICOS Strategy. Participants: murine model. Interventions: conditions related to inflammatory and pathological processes. Comparisons: Hypothalamic microglia status. Outcomes: IL-6 expression. Study design: Experimental studies.

2. Materials and Methods

2.1. Search Strategy. A systematic search was performed in PubMed and Web of Science databases on December 18, 2018, for published studies on the association between IL-6 and microglia in the hypothalamus, with no restrictions with regard to language, timespan, or document type, using mixed strategy keywords. The Systematic Review Protocol was registered in "International Prospective Register of Systematic Reviews" (PROSPERO) through the code CRD42019129248. The following search strategies were used: PubMed, (("Hypothalamus"[Mesh]) AND "Interleukin-6"[Mesh]) AND "Microglia"[Mesh]; Web of Science, ALL FIELDS: ("interleukin 6" and "microglia" and "hypothalamus"). This review follows the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) checklist (*See PRISMA Checklist*, Table S1).

After obtaining the search results, duplicate articles, those that exclusively used an *in vitro* approach, studies without data on IL-6 levels, and those without information regarding microglia in the hypothalamus were identified and excluded from this review (*See Table of included and excluded articles,* Table S2).

2.2. Data Extraction and Classification. The following data were extracted from each study: subject of study, model adopted (*in vivo* and *in vitro*) and intervention (treatment or exposure performed), methods of analysis (mRNA expression and protein expression), IL-6 expression and microglia status (increased/decreased or activated/suppressed in comparison to control) with tissue/location, expression of IL-6 by microglia, and phenotypic outcome. When available, the significance level (p-value) was collected. The evaluation of risk of bias was performed using the SYRCLE's risk of bias tool (*See Evaluation of Risk of Bias, using SYRCLE's risk of bias tool,* Table S3), developed for animal studies.

3. Results

The search strategy identified a total of 25 articles (PubMed, n = 13; Web of Science, n = 13, of which 12 were unique). Twelve papers were excluded based on their title and abstract. The remaining 13 studies were retrieved for a full evaluation and were confirmed to fulfill the inclusion criteria (*See Flow diagram of Systematic Review provided by PRISMA*, Figure S1). Details of these 13 studies are summarized in Table 1.

3.1. Increased Hypothalamic IL-6 Expression and Microglial Status. In this review, the publication search returned eight articles that reported increased IL-6 expression. In five of these articles, increased hypothalamic levels of IL-6 with microglia activation were described. These events were found after (a) exposure to amylin, which is synthesized by pancreatic β-cells and is coreleased with insulin in response to food intake and increased glucose concentrations [31]; (b) lung coinfection, which induces neuroinflammatory events, in part through serum amyloid A production [32]; (c) lipopolysaccharide (LPS) exposure in neonatally

Table 1: Systematic review of data search.

	Source	Subject	Models Interventions	Methods of analysis: mRNA measurement	Methods of analysis: Protein measurement	IL-6 measurement, tissue	Microglia status, tissue	Microglia IL-6 producer	Outcome
IL-6 increased and microglia activated	Wang, H. 2018	Viral-bacterial lung co-infection	mice, adults/ primary microglial cell culture *Influenza A, Streptococcus. pneumoniae*, SAA exposure	qPCR - Hypothalamus and primary cell culture: *Tnf, Il1b, Il6, Ccl2, Fpr2*, S*aa1*	IHC - PVN: IBA1, GFAP, TNF-α, SAA	mRNA expression increased, hypothalamus ($P < 0.05$)	Activated, PVN ($P < 0.05$)	Yes ($P < 0.05$)	Neuroinflammation
	Le Foll, C. 2015	Amylin exposure	rats and mice, adults/ VMH explants/ VMH astrocytes/ VMN neurons/ cortical and hypothalamic microglia Amylin exposure	qPCR - ARC, VMH, VMN, cell culture:: *Il-1b, Il-6, Il-10, Tnf, Lif, Cntf, Gp130, Ctr1a, Ctr1b, Ramp1, Ramp2, Ramp3, Lepr-b, Socs3, Ins-r, Npy, Agrp, Pomc.*	ICC - ARC, VMN: pSTAT3 Immunoassay - cell culture medium: IL-1β, IL-6, IL-10, TNF-α	mRNA expression increased, VMN ($P < 0.05$)	Activated, Cortical ($P < 0.05$)	Yes	Decreased body weight gain
	Ziko, I. 2014	LPS exposure in adults Neonatally overfed	rats, pups and adults Neonatal overfeeding model LPS exposure	qPCR - hypothalamus: *Tlr4, Nfkb,Il1b, Il1ra, Il6, Tnf*	IHC - DMH, LH, VMH: IBA1	mRNA expression increased, hypothalamus ($P < 0.05$)	Activated, PVN ($P < 0.05$)	No data	Increased body weight in small litters and adults

