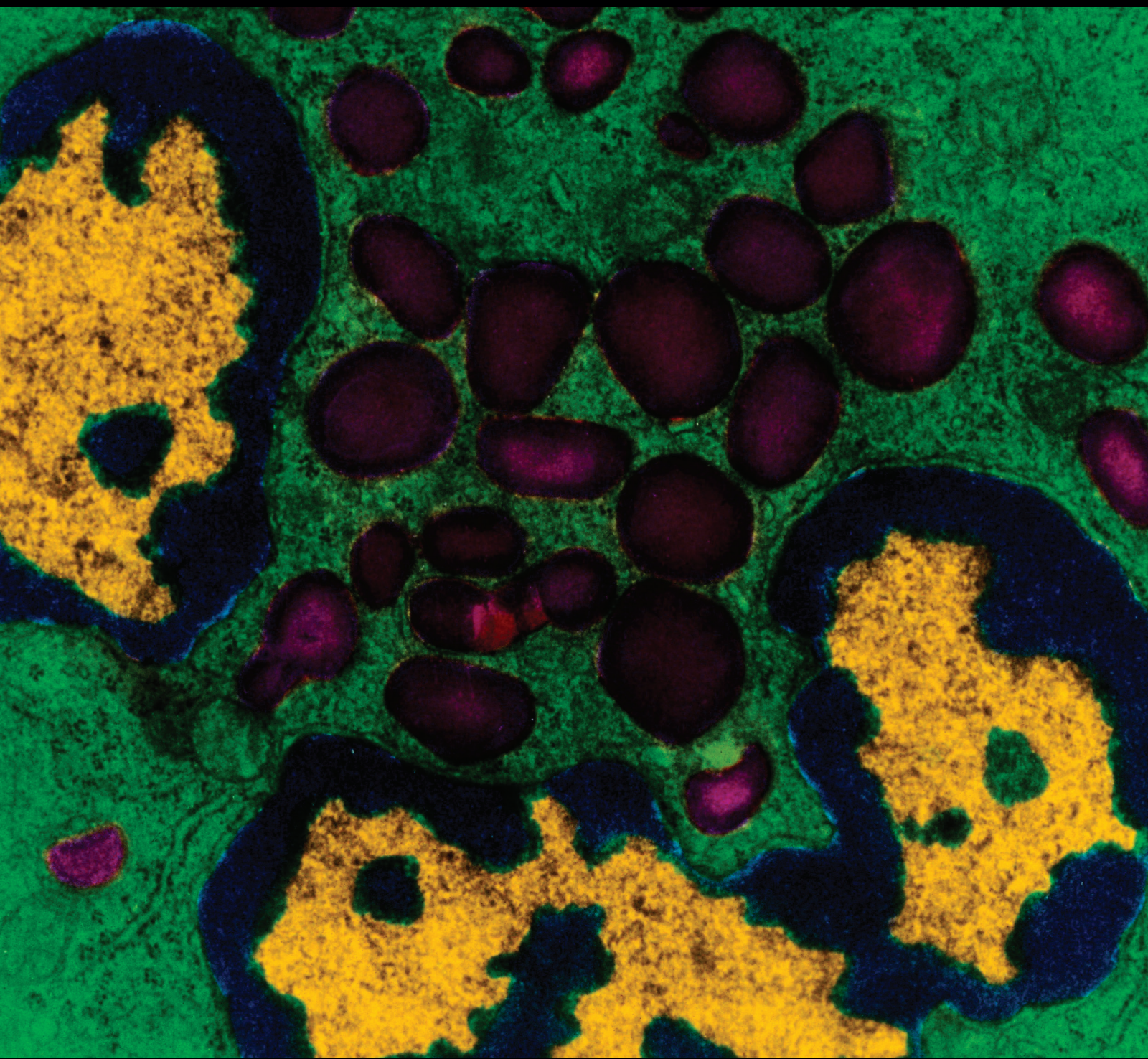
Mediators of Inflammation

Cytokines in Autoimmune Disease

Lead Guest Editor: Qingdong Guan
Guest Editors: Junhui Wang, Yu Sun, and Sudhanshu Shekhar



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Editorial Board

Contents

Hindawi
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Editorial
Cytokines in Autoimmune Disease

Qingdong Guan,[1,2] Xiaoling Gao,[3] Junhui Wang,[4] Yu Sun,[5] and Sudhanshu Shekhar[6]

[1]*Cellular Therapy Laboratory, CancerCare Manitoba, Winnipeg, MB, Canada*
[2]*Institute of Pharmacology, Taishan Medical University, Taian, China*
[3]*The Institute of Clinical Research and Translational Medicine, Gansu Provincial Hospital, Lanzhou, China*
[4]*Department of Physiology, University of Toronto, Toronto, ON, Canada*
[5]*Department of Endocrinology & Metabolism, Qilu Hospital of Shandong University, Jinan, China*
[6]*Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway*

Correspondence should be addressed to Qingdong Guan; qingdong.guan@umanitoba.ca

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The incidence and prevalence of autoimmune disease marked increased over the second half of the 20th century, and it has become a major health problem. These diseases are usually chronic and can be life-threating. The causes of autoimmune disease remain largely unknown. The recent advance in knowledge has greatly increased our understanding of pathogenesis of autoimmune disease, and it is widely accepted that environment, gene, and immunity contributed to the development of autoimmunity. Cytokines, including proinflammatory cytokines and anti-inflammatory cytokines, are important players in the pathogenesis of these diseases through multiple ways, such as regulating inflammation and angiogenesis.

In this special issue, we present original research articles as well as review papers on the role of cytokines in autoimmune diseases.

Psoriasis is a chronic and recurrent dermatitis, mediated by keratinocytes, T cells, and cytokines. In this special issue, P. S. S. de Oliveira et al. showed that the transcript levels of IL-17A, IFNG, and Foxp3, not IL-36A, IL-8, IL-33, IL-10, and IL-22, were increased in the moderate-severe patients with psoriasis. Some studies showed that psoriasis and rheumatoid arthritis (RA) had a common pathogenesis, but the precise molecular mechanism remains unclear. Through RNA sequencing of PBMC of psoriasis and RA patients and healthy controls, Y. Tan et al. showed that the common molecular mechanism of psoriasis and RA was characterized by a cytokine imbalance, in which ERK1/2, MAPK, TNF, CSF3, IL-6, IFN, and canonical signaling pathways played key roles in the pathogenesis of both diseases. The Gαq-containing G protein (Gαq) has been found to play an important role in immune regulation and development of autoimmune disease. D. Wang et al. showed that the expression of Gαq was negatively correlated with the expression of IFN-γ in RA patients and further showed that Gαq deficiency led to enhanced Th1 cell differentiation in animal study. Gαq negatively regulated Th1 differentiation by modulating the expression of T-bet and activity of STAT-4. These results suggested the role of Gαq in the pathogenesis of RA through modulating Th1 cells. The study by B. Wang et al. showed that serum IL-34 level was highly increased in RA patients; the interaction of IL-34 with IL-34R expressed on fibroblast-like synoviocytes (FLS) promoted IL-6 secretion by FLS, which further promoted the differentiation of Th17. Therefore, IL-34 might be involved in the pathogenesis of RA.

Cytokines drive and regulate multiple aspects of inflammatory bowel disease (IBD). Q. Guan et al. reviewed the recent advances of novel proinflammatory and anti-inflammatory cytokines found in IBD with focusing on IL-12 family (IL-12, IL-23, IL-27, and IL-35) and IL-1 family members (IL-1, IL-33, IL-36, and IL-37) as well as their relevance to the potential therapy of IBD.

E. Vasilyeva et al. evaluated the difference of cytokine expression pattern in children and adult with Crohn's disease and found that at acute disease stage, all patients elevated serum levels of CXCL10; besides that, children patients increased serum levels of TNF and IL-6, while adult patient had elevated serum levels of GM-CSF and IFN-γ. B. C. de Sousa et al. explored the effects of *Morinda citrifolia* fruit juice in the treatment of DSS-induced murine colitis and showed that the treatment with this fruit juice played an important role in inhibiting intestinal inflammation during the development of murine colitis.

J. Ma et al. showed that the plasma IL-33 level was significantly increased in the patients of osteonecrosis of femoral head (ONFH) when compared to healthy controls, and interestingly, the level of IL-33 in the patients with late stage was higher than that in the patients with early stage, which indicated that IL-33 may be detrimental during ONFH.

In a review paper, C. Xiaoheng et al. presented a comprehensive summary of the association of various cytokines and related gene polymorphisms with susceptibility to autoimmune thyroid disease (AITD). The review focused on the structure and function of these cytokines and related genes in AITD and attempted to describe their differences in pathogenesis and clinical manifestations.

Adhesion molecules may play an important role in systemic lupus erythematosus (SLE) pathogenesis. But whether the expression of these adhesion molecules was affected by cytokines remains unclear. In this special issue, S. Lin et al. demonstrated that IL-15 significantly increased the expression of CD56 and CD11B but decreased the level of CD62L expression on NKT cells and NKT-like cells from SLE patients.

Different extra-articular clinical symptoms could appear in the course of spondyloarthropathy (SpA) disease, such as acute anterior uveitis (AAU), IBD, psoriasis, and psoriatic onychopathy. H. Przepiera-Będzak et al. showed that increased serum IL-18 and decreased serum endothelial-1 were associated with an increased risk of extra-articular symptoms in SpA patients; increased serum IL-6 and IL-23 also increased the risk of AAU. These results indicated that development of different extra-articular symptoms was relevant to elevated levels of different markers of inflammatory process.

One of the keys to control autoimmune disease is an improved understanding of cytokine responses in these diseases, which will aid the development of effective immunotherapeutic strategies. This special issue encompasses molecular mechanisms of cytokines in immunopathogenesis of autoimmune diseases from bench to bedside.

Overall, we believe that these articles may contribute to improve our knowledge of cytokine-mediated immune mechanisms in autoimmunity, to provide insights into designing of effective immunodiagnostic tools, and to present rational basis for the development of potential therapeutic agents.

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We would like to thank the authors for their cutting-edge research data and thought-provoking reviews. We also express our gratitude to all the reviewers for their kind assistance and valuable insights.

Qingdong Guan
Xiaoling Gao
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Sudhanshu Shekhar

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Research Article

IL-34 Upregulated Th17 Production through Increased IL-6 Expression by Rheumatoid Fibroblast-Like Synoviocytes

Bing Wang,[1] Zijian Ma,[1] Miaomiao Wang,[2] Xiaotong Sun,[1] Yawei Tang,[1] Ming Li,[3] Yan Zhang,[4] Fang Li,[1] and Xia Li[1]

[1]*Department of Immunology, College of Basic Medical Science, Dalian Medical University, Liaoning, China*
[2]*Department of Rheumatology and Immunology, The Third Affiliated Hospital of Hebei Medical University, Hebei, China*
[3]*Department of Microecology, College of Basic Medical Science, Dalian Medical University, Liaoning, China*
[4]*Department of Rheumatology and Immunology, The Second Affiliated Hospital of Dalian Medical University, Liaoning, China*

Correspondence should be addressed to Fang Li; lifang16@hotmail.com and Xia Li; lixia416@163.com

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Rheumatoid arthritis (RA) is a chronic autoimmune disease which is characterized by synovial inflammation and cartilage damage for which causes articular dysfunction. Activation of fibroblast-like synoviocytes (FLS) is a critical step that promotes disease progression. In this study, we aimed to explore the effect of interleukin-34 (IL-34) on RA FLS as a proinflammatory factor and IL-34-stimulated FLS on the production of Th17. We found that serum IL-34 levels were increased compared to those of the healthy controls and had positive correlations with C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), and anticyclic citrullinated peptide (CCP) antibody accordingly. CSF-1R was also highly expressed on RA FLS. The interaction of IL-34 and CSF-1R promoted a dramatic production of IL-6 by FLS through JNK/P38/NF-κB signaling pathway. Further, the IL-34-stimulated IL-6 secretion by RA FLS was found to upregulate the number of Th17. The treatment of IL-6R antagonist could attenuate the production of Th17 mediated by IL-34-stimulated RA FLS. Our results suggest that the increased IL-34 levels were closely related to the disease activity of RA. Additionally, the overexpression of IL-6 in the IL-34-stimulated FLS promoted the generation of Th17. Therefore, IL-34 was supposed to be involved in the pathogenesis of RA. The inhibition of IL-34 might provide a novel target for therapies of RA.

1. Introduction

Rheumatoid arthritis (RA) is a progressive systemic disease involving synovial inflammation and articular destruction [1]. In this disease, RA synovial tissue becomes hyperplastic and forms pannus to invade the cartilage and bone, which eventually leads to restricted movement and disability [2]. Fibroblast-like synoviocytes (FLS) play key roles by local production of cytokines and proteolytic enzymes that degrade the extracellular matrix and cartilage only when they are in an activated condition. Accumulating evidence has indicated that many mediators are involved in the RA-FLS activation, such as TNF-α, IL-1, IL-6, and so on [3]. These cytokines develop a complex network of autocrine and paracrine regulation mode, which causes not only proliferation and migration but also increased resistance to apoptosis of FLS. And among the cytokines network, there must be new cytokines whose actions are still uncertain. Therefore, understanding the effects and mechanisms of new cytokines underlying in FLS remains a very urgent need.