Table 1: Continued.

	Source	Subject	Models Interventions	Methods of analysis: mRNA measurement	Methods of analysis: Protein measurement	IL-6 measurement, tissue	Microglia status, tissue	Microglia IL-6 producer	Outcome
	Tapia-González, S. 2011	Neonatal overfeeding	rats, pups Neonatal overfeeding model		WB - hypothalamus: IL-6, p-IkB IHC - ARC, ME, VMH: MHC-II ELISA - cell culture medium: IL-1β	Protein expression increased, hypothalamus ($P < 0.01$)	Activated, hypothalamus, ($P<0,05$)	No data	Overweight
	Mingam, R. 2008	LPS exposure in P2X7R knockout model	mice, adult/ primary glial cell culture LPS exposure	qPCR - hypothalamus: *Il-6, Il-1b, Tnf*	IHC - cell culture: CD11b, CD68, IL-1β, GFAP, WB - cell culture medium: IL-1β	Protein and mRNA expression increased, whole brain ($P < 0.05$)	Activated ($P < 0.05$)	Yes ($P < 0.01$)	Decreased IL-1β release
IL-6 increased without microglia information	Cao, H. M. 2015	Mimecan exposure	Rats and mice, adults primary neurons cell culture N9 cells Mimecan exposure	qPCR - hypothalamus: *Il1-b, Il-6* qPCR - primary cell culture: *Il1-b, Il-6, Socs3* qPCR qPCR -N9 cell culture: *Il1-b, Il-6, Socs3*	WB - hypothalamus: SOCS3	mRNA expression increased, hypothalamus ($P < 0.05$ / $P < 0.01$)	No data	Yes ($P < 0.01$)	Anorexia
	Roque, A. 2015	Early life stress	rats, pups Maternal separation model	qPCR - hypothalamus: *Il1-b, Il-6, Tnf*	-	mRNA expression increased, hypothalamus ($P < 0.01$)	No data	No data	Stress can dysregulate the HPA axis

Table 1: Continued.

	Source	Subject	Models Interventions	Methods of analysis: mRNA measurement	Methods of analysis: Protein measurement	IL-6 measurement, tissue	Microglia status, tissue	Microglia IL-6 producer	Outcome
IL-6 increase and microglia suppressed	Silva, T.M. 2017	Minocycline in acute hypoxia	rats, adults Pretreatment with Minocycline followed by exposition to acute hypoxia	qPCR - mice PVH: *Il-1b, Il-6, Tnf, Mmp9, Cd3, Hprt*	IHC: TH, c-Fos	mRNA expression increased, PVH ($P < 0.05$)	Suppressed ($P < 0.05$)	No	Disrupted organization of breath activity
IL-6 unaltered and microglia activated	Younes-Rapozo, V. 2015	Long-term effects of nicotine exposure during lactation in offspring	rats, pups Nicotine in dams.	-	IF - ARC, PVN, LH, PE: GFAP, IBA-1, CX3CR1, MCP-1, IL-6	Protein expression had no change, ARC, PE, PVN, LH	Activated, in PVN ($P < 0.05$)	No data	Obesity
	Sugama, S. 2007	Inescapable stress	rats and mice, adults restraint combined with water immersion stress	qPCR - hypothalamus: *Il-1b, Il-6, Inos*	IF: IL1-β, IL-6, iNOS IHC: OX42, CD11b	mRNA expression had no change, hypothalamus	Activated ($P < 0.001$)	No	Hypothalamic microglia activation
	Ye, S. M. 1999	Aging	mice, juvenile, adult, and aged/ primary cell culture of whole brains Basal conditions	RT-PCR - glial cell culture: *Il-6*	ELISA glial cell culture medium: IL-6 IHC: GFAP, MAC-1(CD11B) Flow Cytometry glial cell culture: GFAP, MAC-1 ELISA tissue homogenates: IL-6 Hybridoma bioassay: IL-6	Protein expression had no change in the hypothalamus	Activated, all brain ($P < 0.05$)	Yes ($P < 0.01$)	chronic inflammation in whole brain without changes in the hypothalamus