IL-34 is a newly discovered cytokine, which is consisted of 222 amino acids and shares the same receptor with macrophage colony-stimulating factor (M-CSF) [4, 5], also called colony-stimulating factor-1 receptor (CSF-1R or CD115). In humans, IL-34 mRNA is widely expressed in several of tissues, including heart, brain, lung, liver, kidney, spleen, thymus, testis, ovary, small intestine, prostate, and colon. IL-34 protein was also detected in keratinocytes, epidermis, and neurons [6, 7]. CSF-1R, a member of the platelet-derived growth factor receptor subfamily, is a transmembrane homodimeric type III receptor tyrosine kinase encoded by the c-Fms proto-oncogene [8, 9]. CSF-1R expression is

restricted primarily to cells of the mononuclear phagocytic lineage, including macrophage precursors in bone marrow, monocytes, osteoclasts, and tissue macrophages such as liver Kupffer cells and microglia in the brain [10]. It can be also detected on the surface of some tissue fibroblasts [11]. IL-34 binding with CSF-1R is critical for better survival and differentiation of monocytes and macrophages, as well as Langerhans cells (LC) [12]. High expression of IL-34 has been found to correlate with chronic inflammation and some autoimmune diseases such as Sjogren's syndrome (SS) and mucosa of inflammatory bowel disease (IBD) [13, 14]. IL-34 was also upregulated in RA synovium [15, 16], and there was a positive correlation between synovial IL-34 expression and synovitis severity [17]. Cultured RA-derived FLS were showed to produce IL-34 in response to TNF-α [18]. One year treatment with disease-modifying antirheumatic drugs (DMARDs) decreased the expression of IL-34 in RA [19]. These studies imply an important role of IL-34 in RA pathogenesis and development. However, it is still not fully clear whether IL-34 can modulate FLS and alter its secretion of proinflammatory cytokines.

In this study, we have observed an increased serum level of IL-34 in RA patients, and it was positively correlated with disease activities. The interaction of IL-34 and CFR-1R significantly enhanced IL-6 production of FLS possibly through JNK/P38/NF-κB signaling pathway. Furthermore, IL-34-stimulated FLS in RA patients upregulated Th17 frequency by increased IL-6 production, and the treatment of IL-6R antagonist could attenuate this effect. Our findings will provide an important implication for better understanding the role of IL-34 in RA inflammatory responses.

2. Materials and Methods

2.1. Study Population. 168 RA patients were recruited (male = 40, female = 128, the average age = 50.29 ± 0.93) from the Department of Rheumatology of the Second Affiliated Hospital of Dalian Medical University, Dalian, China. All patients fulfilled the American College of Rheumatology criteria (ACR 1987). For the control group, 85 healthy volunteers (male = 28, female = 57, the average age = 58.84 ± 1.20) were recruited from the healthy physical center, who were matched by age and gender with the RA subjects ($P > 0.05$). Patients with other systemic diseases and using biological agents and high dose prednisolone were excluded from the study. Experiments were approved by the ethics committee of the Second Affiliated Hospital of Dalian Medical University.

2.2. Isolation and Culture of FLS. Synoviocytes were isolated from synovial tissue specimens that were obtained from patients with RA undergoing total joint replacement surgery. Using enzymatic digestion method, the tissue samples were minced into 1-2 mm^3 pieces and treated with 2.5 mg/ml type I collagenase (Gibco, USA) in Dulbecco's modified Eagle's medium (DMEM) for 2–4 h at 37°C with 5% CO_2. Dissociated cells were centrifuged for 5 min at 300 g, and cell pellet was resuspended in DMEM +10% fetal calf serum (FCS) (Gibco, USA), 2 mM L-glutamine, and 100 IU/ml penicillin and streptomycin (Solarbio Life Sciences, China) and seeded in 75 cm^2 flasks and incubated overnight. Synoviocytes from passages 4–6 were used in each experiment. The cells were morphologically homogeneous and exhibited the appearance of synovial fibroblasts. The purity of cells was tested by flow cytometry using anti-CD14-PE (eBioscience, USA), anti-CD68-APC (Miltenyi Biotec, Germany), and anti-vimentin-ALEXA 488 (BD, USA), and 99% of isolated cells was FLS ($CD14^-$ $CD68^-$ $vimentin^+$).

Table 1: Baseline clinical characteristics and medication of RA patients ($\bar{x} \pm$ SEM or n%).

	Baseline value
Age, year	50.29 ± 0.93
Male/female	40/128
Disease duration, years	9.62 ± 2.23
DAS28	4.41 ± 1.39
ESR, mm/h	55.69 ± 3.68
CRP, μg/ml	33.84 ± 3.50
RF, IU/ml	79.01 ± 7.39
Anti-CCP Ab, U/ml	173.80 ± 18.35
Current NSAID users	4 (2.38)
Current DMARD users	21 (12.50)
Current steroid users	2 (11.90)
Two-type drug users	67 (39.88)
Three-type drug users	15 (8.93)
No systemic therapy patients	59 (35.11)

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS: disease activity score; RF: rheumatoid factor; CCP: cyclic citrullinated peptide; NSAIDs: nonsteroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs.

2.3. The Activation of RA FLS by IL-34. 5×10^5/ml FLS ($n = 5$) in 10 cm dish were incubated with FBS-free DMEM for 24 hrs, then stimulated with or without IL-34 (50 ng/ml, R&D Systems, USA) for another 24 h, which were prepared for the detection of IL-6 mRNA expression. FLS (1×10^5/ml) were starved in 6-well plate for 24 h, then stimulated with or without IL-34 (50 ng/ml) for 12, 24, 48, and 72 h ($n = 6$) or pretreated with anti-CSF-1R antibody (25 ng/ml, R&D Systems, USA) for 30 min ($n = 8$). The supernatants of cell culture were collected to measure the levels of IL-6.

2.4. The Expression of CSF-1R on RA FLS. FLS (2×10^5/ml) ($n = 6$) were cultured in 25 cm^2 flask and single cell suspensions were collected. Cells were incubated with anti-human FcR block reagent (10 μl/tube) (Miltenyi Biotec, Germany) in PBS containing 1% BSA for 15 min at 4°C and then stained with APC-labeled anti-CSF-1R (5 μg/ml) or mouse IgG2a, κ isotype antibody (5 μg/ml) (eBioscience, USA) for 30 min at room temperature. Cells were then washed with PBS twice and resuspended in 500 μl PBS. The expression of CSF-1R on FLS was detected using a flow cytometer (Accuri C6, BD Bioscience, USA) and analyzed by CFlow plus software.

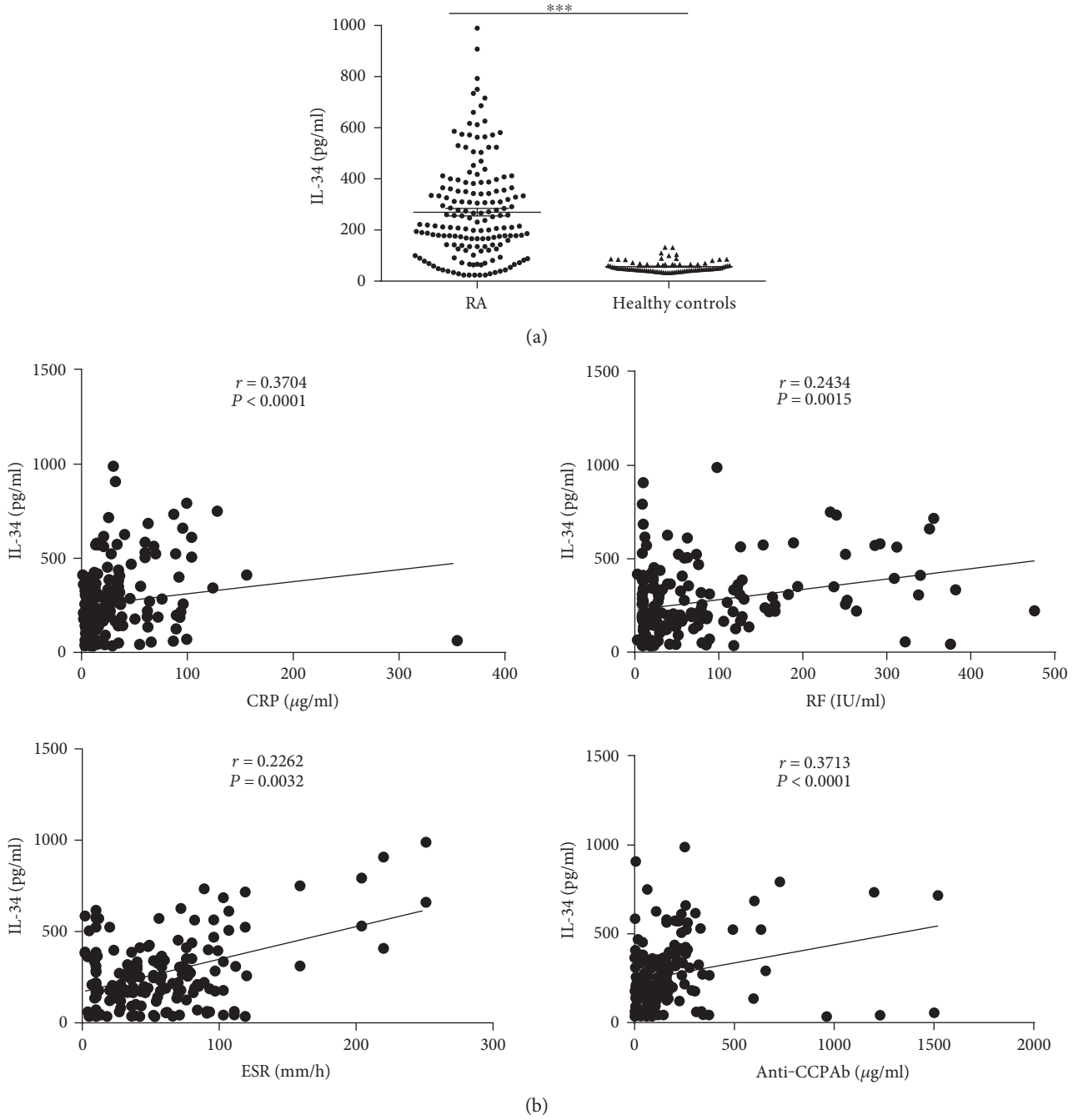

Figure 1: Serum IL-34 levels in RA patients were elevated and correlated with CRP, ESR, RF, and anti-CCP antibody levels. (a) Serum concentrations of IL-34 were measured by ELISA in RA patients ($n = 168$) and healthy controls ($n = 85$). Data were expressed as the mean ± SEM. $^{***}P < 0.0001$. (b) The IL-34 concentrations of RA patients were correlated significantly with CRP ($r = 0.3704$, $P < 0.0001$), ESR ($r = 0.2262$, $P = 0.0032$), RF ($r = 0.2434$, $P = 0.0015$), and anti-CCP antibody ($r = 0.3713$, $P < 0.0001$) ($n = 168$). Correlations between variables were examined using Spearman's rank correlation.

2.5. Inhibitors of Signaling Molecules Used in the Experiment. FLS (1×10^5/ml) ($n = 5$) were pretreated with signaling inhibitors (SP600125 (10 μM, inhibitor of JNK), SB203580 (25 μM, inhibitor of P38 MAPK), IKK-16 (10 μM, inhibitor of NF-κB), and FR180204 (10 μM, inhibitors of Erk1/2) (Selleck Chemicals, USA)) for 1 h and stimulated with IL-34 (50 ng/ml) for 24 h or 72 h at 37°C. The mRNA expression level of IL-6 was measured by RT-PCR, and IL-6 production was measured by ELISA.

2.6. Western Blotting Analysis. FLS (1×10^5/ml) ($n = 3 \sim 5$) were pretreated with or without signaling inhibitors for 1 h and then stimulated by IL-34 (50 ng/ml) for 30 min, the total protein was extracted from FLS on ice by using Whole Cell

Table 2: Correlation analysis for IL-34 and other clinical data in patients with RA.

Variables	n	Spearman rank correlation coefficient (r)	P
IgA	146	0.01771	0.8320
IgG	147	0.03583	0.6666
IgM	145	0.06114	0.4650
IgE	146	0.06142	0.4614
C3	146	0.02767	0.7402
C4	146	−0.07713	0.3548

Lysis Assay (KeyGEN BioTECH, China) according to manufacturer's protocol, then the protein was gotten after centrifugation at 14,000g for 10 min. Western blotting was performed using electrophoresis apparatus (Bio-Rad Co., USA). Briefly, 20 μg of protein in each lane was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co., USA). After blocking with 5% skim milk/TBST, the membranes were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-phospho-JNK antibody (ab124956, Abcam, USA; 1 : 1000), anti-JNK antibody (ab76125, Abcam; 1 : 1000), anti-phospho-NF-κB p65 antibody (ab76302, Abcam; 1 : 2000), anti-NF-κB p65 antibody (ab32536, Abcam; 1 : 2000), anti-phospho-Erk1/2 (ab32538, Abcam; 1 : 500), anti-Erk1/2 antibody (ab184699, Abcam; 1 : 5000), anti-phospho-P38 antibody (ab178867, Abcam, USA; 1 : 1000), and anti-P38 antibody (ab170099, Abcam; 1 : 1000). They were then incubated with the appropriate secondary horseradish peroxidase-conjugated goat anti-rabbit (Abbkine, 1 : 1000) for 2 h. Gel imaging apparatus (Bio-RAD Molecular Imager, ChemiDoc™ XRS+, USA) and analysis software (ImageJ2x, Rawak Software Inc., Germany).