Table 1: Continued.

	Source	Subject	Models Interventions	Methods of analysis: mRNA measurement	Methods of analysis: Protein measurement	IL-6 measurement, tissue	Microglia status, tissue	Microglia IL-6 producer	Outcome
IL-6 decreased and microglia suppressed	Ramirez, K. 2016	Benzodiazepines treatment in Repeated social stress condition	Mice, adult Exposed to stress condition and treated with benzodiazepines	qPCR - hypothalamus: *Il1-b, Il-6, Tnf*	-	mRNA expression decreased, hypothalamus ($P < 0.05$)	Suppressed, whole brain ($P < 0.05$)	Yes ($P < 0.05$)	reversal of stress-induced behavioral
	Santos Masson, G. 2015	Short-term exercise training in arterial hypertension model	Rat, adult 2 weeks of exercise model in spontaneously hypertensive rats	-	WB - PVN: CXCR4, ERK1/2 pERK1/2, HMGB1, IL-6, p-IKBa, SDF-1, TNF-α IF: HMGB1, IBA-1	Protein expression decreased, PVN ($P < 0.01$)	Suppressed, PVN ($P < 0.01$)	No data	Control of arterial hypertension

Legend: ARC: arcuate nucleus, LPS: lipopolysaccharide, HPA: hypothalamic–pituitary–adrenal ME: median eminence, PVN: paraventricular nucleus, SAA: serum amyloid A, VMH: ventromedial hypothalamus, VMN: ventromedial nucleus.

overfed adults [33] and in glial cells from P2X7R-knockout mice (purinoceptor expressed predominantly by cells with immune origin) [34]; and (d) neonatal overfeeding itself [35].

One study showed an increase in hypothalamic IL-6 expression in the context of microglial suppression. Induced hypoxia leads to an increase in IL-6 levels, even with suppression of microglial activity in the CNS (pharmacological suppression). Thus, the increased IL-6 expression was probably from other cell types, like astrocytes. Altered microglial activation was related to alterations in the brain autonomic nuclei responsible for cardiorespiratory control, leading to impairments in breathing [36].

Finally, three studies showed increased hypothalamic IL-6 expression but did not measure microglial activation. Similar to amylin [31], another hormone known as mimecan (also known as osteoglycin) can lead to an increase in hypothalamic IL-6 expression. Using *in vitro* approaches, this study showed that IL-6 is produced by microglia after mimecan stimulus. Mimecan is expressed in adipose tissue, and its action is related to the inhibition of food intake and reduction of body weight in mice [37]. The final study, which employed a model of early-life stress, found increased hypothalamic IL-6 expression [38].

3.2. Unaltered or Decreased Hypothalamic IL-6 Expression and Microglial Status. The expression of IL-6 was unaltered in three studies related to (a) aging as a physiological condition, which leads to an increase in IL-6 levels in other brain areas, but no significant increase was observed in the hypothalamus [39]; (b) a model of inescapable stress, in which microglial activation was not related to increased hypothalamic IL-6 levels [40]; and (c) nicotine exposure during lactation, which promotes paraventricular hypothalamic microglial activation related to obesity in adulthood, but with no alteration in hypothalamic IL-6 expression [41].