2.7. The Coculture of RA FLS and CD4$^+$ T Cells of Healthy Controls. RA FLS (2×10^4/ml) were cultured in a 24-well plate overnight. CD4$^+$ T cells (2×10^5) were isolated from whole blood of healthy individuals ($n = 6$) using immunomagnetic beads (Miltenyi Biotec, Germany) according to the manufacturer's instructions and then CD4$^+$ T cells stimulated with anti-CD3 (3 μg/ml)/CD28 (2 μg/ml) antibody (eBioscience, USA) and brefeldin A (10 ng/ml)/monension (100 ng/ml) (BioLegend, USA) were cocultured with FLS ($n = 6$) for 72 h in the presence or absence of IL-34 (50 ng/ml) in RPMI-1640 medium (Gibco, USA) containing 10% FCS and 1% penicillin-streptomycin solution. Expression frequency of Th17 was measured by a flow cytometer. The IL-6 protein synthesis in the coculture supernatants was detected by ELISA.

To evaluate the effect of IL-6 secreted by FLS on Th17 proportion of CD4$^+$ T cell, IL-34-stimulated FLS ($n = 6$) were cocultured by CD4$^+$ T cells ($n = 6$) with treatment of anti-CD3 (3 μg/ml)/CD28 (2 μg/ml) antibody and brefeldin A (10 ng/ml)/monension (100 ng/ml) in the presence or absence of IL-6R antagonist (1 μg/ml) (R&D Systems, USA) for 72 h, and single cell suspensions were stained with surface FITC-labeled anti-CD4 (5 μg/ml) (eBioscience, USA) for 30 min and then intracellular stained with APC-labeled anti-IL-17A (5 μg/ml) (eBioscience, USA) for 1 h. Cell staining was completed according to the manufacturer's protocol, and the samples were detected and analyzed using a flow cytometer.

2.8. Reverse Transcription PCR (RT-PCR). Total RNA was extracted from cultured FLS ($n = 5$) pellets using RNAisoPlus (Takara Bio, Japan) and then the quantity and purity of RNA was examined by checking A260/A280 and agarose gel electrophoresis. Reverse transcription of 2 μg of total mRNA was performed using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Japan), and the resulting cDNA was subjected to PCR amplified for 33 cycles. Each cycle included 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 30 s of extending at 72°C. GAPDH was amplified as an internal control. The primers used in this study were listed as follows: GAPDH (272 bp): ACCACAGTCCATGCCATCAC (forward), CGCCTGCTTCACCACCTTCTT (reverse). IL-6 (234 bp): CCTTCGGTCCAGTTGCCTTCTC (forward), CC AGTGCCTCTTTGCTGCTTTC (reverse). PCR products of each gene were then observed by electrophoresis on 2% agarose gels.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). IL-6 and IL-34 levels were measured by ELISA kit (BioLegend, USA) according to the manufacturer's instructions. Sensitivity: the minimum detectable concentration of IL-6 for this set is 4 pg/ml, and the minimum detectable concentration of IL-34 is 33.4 pg/ml.

2.10. Serum Profiling on Protein Chip Assay. FLS from RA synovium ($n = 5$) were treated with or without IL-34 (50 ng/ml) for 72 h. The levels of cytokines in supernatants were detected by Protein chip AAH-CYT-G1000 Kit (RayBiotech, Norcross, GA) in accordance with the instructions. InnoScan 300 Microarray Scanner (31390 Carbonne, France) was used for the signal scanning, the median foreground, and the background intensities for each spot in the protein microarrays were obtained and analyzed with AAH-CYT-G6 and AAH-CYT-G7 software. Sensitivity: the minimum detectable concentration is 1 pg/ml.

2.11. Statistical Analysis. All data are expressed as the mean ± standard error of the mean (SEM). Statistical comparison between the two groups was analyzed by paired t-test comparisons or Wilcoxon signed rank test; potential correlations between variables were examined using Spearman's rank correlation through GraphPad Prism 5 (San Diego, CA, USA). Statistical significance was obtained with P values <0.05.

3. Results

3.1. Elevated Serum IL-34 Levels Were Positively Correlated with Disease Activities in RA Patients. We quantified the serum IL-34 levels comparison between 168 RA patients and 85 healthy people by ELISA. These patients' characteristics and drug use situation were shown in Table 1. Amount of IL-34 was significantly higher in RA patients (269.72 ± 14.71 pg/ml) compared to that in healthy controls (56.74 ± 2.30 pg/ml) (Figure 1(a)). In addition, elevated IL-34 was found to positively correlate with CRP, ESR, RF, and

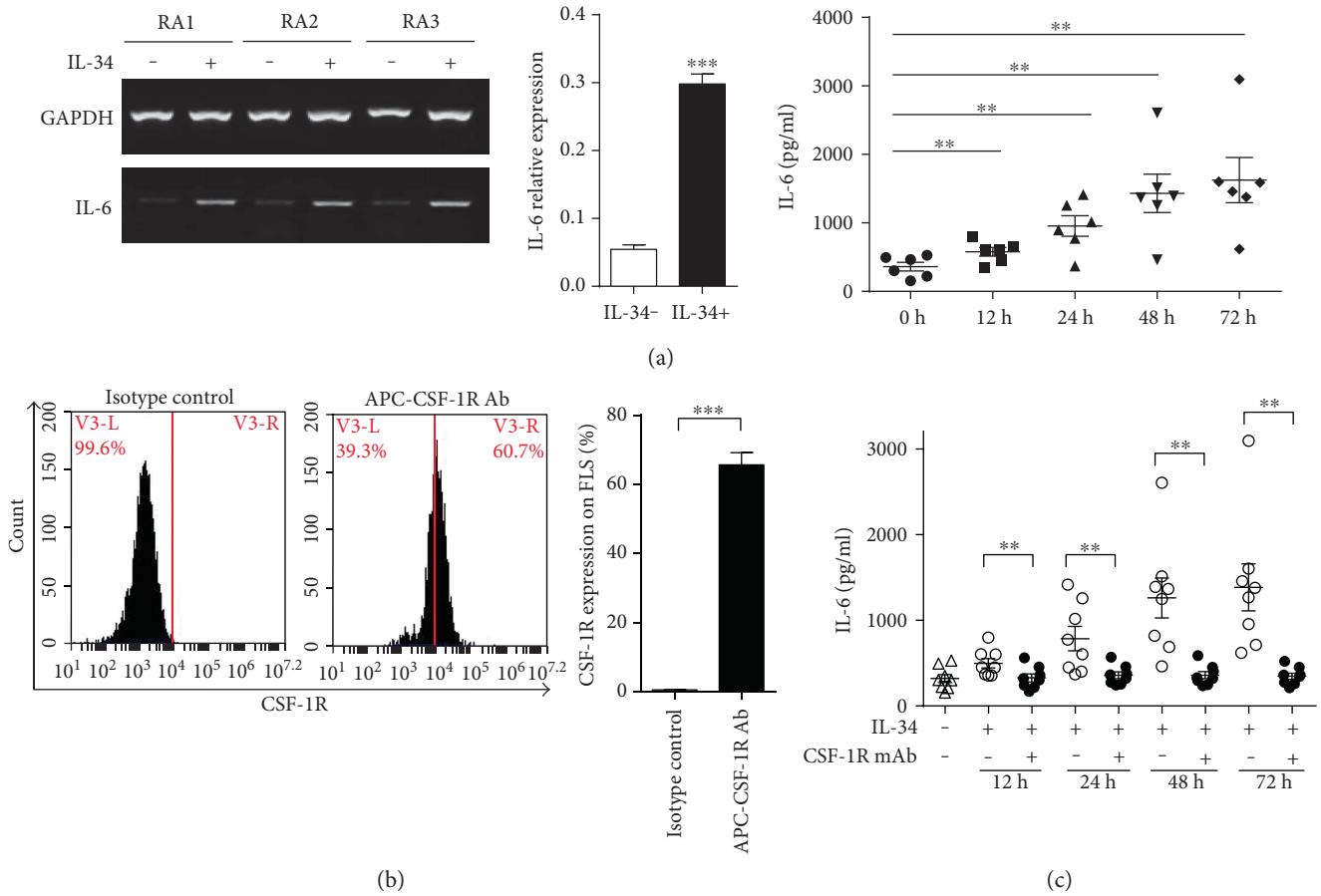

Figure 2: The interaction of IL-34 and CSF-1R promoted the production of IL-6 by RA FLS. (a) RA FLS ($n = 5$) were stimulated with IL-34 (50 ng/ml) for 24 h. IL-6 mRNA expression was measured by RT-PCR (left); data represents the mean ± SEM, $^{***}P < 0.001$ compared to the control group (middle). RA FLS ($n = 6$) were incubated with 50 ng/ml IL-34 for 0, 12, 24, 48 and 72 h. IL-6 levels in the supernatants were measured by ELISA. Data represents the mean ± SEM, $^{**}P < 0.01$ (right). (b) Cultured RA FLS ($n = 6$) were stained with APC-labeled anti-CSF-1R (5 μg/ml) or isotype control antibody (5 μg/ml). CSF-1R expression was detected by flow cytometric analysis. Data represents the mean ± SEM. $^{***}P < 0.0001$ compared with the isotype control antibody group. (c) RA FLS ($n = 8$) were stimulated with IL-34 (50 ng/ml) for 0, 12, 24, 48, and 72 h after 30 min preincubation with 25 ng/ml anti-CSF-1R mAb or not. Data represents the mean ± SEM. $^{**}P < 0.01$ compared with the group incubated with anti-CSF-1R mAb.

anti-CCP antibody (Figure 1(b)), but not with other laboratory indexes including amount of IgA, IgG, IgM, IgE, C3, and C4 (Table 2). These results demonstrate that an overexpression of serum IL-34 in RA patients may be associated with the disease process.

3.2. The Interaction of IL-34 and IL-34 Receptor Promoted the Production of IL-6 by FLS. Evidences showed that RA FLS could keep an excited state for a long time once they were stimulated by proinflammatory mediators and continued to produce a broad range of cytokines including IL-6. In this experiment, we have tested whether IL-34 affects IL-6 synthesis by FLS in RA patients. FLS from six RA patients were stimulated with 50 ng/ml of IL-34 for 24 h, and we found that IL-6 mRNA expression was significantly higher in IL-34-stimulated FLS by RT-PCR analysis. In addition, the IL-34-stimulated FLS produced remarkably higher IL-6 compared to unstimulated FLS (Figure 2(a)). Our results suggest that IL-34 had an inflammatory influence on RA FLS.

In order to investigate whether the enhanced IL-6 is produced by the binding of IL-34 with CSF-1R, we analyzed the expression of CSF-1R on FLS by using flow cytometry. The result showed that CSF-1R was highly expressed on FLS in RA patients (Figure 2(b)). In addition, the anti-CSF-1R mAb had an antagonistic effect on IL-6 production when FLS were preincubated with anti-CSF-1R mAb (25 ng/ml) in the presence of IL-34 (50 ng/ml) for 0, 12, 24, 48, and 72 h (Figure 2(c)), which suggests that the interaction of IL-34 with CSF-1R promoted IL-6 production by RA FLS.