Lastly, two studies showed microglial suppression with decreased IL-6 expression. The first one demonstrated that the stress-induced increase in hypothalamic IL-6 and microglia activation were suppressed following benzodiazepine treatment, ameliorating anxiety, and social avoidance behavior in adult mice [42]. In the same way, exercise can ameliorate hypertension in a murine model, with a marked decrease in IL-6 expression, followed by a reduction in microglial activation in the hypothalamus [43].

Some risks of bias were strongly present in most of the articles analysed, as unclear or not founded information: (a) risk of selection bias, as randomized allocation of animals and cages; (b) risk of performance bias, as blinding of manipulators about interventions performed; (c) risk of detection bias, as random selection of animals to assess outcomes (Table S3).

4. Discussion

In this review, we present a summary of data extracted from studies that evaluated IL-6 in hypothalamic microglia. The models employed in the 13 studies included in this review were divided into those considered "injury-like" stimuli, which can interfere with the hypothalamic–pituitary–adrenal (HPA) axis [32, 33, 38, 42], and others leading to phenotypic manifestations such as an increase in weight gain under a standard diet [33, 35] or anxiety-like behavior [42].

Many signaling pathways are activated by LPS or high-fat feeding [27]. Both LPS and SFAs from the diet are recognized by TLR4 in microglial cells, increasing the production and release of several inflammatory cytokines by these cells [16]. The same happens with overfeeding during lactation in neonates, which causes long-term changes that can lead to the development of obesity [44]. In this case, when adulthood is reached, basal hypothalamic IL-6 levels remain elevated, concomitant with activated hypothalamic microglia [35]. Furthermore, neonatal overfeeding beginning early in life increases hypothalamic TLR4 expression and the number of Iba-1 (microglia/macrophage-specific protein) positive cells, followed by increased expression of IL-6 following LPS challenge [33].

In addition to TLR4, microglial receptors dependent on ATP binding are also important to trigger cytokine production. Evidence indicates that microglial IL-6 production is more strongly associated with the activation of P2Y receptors [45]. A study by Mingam et al. (2008) confirmed the specificity of P2 purinoceptors to the production of cytokines, as absence of the P2X7 receptor leads to impairment only in IL-1β production by activated microglia but does not interfere with IL-6 production after LPS stimulus [34].

Neuroinflammation driven by infections and other systemic inflammatory events can be modulated by several ligands and specific receptors. Mice submitted to viral or bacterial lung infection drive the hepatic release of circulating amyloids, which activate PVN microglia through binding to formyl peptide receptor 2 (Fpr2), leading to a marked increase in hypothalamic IL-6 expression and exacerbated neuroinflammation [32]. Other evidence suggests that the hypothalamic distribution of receptors with multiple ligands, such as Fpr2, can contribute to these effects [46].

Although diverse pathways are related to microglial activation related to inflammation, different conditions, such as hormonal stimuli, can also result in microglial activation and increased IL-6 expression [31, 37, 47]. As shown by Ropelle et al. (2010), physical exercise increases hypothalamic IL-6 expression, which improves insulin and leptin signaling in the hypothalamus, leading to decreased food intake in rats fed with a high-fat diet [48]. Two of the reviewed studies demonstrated that IL-6 can interfere with energy balance, inhibiting food intake or reducing body weight gain, and that hormonal signaling is related to hypothalamic IL-6 expression through different mechanisms. Amylin stimulus increases IL-6 in the ventromedial hypothalamus (VMH) through binding to the microglia. Elevated IL-6 can improve leptin signaling in neurons via the phosphorylation of STAT-3, thereby reducing body weight gain [31]. Leptin itself can drive this event, as IL-6 is produced by microglia after a leptin stimulus through activation of microglial leptin receptor isoforms [47]. Furthermore, IL-6 can interact with leptin in the parabrachial nucleus, leading to reduced food intake [24]. On the other hand, mimecan can reduce food intake independent of leptin signaling, and its action is related to microglial IL-6 release in the hypothalamus [37]. Although

the increases in hypothalamic IL-6 are primarily associated with inflammatory events and responses, growing evidence suggests a relationship between IL-6 and hormones involved in energy balance, which can occur in a leptin-dependent or leptin-independent manner.