3.3. IL-34 Dramatically Promoted IL-6 Production of FLS via JNK/P38/NF-κB Signaling Pathways. Previous studies have demonstrated that some inflammatory diseases are associated with dysregulation of the IL-34/CSF-1R axis in humans. IL-34 stimulation might promote phosphorylation of several tyrosine residues in the kinase domain of CSF-1R. In the current study, we would like to confirm whether IL-34 induced IL-6 production through MAPKs and NF-κB signal pathways that

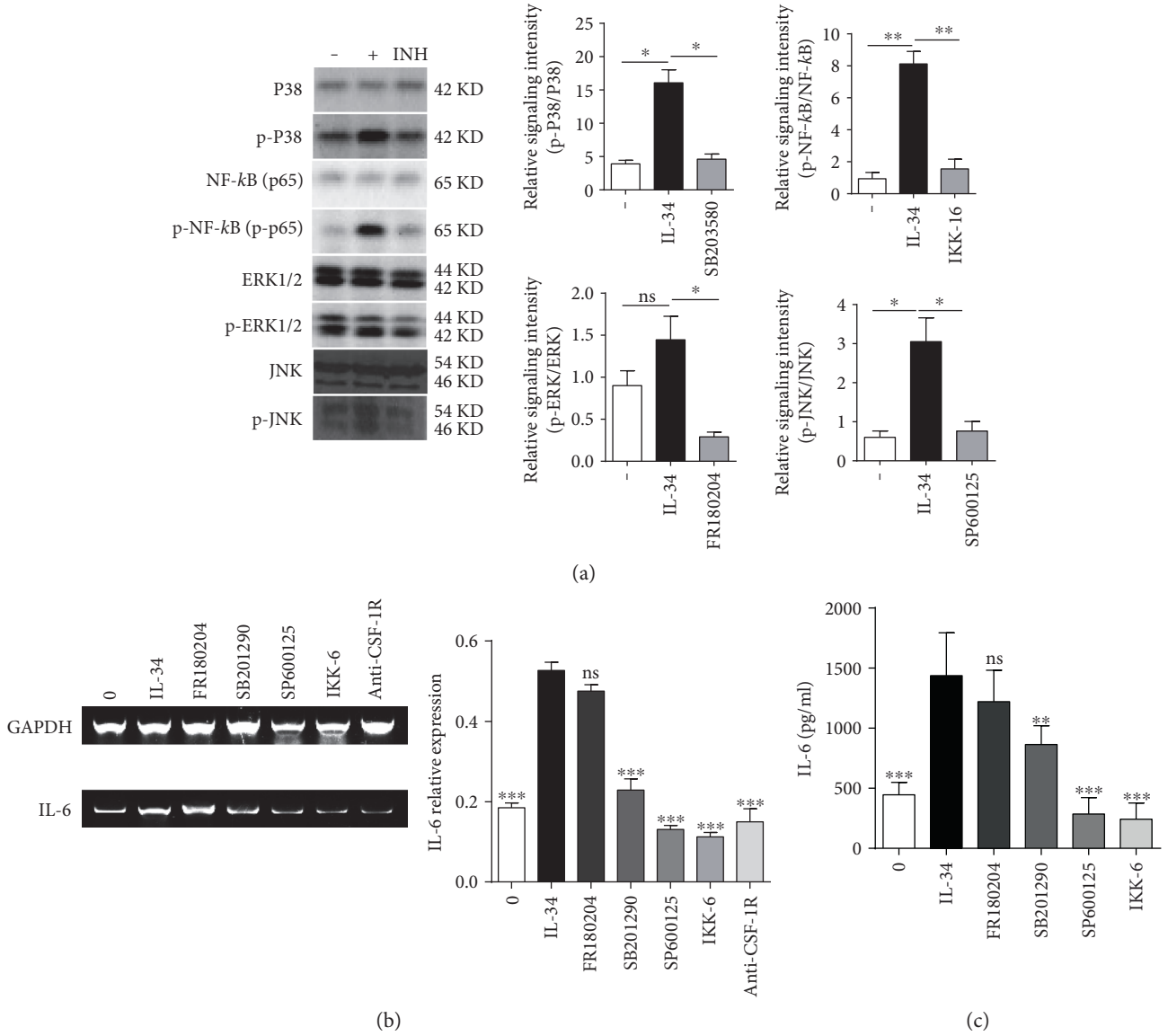

Figure 3: IL-34 dramatically promoted IL-6 production of FLS through JNK/P38/NF-κB signaling pathway. (a) RA FLS ($n = 3 \sim 5$) were pretreated with or without inhibitors of signaling molecules for 1 h and then stimulated by IL-34 (50 ng/ml) for 30 min. Protein was acquired in whole cell lysis buffer (20 μg/lane); meanwhile, phosphorylations of P38, NF-κB, ERK1/2, and JNK were analyzed by Western blotting using anti-phospho-specific antibody. Total P38, NF-κB, ERK1/2, and JNK (20 μg/lane) were determined by Western blotting using corresponding antibodies, respectively. The expression ratios of p-P38 to P38, p-NF-κB to NF-κB, p-ERK1/2 to ERK1/2, and p-JNK to JNK were represented in a bar graph. Data represents the mean ± SEM. $^{*}P < 0.05$, $^{**}P = 0.0074$, ns: no significant compared with the untreated cells. (b) FLS isolated from RA patients ($n = 5$) were pretreated with inhibitors of signaling molecules for 1 h or pretreated with anti-CSF-1R mAb (25 ng/ml) for 30 min, respectively, and then incubated with IL-34 (50 ng/ml) for 24 h. The expression of IL-6 mRNA was detected by RT-PCR. Data represents the mean ± SEM. $^{***}P < 0.0005$, ns: no significant compared to the group incubated with IL-34 only. (c) FLS isolated from RA patients ($n = 5$) were pretreated with inhibitors of signaling molecules for 1 h and then incubated with IL-34 (50 ng/ml) for 72 h. IL-6 levels in the supernatants were measured by ELISA. Data represents the mean ± SEM. Statistical analysis was using the paired t-test. $^{***}P < 0.0001$, $^{**}P = 0.0017$, ns: no significant compared with the IL-34 group.

have been known to associate with cell proliferation. Results showed that the phosphorylation of P38, NF-κB, and JNK but not ERK1/2 in the cytoplasm of FLS was significantly increased after FLS was treated with IL-34 (50 ng/ml) for 30 min. Further, when RA FLS were stimulated with four signal molecules' inhibitors including SP600125, SB203580, FR180204, and Ikk-16, which inhibited JNK, P38 MAPK, ERK1/2, and NF-κB activation, respectively, the phosphorylation of P38, NF-κB, ERK1/2, and JNK was suppressed apparently (Figure 3(a)). Accordingly, the expression of IL-6 mRNA and protein in IL-34-stimualted FLS were significantly decreased in the presence of SP600125, SB203580, and IKK-6, but FR180204 impaired the production of IL-6 only to a small extent (Figures 3(b) and 3(c)). Based on these results, it is concluded that IL-34 induced IL-6 production possibly through JNK/P38 MAPK and NF-κB signaling pathways.

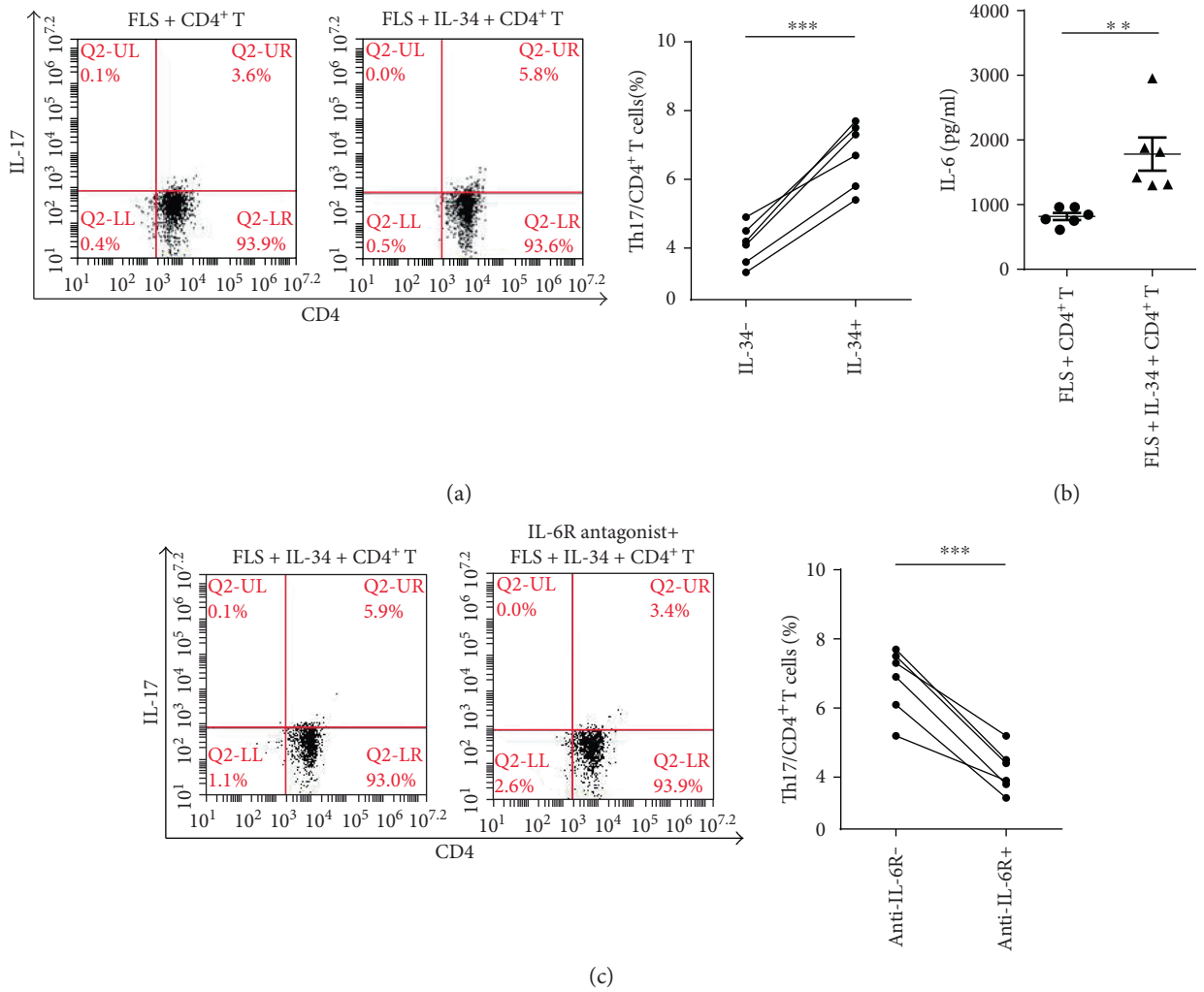

FIGURE 4: IL-34 upregulated the production of Th17 through increased IL-6 expression by RA FLS. (a) RA FLS and $CD4^+$ T cells from healthy controls ($n = 6$) were incubated with anti-CD3 (3 μg/ml)/CD28 (2 μg/ml) antibody, brefeldin A (10 ng/ml)/monension (100 ng/ml), and IL-34 (0 or 50 ng/ml) for 72 h, $CD4^+$ $IL\text{-}17^+$ T cells were analyzed by flow cytometric analysis. (b) $CD4^+$ T cells and RA FLS ($n = 6$) were cocultured with anti-CD3 (3 μg/ml)/CD28 (2 μg/ml) antibody and IL-34 (0 or 50 ng/ml) for 72 h. IL-6 levels in the supernatants of coculture system were measured by ELISA. (c) RA FLS and $CD4^+$ T cells ($n = 6$) were preincubated with anti-IL-6R antibody (1 μg/ml) for 1 h and then stimulated with anti-CD3 (3 μg/ml)/CD28 (2 μg/ml) antibody, brefeldin A (10 ng/ml)/monension (100 ng/ml), and IL-34 (50 ng/ml) for 72 h. $CD4^+$ $IL\text{-}17^+$ T cells were analyzed by flow cytometry. Data represents the mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$.

3.4. IL-34 Upregulated Th17 Production through Increased IL-6 Expression by RA FLS. As IL-6 is critical in maintaining a balanced Th17 immune response, and an elevated IL-6 was observed in IL-34-stimulated FLS in RA patients; we then tested whether this increased IL-6 by FLS could alter Th17 production. We have detected that T and B lymphocytes did not express CSF-1R (data not shown), so we cultured RA FLS with purified $CD4^+$ T cells in a 24-well plate together and stimulated them by IL-34. Results showed that the amounts of Th17 in the IL-34-stimulated coculture system were higher compared to that of the unstimulated system, accompanied by an increased IL-6 (Figures 4(a) and 4(b)). However, the elevated Th17 response was impaired when anti-IL-6R antibody was added into the coculture system (Figure 4(c)). These data indicate that IL-34-stimulated FLS facilitated Th17 generation via increased IL-6 production in vitro.

3.5. Chemokines Expression on IL-34-Stimulated FLS in RA Patients. We also tested whether IL-34 stimulation affected the expression of some chemokines. By using protein chip AAH-CYT-G1000 kits, we found that IL-34-stimulated FLS exhibited significantly increased epithelial neutrophil-activating peptide-78 (ENA-78), interleukin-8 (IL-8), growth-related oncogene (GRO), and macrophage chemoattractant protein-1 (MCP-1) compared to the unstimulated control (Figures 5(a) and 5(b)).

4. Discussion

Some researchers have revealed that IL-34 has a role in immunological regulation, proinflammation, and nerve protection. It has been confirmed that IL-34 is secreted by neurons, keratinocytes, and osteoblasts; also, FLS in RA

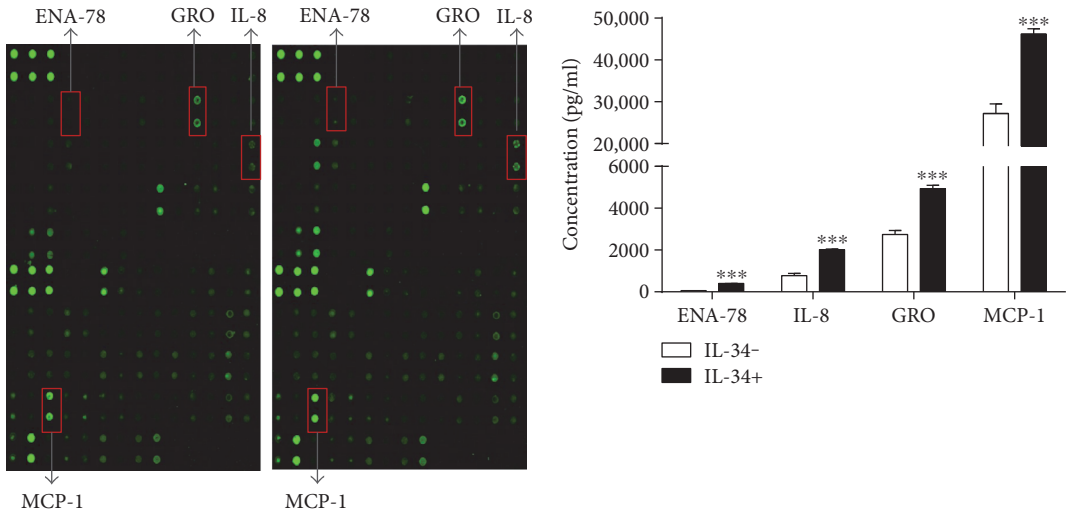

Figure 5: IL-34 increased the expression of inflammatory chemokines in RA FLS. RA FLS ($n = 5$) were stimulated with or without IL-34 (50 ng/ml) for 72 h, and the supernatants of RA FLS were detected with the protein chip AAH-CYT-G1000 kits. (a) Four chemokines (ENA-78, GRO, IL-8, and MCP-1) with obvious enhanced brightness after being incubated with IL-34 (right part) for 72 h were picked out for further analysis. (b) The secretion levels of the four chemokines were analyzed in RA FLS supernatant with or without IL-34 stimulation. Data represents the mean ± SEM. $^{***}P < 0.001$.

patients produce IL-34 following TNF-α and IL-1β stimulation [18, 19]. The elevated IL-34 in serum has been observed in RA patients, which is associated with radiographic progression, RF, and anti-CCP antibody titers [20, 21]. IL-34 greatly increases the activation and proliferation of osteoclast, suggesting that IL-34 may have a potential role in bone destruction in RA [22]. In the current study, we confirmed that IL-34 was overexpressed in RA patients and had a substantial correlation with disease activities.