Beyond the regulation of energy balance, we found several studies that reported noninflammatory outcomes of IL-6 in the CNS. One of these studies was focused on neuroplasticity via the PI3k-AKT pathway [49], while the others assessed neuroprotection and repair events [19, 50], the maintenance and control of proliferative niches close to ventricles [51], and neurogenesis in the subventricular zone mediated by IL-6 and other cytokines of microglial origin [52].

One of the reviewed studies adopted an aerobic-training protocol in spontaneously hypertensive rats presenting with hypothalamic inflammation and found a decrease in high mobility group box-1 (HMGB1, related to the injury-induced inflammatory response) and CXCR4 signaling, which ameliorates the autonomic control of blood pressure due to a reduction in microglia activation and hypothalamic IL-6 expression [43]. Indeed, anti-inflammatory events have been found to be associated with physical exercise protocols and an increase in circulating IL-6 released by the muscle [53, 54], thus reinforcing the anti-inflammatory effect of exercise and its central outcomes.

Not every inflammatory stimulus or condition is related to increased IL-6 expression in the hypothalamus, although it is classically associated with microglial activation along with IL-1β and TNFα expression. In a model of inescapable stress, microglial activation was not found to be related to an increase in hypothalamic IL-6 levels [40]. A similar finding was reported for nicotine exposure during lactation, with no relationship between long-term obesity and increased hypothalamic IL-6 expression [41]. Finally, during the aging process, an increase in microglial IL-6 production was observed in the cerebellum, cortex, and hippocampus, but not in the hypothalamus. Increased IL-6 expression in the hypothalamus did not show an age dependence [39].

Given the complexity of homeostatic maintenance and inflammatory events, an absence of microglial activity or IL-6 production can lead to impaired phenotypes. Under basal conditions, microglia ablation leads to a variety of events, including reduced neuroblast survival within the dentate gyrus of the hippocampus [55]. In the presence of severe injury, such as brain ischemia, microglia have been described as an important producer of neurotrophic factors [56] and other proteins. In a physiological context, according to the diverse microglial hypothalamic signatures, it acts like a "sentinel," functioning as an environmental sensor and regulator of hypothalamic metabolic control [28]. As demonstrated by Silva et al. (2018), pharmacological inhibition of microglia in the CNS combined with hypoxia leads to an increase in IL-6 expression, probably due to a different cell type, such as astrocytes, resulting in alterations in the brain autonomic nuclei responsible for cardiorespiratory control [36]. Furthermore, ablation of IL-6 (knockout IL-6 mice) is related to weight gain and disturbance in glucose homeostasis during adulthood [57]. These knockout mice have lower neuronal protection in the dentate gyrus. Conversely, elevated expression of hippocampal IL-6 was found to be related to better neuronal regeneration and a better neuroprotective effect in acute lesions [58]. Furthermore, IL-6 plays a critical role in neuronal survival during early life development and adulthood [59]. Thus, there is evidence that IL-6 can exert central and peripheral functions through modulation of metabolic events, neuroprotection, and participation in regenerative/proliferative processes.

Given the plasticity of microglia [60] and the pleiotropy of IL-6 [22], studies that evaluate both require specific and accurate approaches such as conditional knockouts. In light of this, future studies should be conducted with the purpose of clarifying the behavior of microglia, as well as the effect of IL-6, under different conditions. Understanding this relationship could lead to more specific pharmacological approaches to acute and/or chronic conditions in the future, such as the management of obesity and metabolic diseases. This refinement is important in order not to disturb the homeostasis of the hypothalamic environment, as IL-6 and microglia make a remarkable contribution under normal physiological conditions.