Meanwhile, CSF-1R was highly expressed on RA FLS. The IL-34/CSF-1R axis promoted a dramatic production of IL-6 by FLS through JNK/P38/NF-κB signaling pathways. Further, IL-6 secreted by IL-34-stimulated RA FLS was found to upregulate the number of Th17.

IL-6 is a pleiotropic cytokine, which is produced mainly by lymphocytes, macrophages, epithelial cells, tumor cells, and also FLS in humans [23]. Accumulated evidence potentiated that FLS can be activated under inflammation. Here, we showed that IL-34 could promote FLS to produce IL-6 in RA patients. IL-6 can not only contribute to the production of matrix metalloproteinases (MMPs) but also induce the differentiation and activation of osteoclasts [24]. IL-6 can also lead to human Th17 generation [25, 26]. In our study, we found that IL-34-stimulated FLS in RA patients could enhance the numbers of Th17 and addition of IL-6R antagonist reversed Th17 response, which indicated that the combination of IL-6/IL-6R mediated Th17 production in $CD4^+$ T cells. Our data suggests that IL-34 could have direct effects on the secretion of IL-6 by FLS as an upstream proinflammatory cytokine. Also, IL-34 was able to upregulate Th17 production through the overexpressed IL-6 on FLS in RA patients.

IL-34 acts on macrophages and Langerhans cells upon binding CSF-1R [12]. CSF-1R is mainly found in myeloid cells. In the present study, we also got the results that CSF-1R was highly expressed on FLS and IL-34 might affect the function of FLS via binding with CSF-1R. CSF-1R signaling pathways are often involved in the survival, proliferation, and differentiation of myeloid cells, and there are a wide variety of downstream proteins participating in the process [10, 27]. JNK/P38 MAPK/ERK1/2 signal pathways take part in cell cycle regulation and cell apoptosis. Furthermore, NF-κB pathway, an important transcription factor, is involved in the regulation of gene transcription related to immune response, inflammation, and cell differentiation [28–31]. In this study, we found that IL-34 induced the phosphorylation of P38, NF-κB, and JNK compared with the controls. However, the phosphorylation of ERK1/2 itself kept a relatively high level in the cytoplasm of RA FLS when they were not treated with IL-34. Additionally, the amounts of IL-6 secreted by IL-34-stimulated RA FLS were decreased in the presence of SB201290, SP600125, andIKK-6, except for FR180204. These data indicated that IL-34/CSF-1R axis favored the generation of IL-6 via JNK/P38 MAPK/NF-κB pathways, but not ERK1/2.

CSF-1R signaling pathway also mediates the chemotaxis of microglia and monocytes [32]. Several chemokines and their receptors were implicated in FLS infiltration, macrophage recruitment, and angiogenesis in RA [33]. Our results by protein chips showed that the levels of ENA-78, IL-8, GRO, and MCP-1 were significantly increased in the FLS culture supernatants after IL-34 stimulation. ENA-78, IL-8, and GRO belong to the C-X-C subgroup. They can combine with CXCR2 then promote neutrophil attachment and exudation, mediate the inflammation and immune mediation, and delay apoptosis [34, 35]. MCP-1 belongs to the CC subgroup, whose receptors are CCR5 and CCR2. In most cases, it can bind with CCR2 specifically. MCP-1 has the function of

chemotaxis and activation of monocytes, macrophages, and T lymphocytes [36]. The findings that IL-34 could promote the production of some chemokines associated with inflammation and autoimmune diseases indicated that IL-34 might play multiple roles in the pathology of RA.

In summary, we found that IL-34 could combine with CSF-1R on RA FLS to elevate the expression of IL-6, which subsequently promoted the Th17 production. Therefore, IL-34 might constitute a key cytokine that affect the interactions between inflammatory cells in RA. Targeting of IL-34 might represent a useful therapeutic strategy for RA. However, the mechanism about how IL-34 reacts with FLS and T cells will need further exploration.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Bing Wang researched the data and wrote the manuscript. Zijian Ma collected the clinical data. Miaomiao Wang, Xiaotong Sun, and Yawei Tang researched the data and treated the clinical samples. Ming Li and Fang Li reviewed/edited the manuscript. Yan Zhang collected the clinical samples. Xia Li conceived the study and reviewed/edited the manuscript. Bing Wang and Zijian Ma contributed equally to this study.

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Review Article

Recent Advances: The Imbalance of Cytokines in the Pathogenesis of Inflammatory Bowel Disease

Qingdong Guan and Jiguo Zhang

Institute of Pharmacology, Taishan Medical University, Tai'an, Shandong Province 271016, China

Correspondence should be addressed to Qingdong Guan; qdguan@gmail.com and Jiguo Zhang; jgzhang@tsmc.edu.cn

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Cytokines play an important role in the immunopathogenesis of inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, where they drive and regulate multiple aspects of intestinal inflammation. The imbalance between proinflammatory and anti-inflammatory cytokines that occurs in IBD results in disease progression and tissue damage and limits the resolution of inflammation. Targeting cytokines have been novel strategies in the treatment of IBD. Recent studies show the beneficial effects of anticytokine treatments to IBD patients, and multiple novel cytokines are found to be involved in the pathogenesis of IBD. In this review, we will discuss the recent advances of novel biologics in clinics and clinical trials, and novel proinflammatory and anti-inflammatory cytokines found in IBD with focusing on IL-12 family and IL-1 family members as well as their relevance to the potential therapy of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, which clinically contains Crohn's disease (CD), ulcerative colitis (UC), and other conditions [1, 2]. The inflammation of the intestinal mucosa in IBD is characterized by episodes of abdominal pain, diarrhea, bloody stools, weight loss, and the influx of neutrophils, macrophages, and other immune cells that produce cytokines, proteolytic enzymes, and free radicals that result in inflammation and ulceration [1, 3].

IBD is a lifelong disease occurring early in life in both males and females. The incidence and prevalence of IBD markedly increased over the second half of the twentieth century, and since the beginning of the twenty-first century, IBD has been considered one of the most prevalent gastrointestinal diseases [4–7]. Estimates indicate that as of 2005, about 1.4 million Americans and several millions persons worldwide have been diagnosed with IBD. Roughly 30% are children and adults between 10 and 30 years of age [8]. The incidence of CD in North America has been estimated at between 3.1 and 14.6 per 100,000, with a prevalence of between 26.0 and 198.5 per 100,000 [1]. For UC, both incidence and prevalence are estimated at between 2.2 and 14.3 and 37.5 and 229 per 100,000, respectively [1].

Although the cause of IBD remains unknown, considerable progress has been made in the recent years to unravel the pathogenesis of this disease. Studies have provided evidence that the pathogenesis of IBD is associated with genetic susceptibility of the host, intestinal microbiota, other environmental factors, and immunological abnormalities [9–11]. The immunological dysregulation in IBD is characterized by epithelial damage (abnormal mucus production, defective repair); expansion of inflammation driven by intestinal flora and a large number of cells infiltrating into the lamina propria including T cells, B cells, macrophages, dendritic cells (DC), and neutrophils; and a failure of immune regulation to control the inflammatory response [2, 4, 12]. A large number of soluble mediators are actively secreted by the activated lamina propria cells in the local tissue, including proinflammatory cytokines (TNF, IFN-γ, IL-6, IL-12, IL-21, IL-23, IL-17, integrin, etc.) and anti-inflammatory cytokines (IL-10, TGFβ,

IL-35, etc.) [2, 11]. CD is usually designated as a Th1 and Th17 condition with elevated production of IL-12, IL-23, IFN-γ, and IL-17, whereas UC is usually characterized as a Th2 and Th9 condition with increased production of IL-13, IL-5, and IL-9 [2]. The roles of cytokines in initiating, mediating, perpetuating, and controlling intestinal inflammation and tissue injury have been intensely studied because they are the key players in the pathogenesis of IBD and they may be the potential therapeutic targets [11, 13]. Antibodies against TNF, IL-12/IL-23p40, IFN-γ, IL-6R, IL-11, IL-13, IL-17A, integrin, and recombinant IL-10 and IFN-β have been tested or applied in clinics to treat IBD patients [11]. This review will describe recent advances in biologics treatment or clinical trials for IBD patients and novel cytokines found in the pathogenesis of IBD with focusing on IL-12 family and IL-1 family members.

2. Recent Advances in Biologics Treatment and Clinical Trials in IBD

Monoclonal antibodies against TNF are the first biologics approved and widely used for the treatment of both CD and UC, including infliximab, adalimumab, and certolizumab pegol, which have demonstrated good clinical efficacy in their abilities to induce remission and maintain steroid-free remission [14, 15]. However, around 20% of patients do not respond to anti-TNF, and over 30% eventually lose response [16]. Moreover, these antibody treatments can increase the risk of infections and malignancies [16]. Therefore, other new biologics are currently being developed for both anti-TNF-naive and TNF-resistant IBD patients [16]. In 2014, monoclonal antibody against integrin $\alpha4\beta7$, vedolizumab, was approved to treat adults with moderate-to-severe active CD or UC. Vedolizumab also benefits one-third of patients with IBD who failed to previous anti-TNF therapy in a phase III trial [17]. Vedolizumab is efficacious and safe in pediatric IBD patients too, with UC patients experiencing earlier and higher rates of remission than CD patients in a multicenter experience trial [18]. Mongersen, an oral SMAD7 antisense oligonucleotide, which targets SMAD7 to increase the activity of TGFβ, induces significantly higher rates of remission and clinical response than placebo in patients with active CD in a phase II trial [19]. Tofacitinib is an oral inhibitor of JAK1, 2, and 3 to block signaling pathways of γ chain-containing cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [16]. In a phase II trial, tofacitinib induces clinical responses and remission in patients with moderate-to-severe UC, not for CD patients [20]. Add-on therapy with tralokinumab targeting IL-13 does not significantly improve clinical response but induces a higher clinical remission rate than placebo in a phase IIa trial, suggesting that tralokinumab may benefit some patients with UC [21]. But not all these biological agents achieve clinical responses. Another monoclonal antibody against IL-13, anrukinzumab, does not induce clinical benefit for patients with active UC in a phase IIa trial [22]. Several other biologics are under the clinical evaluation, such as anti-IL-12/IL-23p40.

3. IL-12 Family

IL-12 family of cytokines contains four cytokines, IL-12, IL-23, IL-27, and IL-35. Each member is composed of a helical α subunit (p35, p19, and p28) and a β-subunit (p40 and EBI3) structurally similar to the extracellular domain of type 1 cytokine receptors [23, 24]. IL-12 is composed of p35 and p40, IL-23 is composed of p19 and p40, IL-27 is composed of p28 and EBI3, and IL-35 contains p35 and EBI3 [23, 24]. The binding of the IL-12 family of cytokines to their corresponding receptors will activate JAK/STAT signaling pathways, leading to transcription of target genes that mediate biological activities. The IL-12 family of cytokines has emerged as important regulators of host immunity [23, 24].

IL-12 is predominantly produced by DC, monocytes, and macrophages following recognition of pathogenic structures by toll-like receptors and other receptors [25, 26]. IL-12 induces the production of IFN-γ, favours the differentiation of Th1, and forms a link between innate and adaptive immunity [27]. IL-23 is also predominantly produced by activated dendritic and phagocytic cells [26, 28, 29]. IL-23 plays an important role in stabilizing/amplifying Th17 proliferation [30, 31].

Studies have demonstrated that IL-12 and IL-23 play important roles in the pathogenesis of CD. Multiple studies indicate that IL-12 is overproduced in the gastric mucosa, lamina propria mononuclear cells, and macrophages in CD, and macrophages isolated from the inflammatory lesions of patients with CD produce increased amounts of IL-12 ex vivo [32–35]. Recent studies have highlighted the roles of IL-23 in the pathogenesis of CD [4, 36–38]. The colonic level of IL-23 is increased in patients with CD [39]. The myeloid DC from the mesenteric lymph nodes of CD patients secretes high levels of IL-23 [40]. The expression of IL-23R is upregulated in the lamina propria isolated from CD [41]. And the upregulated expression of IL-23R is correlated with IFN-γ [41]. Recently, a genome-wide association study has identified numerous SNP in *IL-23R*, with high association for CD and UC [42, 43]. Of interest, the G149R, V362I and R381Q IL-23Rα chain variants, confers the protective effects in patients with CD and UC [43, 44]. These protective effects are due to impaired protein stability and intracellular trafficking, then leading to decrease the surface receptor expression and further reduce STAT signaling pathway [44]. Yen et al. used IL-10 knockout mice, a spontaneous IBD model, and showed that the development of colitis was suppressed by IL-23p19 deficiency but not IL-12p35 deficiency in IL-$10^{-/-}$ mice; administration of IL-23 accelerated the onset of colitis and promoted inflammation through IL-17- and IL-6-dependent mechanisms [36]. As IL-12 and IL-23 share p40 subunit, targeting IL-12/IL-23p40 has been widely tested to improve intestinal inflammation in preclinical studies and clinical trials. Briakinumab and ustekinumab are human monoclonal antibodies against IL-12/IL-23p40 which induced clinical response and remission in a certain subtype of patients with CD [45–48]. In a phase IIb trial, briakinumab induced numerically greater rates of remission and response in moderate-to-severe active CD patients when compared to

placebo treatment [47]. In a phase III-randomized trial, ustekinumab treatment induced significant clinical response and remission in moderate-to-severe CD patients refractory to prior TNF antagonists [48]. Since CD is a chronic inflammatory disease, it usually needs long-term treatment while monoclonal antibodies have short half-life. To overcome these disadvantages, we developed an IL-12/IL-23p40 peptide-based vaccine which induced relative long-lasting antibodies against IL-12/IL-23, and immunization with this vaccine improved TNBS-induced acute and chronic murine colitis by downregulating IL-12, IL-23, TNF, and IFN-γ and reducing fibrosis [49, 50]. Interestingly, immunization with the same vaccine can also ameliorate allergic murine skin and airway inflammation [51].

IL-27 is predominately produced by DC, macrophages and monocytes following stimulation by different immune stimuli [52, 53]. IL-27 regulates both innate and adaptive immune responses, including activating innate immune cells (e.g., macrophages), promoting Th1 and type 1 regulatory T cell differentiation, and inhibiting the differentiation of Th2, Th17, and Treg cells [52, 53]. However, under certain conditions, the regulatory function of IL-27 can be deviated, such as inhibiting Th1 but promoting Treg development [52, 53].

The important role of IL-27 in the pathogenesis of IBD has been indicated. Multiple studies have implicated IL-27 gene polymorphism and mutation are associated with the risk of IBD [54]. IL-27 gene expression level is increased in the local colon tissue of patients with active UC or active CD [55]. The proinflammatory and anti-inflammatory effects of IL-27 have been observed in IBD [54]. Oral delivery of recombinant IL-27 food-grade bacterium *Lactococcus lactis* ameliorates the murine colitis in a T cell-dependent colitis model, including improving survival, decreasing clinical score and pathologic score, downregulating inflammatory cytokines, and increasing IL-10 [56]. Subcutaneous administration with IL-27 also attenuates TNBS-induced intestinal inflammation with reducing pathologic score, decreasing inflammatory cytokines, and inhibiting Th17 cells [57]. IL-27R$^{-/-}$ mice exhibited earlier onset and significantly increased severity of intestinal inflammation when compared to wild-type controls after DSS treatment [58]. These studies demonstrated the protective role of IL-27 in IBD.

On the other hand, some studies indicated the proinflammatory roles of IL-27 in IBD based on blocking IL-27 receptor signaling pathway [54]. IL-27R$\alpha^{-/-}$ mice developed less severe intestinal inflammation after DSS treatment when compared to wild-type mice, characterized by reducing inflammatory cytokines (IL-6, TNF, and IFN-γ). Another study showed that IL-27R$\alpha^{-/-}$ effector T cells had poor proliferation and less IFN-γ secretion; transfer of IL-27R$\alpha^{-/-}$ T cells results in diminished weight loss and reduced intestinal inflammation when compared to transferring of wild-type T cells [59]. Visperas et al. showed that IL-27R$\alpha^{-/-}$TCR$\beta^{-/-}$ recipients did not develop intestinal inflammation after naive CD4^{+}T cell infusion because these recipient mice had poor Th17 differentiation and lower expression of IL-6 and IL-1β by antigen-presenting cells; while IL-27R$\alpha^{+/+}$TCR$\beta^{-/-}$ recipients developed severe colitis after naive CD4^{+}T cell infusion [60]. These results indicate the complicated roles of IL-27 in IBD, which requires further investigation.

IL-35 is a newly identified cytokine of the IL-12 family, which is mainly produced by Treg, activated B cells, and DC [11, 23, 61]. IL-35 can induce naive human and mouse T cells to differentiate into regulatory T cells (iTr35 cells), which do not express Foxp3, IL-10, and TGF but suppress T cell responses through IL-35 [23]. IL-35 suppresses T cell proliferation by inducing cell cycle arrest in G1 phase without inducing apoptosis [23]. In addition, IL-35 promotes the differentiation of human B cells into Breg which produces IL-35 and IL-10, and IL-35-producing B cells play an important role in the suppressive regulation of immunity [62]. Multiple studies have demonstrated the important regulatory role of IL-35 in IBD. Recently, two groups showed that IL-35 levels are significantly decreased in the serum but increased in the local colon tissue, and high IL-35 secreting intestinal Breg and circulating regulatory CD4^{+}T and CD8^{+}T cells are found in patients with active IBD [63, 64]; the level of IL-35 in the serum is inversely correlated with that of the activity of UC [64]. In T cell-dependent murine colitis model, EBI3$^{-/-}$ mice (lacking both IL-27 and IL-35) develop early onset and severe colitis with shorten survival time, when compared to IL-27p28$^{-/-}$ mice (lacking IL-27 only) [65], while recombinant IL-35 treatment significantly limited the development of several forms of experimental colitis and decreased levels of markers of Th1 and Th17 cells [65]. Adoptive transfer of IL-35-induced iTr35 can ameliorate the development of murine colitis [23]. These results suggest IL-35 may be an important regulator of IBD, with the potential for the treatment of IBD.

4. IL-1 Family

IL-1 family includes seven agonistic cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), three receptor antagonists (IL-1Rα, IL-36Rα, and IL-38), and one anti-inflammatory cytokine (IL-37) [66]. The IL-1 receptor family members include 10 molecules, from IL-1R1 to IL-1R10 [66]. Upon cytokine binding, IL-1 receptors heterodimerize, which recruit intracellular signaling molecules, including MyD88, IRAK, and TRAF6, then activate NF-κB, p38, JNK, and/or MAPK transcription factors, leading to transcription of target genes (such as IL-6, IL-5, IL-4, IL-8, MCP-1, and COX-2) [67]. All innate immune cells express and/or are affected by the IL-1 family members. Moreover, the IL-1 family members play an important role in the differentiation and function of polarized innate and adaptive lymphoid cells [66]. Here, we will focus on the review of the roles of novel cytokines IL-33, IL-36, and IL-37 in the pathogenesis of IBD.

IL-33 is widely expressed by many cell types, such as epithelial cells, fibroblasts, smooth muscle cells, and endothelial cells [66, 68]. IL-33 exerts its biological function through binding of its receptor T1/ST2 [69]. Many immune cells are responsive to IL-33 and express T1/ST2 on their surface, and the main effects of IL-33 are involve in inflammation and type 2 immunity, including activation and accumulation of type 2 innate lymphoid cells, Th2, and M2 polarized macrophages [66, 68]. It has shown that IL-33 plays a role in the

pathogenesis of many diseases, such as infection and autoimmune diseases [69].

IL-33 is constitutively expressed in epithelial cells at barrier sites of the gut, which can be regulated by epidermal growth factor [70]. In response to tissue damage, IL-33 functions as an alarmin by driving Th2, Th17, and Treg responses and influencing wound healing of damaged tissue [68, 71]. Through screening over 1500 IBD patients, a recent study shows that IL-33 polymorphisms contribute to the risk of IBD [72]. IL-33, along with ST2, is significantly increased in the inflamed IBD biopsy samples, especially in UC [72–74]. Furthermore, the expression of IL-33 is correlated with the inflammatory status [69]. At the disease remission stage of UC after anti-TNF treatment, IL-33 loses its expression in colonic crypts [75]. Soluble ST2 is a sensitive marker of treatment response and clinical outcome of UC [76]. IL-33 is also found to be increased in the colon tissue of animal colitis models [77, 78]. Deficiency of ST2 protects mice from TNBS- and DSS-induced murine colitis [79]. Administration of recombinant IL-33 exaggerates the severity of DSS-induced acute colitis, which is associated with marked elevation of IL-4, IL-5, and IL-13, significant reduction of IL-17 and IFN-γ in the colon tissue, impairment of the epithelial barrier, and delay of wound healing of the injured colonic epithelia [79–81]. But another group shows that administration of IL-33 protects DSS-induced acute colitis through inducing group 2 innate lymphoid cells with the expression of IL-4 and IL-5 and growth factor amphiregulin [78]. In TNBS-induced acute colitis, IL-33 administration protects the intestinal inflammation by promoting Th2/Foxp3$^+$Treg cells [77]. In DSS-induced chronic colitis, IL-33 ameliorates the intestinal inflammation through suppressing Th1 and Th17 responses [82]. However, in SAMP1/YitFc spontaneous chronic murine colitis model, IL-33 administration worsens the chronic intestinal inflammation by enhancing eosinophil infiltration and increasing pathogenic Th2 response [83]; these effects can be reversed by blockade of IL-33 signaling or depletion of eosinophils and required gut microbiomes [83, 84]. These animal findings indicate that IL-33 may confer protection from injury or lead to inflammation although the behind mechanisms remain largely undefined.

IL-36 contains three agonistic ligands (IL-36α, IL-36β, and IL-36γ) and one antagonist ligand (IL-36Ra) [85], which bind to the same heterodimeric receptor. Not like other members of the IL-1 family, IL-36 is mainly expressed in keratinocytes, bronchial epithelia, brain tissues, and monocytes/macrophages [85]. IL-17 and TNF can induce the expression of IL-36 in keratinocytes, and IL-22 can synergize these effects [86]. IL-36 receptor signaling activates DC and plays a role in polarizing helper T cell responses [85]. IL-36 induces the secretion of proinflammatory cytokines (IL-12, IL-6, IL-1β, and TNF) from bone marrow-derived DC, IL-4, IL-17, and IFN-γ from CD4$^+$T cells [87]. IL-36β induces the secretion of IL-12 and IL-18 from human monocyte-derived DC, which subsequently leads to the proliferation of IFN-γ^+T lymphocytes [88]. Studies have shown that IL-36 plays a role in the pathogenesis of asthma, autoimmune diseases, psoriasis, and other diseases [85].

The mRNA expression of IL-36α and IL-36γ, not IL-36β, is increased in the inflamed mucosa of IBD patients, especially in UC and in DSS-induced murine colitis [89, 90]. Further, it finds that T cells, monocytes, and plasma cells in inflamed mucosa of IBD patients are the source of IL-36α and IL-36γ [89]. IL-36α and IL-36γ induce the expression of CXC chemokines on human intestinal epithelial cell line HT-29 cells in dose-dependent and time-dependent manners [89]. IL-36R$^{-/-}$ mice reduced the intestinal inflammation in DSS-induced acute colitis, associated with decreased innate inflammatory cell infiltration into the colon lamina propria [91]. Similarly, after infection with the enteropathogenic bacteria *Citrobacter rodentium*, IL-36R$^{-/-}$ mice reduced innate inflammatory cell infiltration and increased bacterial colonization in the colon, with enhanced Th17 and reduced Th1 responses [91]. Another group shows that IL-36 signaling may be important in the resolution of intestinal damage [92]. After DSS treatment, IL-36R-deficient mice reduce intestinal inflammation but significantly delay the wound healing of colonic mucosa, which is associated with the reduction of neutrophil infiltration into the colonic mucosa and reduction of barrier-protective cytokine IL-22 in the colon [92]. Administration of an aryl hydrocarbon receptor agonist restores IL-22 expression and promotes full recovery from DSS treatment in IL-36R-deficient mice [84, 92]. These studies indicate that IL-36 signaling plays a role in the pathogenesis of IBD and post injury healing, with the potential to be the target for the treatment of IBD.

IL-37 is expressed in diverse human tissues, such as skin, tonsil, placenta, breast, and melanoma [93–95]. IL-37 is induced in DC and peripheral blood mononuclear cells stimulated by TNF, IFN-γ, IL-1β, and several toll-like receptor agonists [96]. The binding of IL-37 to IL-18Rα/IL-1R8 controls the regulators of cellular adhesion and migration such as FAK, Pyk2, and transcription factors such as NF-kB and MAPK to display the anti-inflammatory activities mediated via Smad3 and caspase-1 [93, 94]. Increased levels of IL-37 have been reported in multiple diseases by measuring IL-37 mRNA or protein in cells derived from patients or in serum, such as rheumatoid arthritis, melanoma, atopic dermatitis, and ankylosing spondylitis [94]. In some other diseases, IL-37 levels are found to be decreased, such as psoriasis, asthma, and allergic rhinitis [94].