5. Conclusions

Advances in our understanding of microglial IL-6 hypothalamic expression and its functions are important for the interpretation of hypothalamic responses under diverse stimuli. Taken together, our review identified three main contexts where microglial activity and IL-6 expression are strongly related: (1) basal levels of hypothalamic IL-6 and microglial function are important to maintain environmental homeostasis; (2) some hormones can activate microglia and increase IL-6 expression to improve hormonal signaling in the hypothalamus; and (3) under conditions of acute or sustained inflammatory conditions, IL-6 expression and microglia activation will be increased together with other inflammatory markers such as TNFα and IL-1β, generating neuroinflammatory responses (Figure 1).

Disclosure

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Conflicts of Interest

The authors declare no conflicts of interest.

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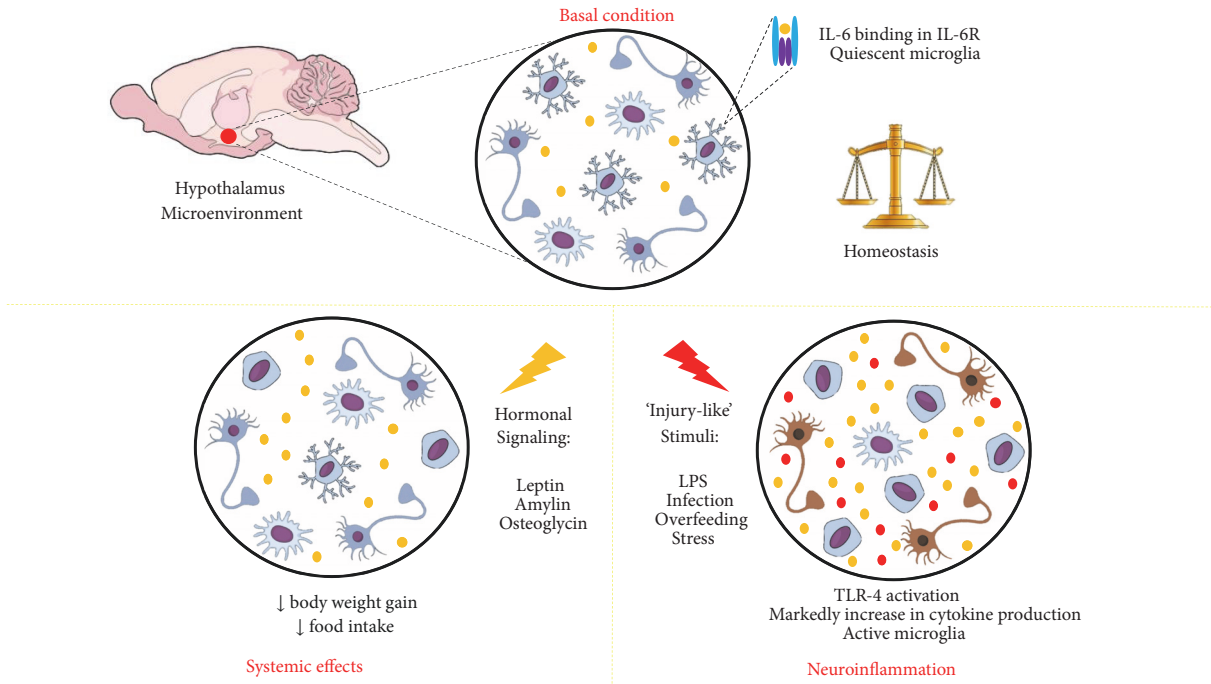

Figure 1: Summary of findings. IL-6 (represented by yellow dots) in hypothalamic microenvironment. In basal conditions, binding in its receptor have correlation with homeostatic maintenance of microenvironment. During hormonal signaling, temporary increases in IL-6 levels were related to systemic effects provide by hormonal signaling. During "injury-like" stimuli, neuroinflammation is characterized by marked increase in IL-6 and other cytokine production, microglial activation, and TLR-4 activation.

Supplementary Materials

Figure S1: PRISMA flow diagram. Table S1: Excluded and included articles. Table S2: Evaluation of Risk of Bias, using SYRCLE's risk of bias tool for animal studies. Table S3: PRISMA Checklist. *(Supplementary Materials)*

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