There are several reports that evaluated the levels of IL-37 in IBD patients. The percentage of IL-37-secreting cells is higher in the inflamed intestine of active CD patients than that in active ulcerative and noninflamed control tissues [63]. Levels of IL-37 in the sera are increased in active IBD patients, which are conspicuously produced by circulating B cells, active natural killer cells, and monocytes [63, 64]. In pediatric IBD patients, IL-37 protein expression is increased in submucosal lymphoid cells and correlated with histological severity score of intestinal inflammation; another IL-1 family member, IL-18, shows the similar pattern as IL-37 in these patients [97]. In vitro experiment shows that IL-37b inhibits the TNFα-induced chemokine IP-10 expression in human colonic subepithelial myofibroblasts [98]. Animal studies demonstrate anti-inflammatory roles of IL-37 in colitis. Transgenic mice overexpressing human IL-37b (IL-37b-tg) exhibits

reduction of DC activation and proinflammatory cytokine secretion after LPS stimulation. IL-37b-tg mice protect from DSS-induced colitis, characterized by decreasing clinical disease score, histological score, and TNFα and IL-1β production, but increasing IL-10 production in colon tissue [99]. If overexpressing IL-37b on mesenchymal stromal cells, it will greatly increase the therapeutic efficacy of mesenchymal stromal cells in DSS-induced murine colitis [100]. These results indicate that IL-37 may be useful for the treatment of IBD.

5. Conclusion and Prospective

Cytokines play a crucial role in driving, perpetuating, resolving, and wound healing of intestinal inflammation in IBD. Novel biologics that targeting cytokines or cytokine signaling pathway cascades are being used in clinics or being tested in clinical trials. However, these biologics only seem to have beneficial clinical effects in certain subgroups of IBD patients. This may reflect the complex of cytokine networks in the inflamed colon tissue, which are subject to types of inflammation, location, microbiota, genetic, immune cell plasticity, and others [11]. Blockade of a single cytokine in IBD patients may drive other proinflammatory cytokine pathways. Therefore, to optimize the clinical response and remission rate in IBD patients, it may require using multiple cytokine inhibitors that simultaneously block several cytokines or common cytokine signaling pathway—the JAK-STAT pathway. Taken together, anti-TNF and anti-integrin $\alpha 4\beta 7$ have been the mainstay of biological therapy in IBD. New cytokine targets (e.g., IL-12/IL-23p40 and SMAD7), novel anti-inflammatory cytokines (e.g., IL-35 and IL-37), and personalized medicines may provide potential treatment for IBD patients.

Abbreviations

CD: Crohn's disease
DC: Dendritic cells
DSS: Dextran sulfate sodium
IBD: Inflammatory bowel disease
IFN: Interferon
IL: Interleukin
TNBS: 2,4,6-Trinitrobenzenesulfonic acid
TNF: Tumor necrosis factor
UC: Ulcerative colitis.

Conflicts of Interest

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

Authors' Contributions

Qingdong Guan proposed the idea and drafted the manuscript. Jiguo Zhang extensively reviewed and edited the manuscript. Qingdong Guan and Jiguo Zhang designed the outline of the manuscript and had final approval of the version submitted for publication.

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Research Article

Cytokine Imbalance as a Common Mechanism in Both Psoriasis and Rheumatoid Arthritis

Yong Tan,[1] Qiu Qi,[2] Cheng Lu,[1] Xuyan Niu,[1] Yanping Bai,[3] Chunyan Jiang,[4] Yang Wang,[5] Youwen Zhou,[6,7] Aiping Lu,[1,8,9] and Cheng Xiao[10]

[1] *Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[2] *Institute of Clinical Pharmacology, Beijing An Zhen Hospital, Capital Medical University, Beijing 100029, China*
[3] *Department of Dermatology, China-Japan Friendship Hospital, Beijing 100029, China*
[4] *Department of Dermatology, Beijing Hospital of Traditional Chinese Medicine, Beijing 100010, China*
[5] *Department of Dermatology and Venerology, Peking University First Hospital, Beijing 100034, China*
[6] *Department of Dermatology and Skin Science, University of British Columbia, Vancouver, BC, Canada*
[7] *Molecular Medicine Laboratory and Chieng Genomics Center, Vancouver Coastal Health Research Institute, Vancouver, BC, Canada*
[8] *Institute for Advancing Translational Medicine in Bone & Joint Diseases, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong*
[9] *E-Institute of Chinese Traditional Internal Medicine, Shanghai Municipal Education Commission, Shanghai 201203, China*
[10] *Institute of Clinical Medicine, China-Japan Friendship Hospital, Beijing 100029, China*

Correspondence should be addressed to Youwen Zhou; youwen.zhou@ubc.ca, Aiping Lu; aipinglu@hkbu.edu.hk, and Cheng Xiao; xc2002812@126.com

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Psoriasis (PS) and rheumatoid arthritis (RA) are immune-mediated inflammatory diseases. Previous studies showed that these two diseases had a common pathogenesis, but the precise molecular mechanism remains unclear. In this study, RNA sequencing of peripheral blood mononuclear cells was employed to explore both the differentially expressed genes (DEGs) of 10 PS and 10 RA patients compared with those of 10 healthy volunteers and the shared DEGs between these two diseases. Bioinformatics network analysis was used to reveal the connections among the shared DEGs and the corresponding molecular mechanism. In total, 120 and 212 DEGs were identified in PS and RA, respectively, and 31 shared DEGs were identified. Bioinformatics analysis indicated that the cytokine imbalance relevant to key molecules (such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), tumor necrosis factor (TNF), colony-stimulating factor 3 (CSF3), interleukin- (IL-) 6, and interferon gene (IFNG)) and canonical signaling pathways (such as the complement system, antigen presentation, macropinocytosis signaling, nuclear factor-kappa B (NF-κB) signaling, and IL-17 signaling) was responsible for the common comprehensive mechanism of PS and RA. Our findings provide a better understanding of the pathogenesis of PS and RA, suggesting potential strategies for treating and preventing both diseases. This study may also provide a new paradigm for illuminating the common pathogenesis of different diseases.

1. Introduction

Psoriasis (PS) and rheumatoid arthritis (RA) are immune-mediated chronic inflammatory diseases. PS is characterized by epidermal hyperplasia, and the predominant pathological feature of RA is the destruction of synovial joints. Studies have increasingly suggested that patients suffering from PS or RA have similarly increased risks of certain disorders, such as major adverse cardiovascular events, malignancy, and liver fatty changes, compared to the general population [1–6]. Additionally, these two diseases have a similar pathogenesis. Previous studies have indicated that the chronic inflammation mediated by T helper (Th) 17 and Th1 cells plays a key role in PS [7, 8]. Cytokines, including Th1-related (tumor necrosis factor- (TNF-) α, interferon gamma (IFNγ), and interleukin- (IL-) 2) and Th17-related (IL-17A, IL-17F, IL-22,

IL-26, and TNF-α) proteins, together with IL-23, IL-20, and IL-15 were increased in the sera of PS patients [8, 9]. For RA, the concerted interaction of proinflammatory cytokines also plays a dominate role in its pathogenesis [10]. An increasing number of clinical and histopathological features of PS and RA are explained by an imbalance in particular cytokines, which is one of the most fascinating research topics inspiring researchers [9]. The similarities between the mechanisms involved in PS and RA imply an underlying genetic homogeneity of these two diseases. By exploring the genetic overlap of PS and RA, we seek to provide a better understanding of their molecular correlation and their shared mechanisms. The genetic commonality of PS and RA may provide increasing evidence for developing combined treatment targets for both diseases. These targets will eventually complement the traditional systemic treatments and biological agents that are currently available.

Due to the progress in high-throughput techniques for biological research, next-generation sequencing (NGS) platforms are often used to explore the gene profile; these platforms have the advantages of greater sensitivity and more precise quantification, thus providing a more complete picture of the transcriptome in studies of gene expression than that obtained by microarrays [11]. Measurements of mRNA expression by RNA sequencing are valuable for identifying the molecular changes that occur in cells, thus providing clues regarding the molecular networks involved in disease processes [12]. Studies have focused on molecular changes in PS or RA independently using transcriptome or gene expression profile technology [12, 13], but few reports have been published concerning the correlations between PS and RA at the transcriptome level, including in-depth studies of the mechanisms and molecular networks involved in the pathogenesis common to RA and PS.

The present study applied RNA sequencing technology to the peripheral blood mononuclear cell (PBMC) RNA of PS and RA patients and healthy volunteers, and differentially expressed genes (DEGs) were explored among the groups. Furthermore, bioinformatics analysis was performed to identify the key molecules and signaling pathways relevant to RA and PS as well as the upstream regulators related to the identified genes. This study aimed to obtain a comprehensive understanding of the cytokine imbalance in RA and PS based on DEGs, which may provide new insights into the pathogenesis of and suitable prevention strategies for these two diseases.

2. Materials and Methods

2.1. Patients. PS patients, RA patients, and healthy volunteers were recruited from the China-Japan Friendship Hospital in Beijing City of China at the dermatology clinic, the rheumatology clinic, and the health screening center, respectively. The diagnosis of PS was consistent with the guidelines of the care for the management of psoriasis from the American Academy of Dermatology and the guidelines for the treatment of psoriasis from the Psoriasis Study Group of Chinese Medical Association [14, 15]. Additionally, enrolled patients had no symptoms or signs of psoriatic arthritis. These patients had a psoriasis area severe index (PASI) greater than 10 or body surface area (BSA) greater than 10% but a PASI less than 30 and BSA less than 30%. A diagnosis of RA was based on the 1987 American College of Rheumatology revised criteria and the 2010 American College of Rheumatology/European League against Rheumatism classification criteria for RA. Disease activity was assessed by the Disease Activity Score in 28 joints (DAS28). For inclusion, the control subjects could not have a history of an arthritic disorder and were subject to the same exclusion criteria as the PS and RA patients. Given that RA is two- to threefold more common in females than males, only females were chosen as the observed subjects in this study [16].

The following subjects were excluded: individuals who were $\geq$65 years old and $\leq$18 years old; individuals with complications, such as cardiovascular and cerebrovascular diseases, respiratory, digestive, urinary, and hematological diseases, metabolic syndrome, and mental disturbances; individuals who were pregnant, lactating, or who planned to become pregnant within a year; individuals who received topical treatments (such as corticosteroids or retinoic acid) within 2 weeks, systemic therapy within 4 weeks, or biological therapy within 12 weeks; PS patients with a concurrent RA diagnosis; and RA patients diagnosed with any type of PS.

In summary, 10 female PS patients, 10 female RA patients, and 10 female healthy controls were enrolled into this study. All protocols involving human subjects were approved by the ethics committee of the China-Japan Friendship Hospital (ethics ID: 2014-58), and informed consent was signed by all participants before the study began.

2.2. PBMC Isolation and Total RNA Extraction. In all, 3 mL peripheral fasting blood samples were collected from all subjects in the morning. PBMCs were isolated using density gradient centrifugation. Specifically, based on Ficoll-Hypaque gradient solution (Histopaque-1077, Sigma-Aldrich, USA), 3 mL of heparinized whole blood was diluted to 6 mL with phosphate-buffered saline (PBS, pH 7.4), layered on top of 3 mL of Histopaque and centrifuged for 30 min at 400 ×g. PBMCs were aspirated, washed twice, suspended in PBS, and counted with a hemocytometer. PBMCs were lysed in Trizol reagent (1 mL/1 $\times$ 10^7 PBMCs) (Invitrogen, Karlsruhe, Germany; Carlsbad, CA) and stored at -80°C for the subsequent testing. Total RNA in PBMC sample was isolated using the Trizol extraction method, and it was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Marietta, OH, USA). The RNA Integrity Number was greater than 7.0, and acceptable quality values accorded with A260/A280 ratios ranging from 1.8 to 2.2 for each total RNA sample.

2.3. Identification of DEGs. Total RNA of each sample was purified by adsorption of biotin oligo magnetic beads. cDNA synthesis was conducted after the binding of mRNA. Double-stranded cDNA was introduced to the cDNA fragment digested by NlaIII endonuclease, and the bound fragments contained CATG sites and adjacent poly A tails at the 3′ end. After precipitation of the 3′ cDNA fragment, Illumina adaptor 1 was added to the 5′ end. Both the adaptor 1

and CATG sites are recognized by MmeI, which cuts at a downstream CATG site and produces fragments of 17-bp tags with adaptor 1. Adaptor 2 was added to the 3′ end of these tags after the fragment was removed using beads attached to the 3′ end. Then, these sequences were prepared for Solexa sequencing [17].

Clean tags were produced by filtering the adaptor sequences and removing low-quality sequences (containing ambiguous bases). Only the tags with perfect matches or one mismatch were further considered and annotated based on the reference genes. The expression level of each gene was estimated by the frequency of clean tags and then normalized to TPM (number of transcripts per million clean tags), which is a standard method extensively used in DEG analysis [18]. The number of tags mapped to a given gene represented the expression level of this gene. Expression levels of a gene from two different samples were compared to provide an expression difference. Significance values for differences in expression were determined using a modified exact test. The gene was classified as differentially expressed only when the expression difference was greater than 1.2-fold with a p value less than 0.01.

2.4. Bioinformatics Analysis about DEGs. The information of shared DEGs identified in PS and RA was uploaded into the Ingenuity Pathways Analysis system (IPA, Ingenuity Systems, http://www.ingenuity.com). The "Core Analysis" module in IPA was utilized to analyze and visualize interactions of the shared DEGs. These interactions were characterized by specific canonical pathways and molecular networks. Analytical score was the negative base 10 logarithm of Fisher's exact test p value in canonical pathway analysis. Significance for biological functions of each network was symbolized by a p value for the enrichment of the genes in the network by comparison with the entire Ingenuity Pathway Knowledge Base.

3. Results

3.1. Baseline Characteristics of Study Subjects. The characteristics of the enrolled subjects, including age, disease duration, BMI, PASI, BSA, and results of blood routine and biochemical tests, are presented in Table 1. No significant differences in any of the examination indicators were noted among the groups.

3.2. Identified Shared DEGs between PS and RA and the Corresponding Functions. One hundred and twenty genes in PS and 212 genes in RA were identified as DEGs when compared with the controls (Figure 1, Tables S1 and S2 in Supplementary Material available online at https://doi.org/10.1155/2017/2405291). As shown in Figure 1 and Table 2, there were 31 shared genes between PS and RA, including 20 upregulated and 11 downregulated DEGs, which reflects the complex association of PS and RA at the transcriptome level. The biological functions corresponding to the shared DEGs mainly include cell-to-cell signaling, systemic autoimmune syndrome, cell death and apoptosis, inflammatory dermatoses, and rheumatic arthritis (Figure 2, Table S3).

Table 1: Characteristics of the enrolled subjects for the three groups.

Characteristic	Control (n = 10)	PS (n = 10)	RA (n = 10)
Age (years)	45.80 ± 3.50	48.60 ± 5.20	54.50 ± 7.10
Disease duration (years)	/	3.70 ± 1.20	2.10 ± 1.10
BMI (kg/m^2)	22.34 ± 1.83	23.23 ± 4.71	25.81 ± 2.62
PASI	/	9.17 ± 8.56	/
BSA (%)	/	19.40 ± 4.14	/
ESR (mm/h)	/	/	39.14 ± 29.53
CRP (mg/L)	/	/	15.48 ± 15.40
RF (IU/mL)	/	/	63.76 ± 71.81
WBC (×10^9/L)	4.92 ± 1.10	5.33 ± 0.98	6.09 ± 1.50
HGB (g/L)	126.20 ± 13.89	124.72 ± 11.54	122.38 ± 14.74
PLT (×10^9/L)	228.70 ± 28.69	251.19 ± 34.56	246.75 ± 75.06

Note. Comparisons of clinical indicators of the PS group, RA group, and control group. An unpaired t-test was used for continuous variables analysis, and the data are expressed as the mean ± SD when appropriate (95% CI).

3.3. Networks of the Shared DEGs and the Corresponding Functions. To reveal the connections between the shared DEGs, the biomolecular networks of these DEGs were constructed using IPA. As shown in Figure 3, these DEGs were associated with one another directly or indirectly, and three networks were established. Highly linked molecules of the networks included extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), interferon gene (IFNG), and Ca^{2+}. The network functions included organismal injury and abnormalities, cell death and survival, and cellular function and maintenance.

3.4. Signaling Pathways Relevant to the Merged Bionetwork. The three networks were merged and formed a large network that was associated with 42 signaling pathways. TNF was highly linked molecule of the network. The main categories corresponding to these signaling pathways were cytokine signaling, cellular immune response, and humoral immune pathways. Signaling pathways with −log(p-value) more than $2.00E + 00$ represented the most significantly relevant pathways related to the merged network and included the complement system, antigen presentation, macropinocytosis signaling, acute phase response signaling, nuclear factor-kappa B (NF-κB) signaling, IL-6 signaling, IL-17 signaling, and p38 MAPK signaling (Figures 4 and 5 and Table S4). Top five pathways of these pathways were associated with the shared DEGs.

3.5. Upstream Regulators of the Shared DEGs. Thirty-six upstream regulators were identified by biomolecular network analysis, with the majority being cytokine molecules, that is, 52.78% of them (Figure 6(a), Table S5). The regulators with p values less than $1.00E - 04$ included colony-stimulating factor 3 (CSF3), IL-6, FOS, p38 MAPK, and TNF, and the connection between every regulator and the corresponding target molecule is presented in Figure 6(b). The main biofunctions of the regulated effect networks corresponding

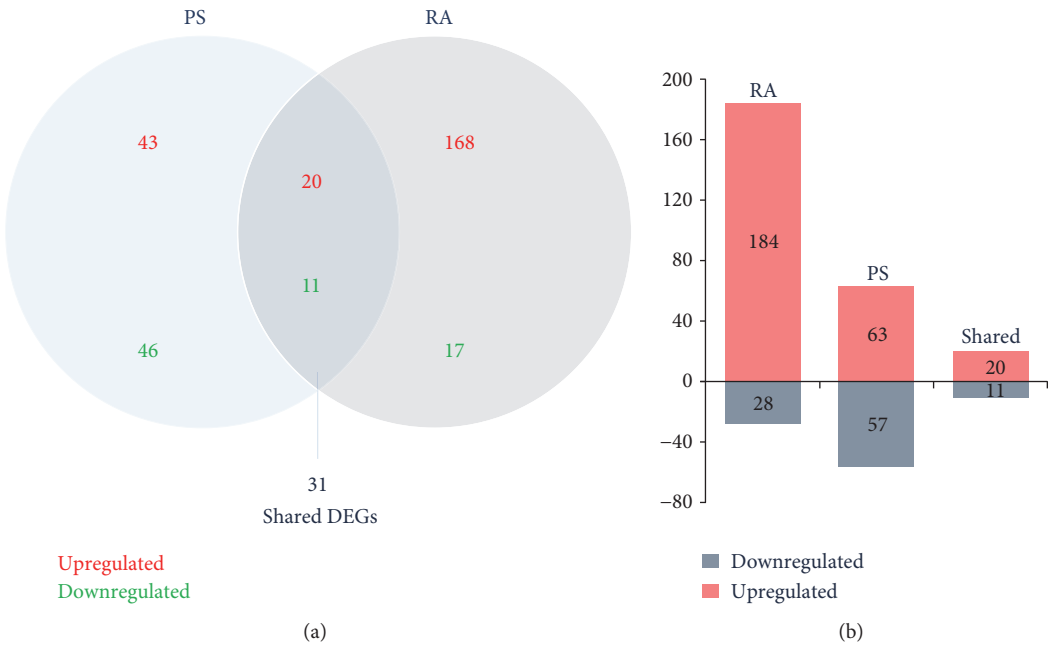


Figure 1: Number of DEGs in PS and RA. (a) The Venn diagram indicates the number of uniquely upregulated (red) or downregulated (green) genes from the comparisons of PS and RA with control and the number of shared DEGs. (b) The bar diagram shows the number of DEGs in PS, RA, and the shared DEGs between them.

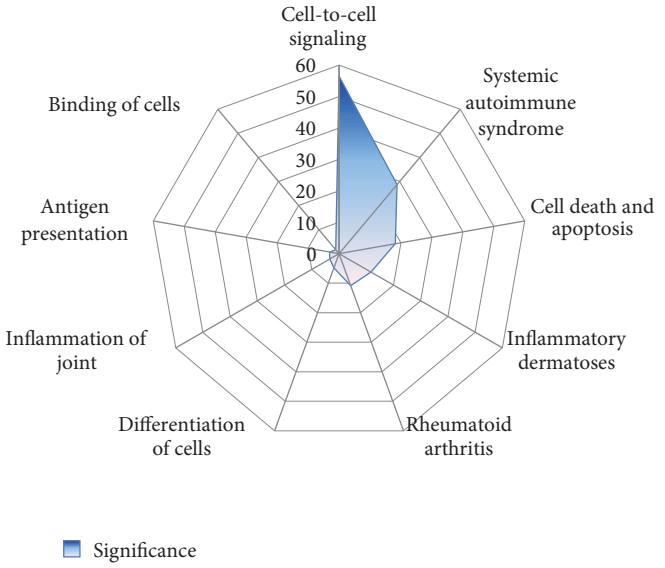


Figure 2: Biological functions corresponding to the shared DEGs.

to those regulators were inflammation or immune-related processes.

4. Discussion

PS and RA are immune-mediated inflammatory diseases. An increasing number of studies have reported a correlation between PS and RA, but the exact common molecular mechanisms have not been elucidated. With the development of high-throughput detection and analysis techniques, including genomics and bioinformatics, the exploration of these comprehensive mechanisms has become feasible. PBMCs can be extracted from whole blood and consist of lymphocytes (T cells, B cells, and NK cells) and monocytes. Identifying gene expression in PBMCs is an important strategy to determine disease-specific genes in holism [19]. Methodologically, by comparison of gene profiles of patients with particular disease and healthy persons, the disease-specific genes can be found [20]. In this study, the PBMC gene profiles of PS patients, RA patients, and healthy

Table 2: The shared DEGs in PS and RA.

n	Symbol	Entrez gene name	Fold change	
			PS	RA
1	AEBP1	AE binding protein 1	2.615	2.644
2	ARG1	Arginase 1	1.295	2.224
3	C1QB	Complement component 1, q subcomponent, B chain	1.153	2.356
4	C1QC	Complement component 1, q subcomponent, C chain	1.177	3.000
5	C4BPA	Complement component 4 binding protein alpha	1.504	3.322
6	CACNG6	Calcium voltage-gated channel auxiliary subunit gamma 6	−1.329	−1.445
7	CCNB2	Cyclin B2	1.479	2.390
8	CD177	CD177 molecule	1.000	2.404
9	CEP55	Centrosomal protein 55	1.303	1.554
10	CHI3L1	Chitinase 3 like 1	1.095	1.258
11	CTGF	Connective tissue growth factor	4.700	4.954
12	DAAM1	Dishevelled associated activator of morphogenesis 1	−1.158	−1.489
13	EGR2	Early growth response 2	1.413	2.495
14	FHDC1	FH2 domain containing 1	1.122	1.631
15	FOLR3	Folate receptor 3 (gamma)	1.787	1.853
16	HLA-DQA2	Major histocompatibility complex, class II, DQ alpha 2	1.548	1.305
17	HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	1.884	1.711
18	IFI27	Interferon alpha inducible protein 27	1.948	2.764
19	ITGB4	Integrin subunit beta 4	−1.531	−2.478
20	KRT1	Keratin 1	−1.142	−1.415
21	LTF	Lactotransferrin	1.526	1.708
22	MET	MET protooncogene, receptor tyrosine kinase	4.700	4.459
23	MT2A	Metallothionein 2A	−1.705	−1.253
24	PGLYRP1	Peptidoglycan recognition protein 1	1.535	1.907
25	RNF182	Ring finger protein 182	3.138	3.459
26	SLC26A8	Solute carrier family 26 member 8	1.890	3.021
27	SNAI1	Snail family zinc finger 1	−2.149	−1.672
28	TECPR1	Tectonic beta-propeller repeat containing 1	−1.027	−1.240
29	THEM5	Thioesterase superfamily member 5	−1.476	−1.306
30	TNFSF11	Tumor necrosis factor superfamily member 11	−2.000	−3.000
31	YEATS2	YEATS domain containing 2	−1.631	−1.304

volunteers were evaluated by determining the DEGs, and 31 gene expression signatures commonly shared between PS and RA were identified. Based on these shared DEGs, the pathogenesis common to both PS and RA was elucidated at the transcriptome level. The discoveries of this study suggest that the common mechanism of PS and RA mainly involves inflammation and an abnormal immune response characterized by a cytokine imbalance. Specifically, the identified highly linked molecules, significant signaling pathways, and upstream regulators were directly or indirectly associated with the regulation of a variety of cytokines. As shown in Figure 7, certain key molecules (ERK1/2, CSF3, FOS, IFNG, and TNF) and significant signaling pathways (the complement system, antigen presentation, macropinocytosis signaling, acute phase response signaling, NF-κB signaling, IL-6 signaling, IL-17 signaling, and p38 MAPK signaling) were associated with an imbalance of cytokines; this imbalance may provide new clues for a better understanding of PS and RA.

The complement system is an essential component of innate immunity, and it plays an important role in modulating adaptive immunity. Its activation contributes to the pathogenesis of autoimmune and inflammatory diseases, such as PS and RA [21]. Reduction of complement activation is one of the mechanisms by which TNF-α inhibitors exert their effectiveness in these two diseases [22]. In this study, the upregulation of three DEGs (C4BPA, C1QB, and C4BPA) implied that the complement system was activated, which was consistent with previous studies. This study further confirmed that the complement system is indeed an attractive therapeutic target for both PS and RA. Professional antigen-presenting cells, such as dendritic cells (DCs), macrophages, and B cells, play a key role in triggering and/or maintaining the chronic inflammatory process in RA [23]. Increasing evidence indicates that RA treatment may occur through the manipulation of antigen presentation [24]. This study found that the activated antigen presentation pathway is characterized by upregulation of two DEGs, HLA-DQA2 and

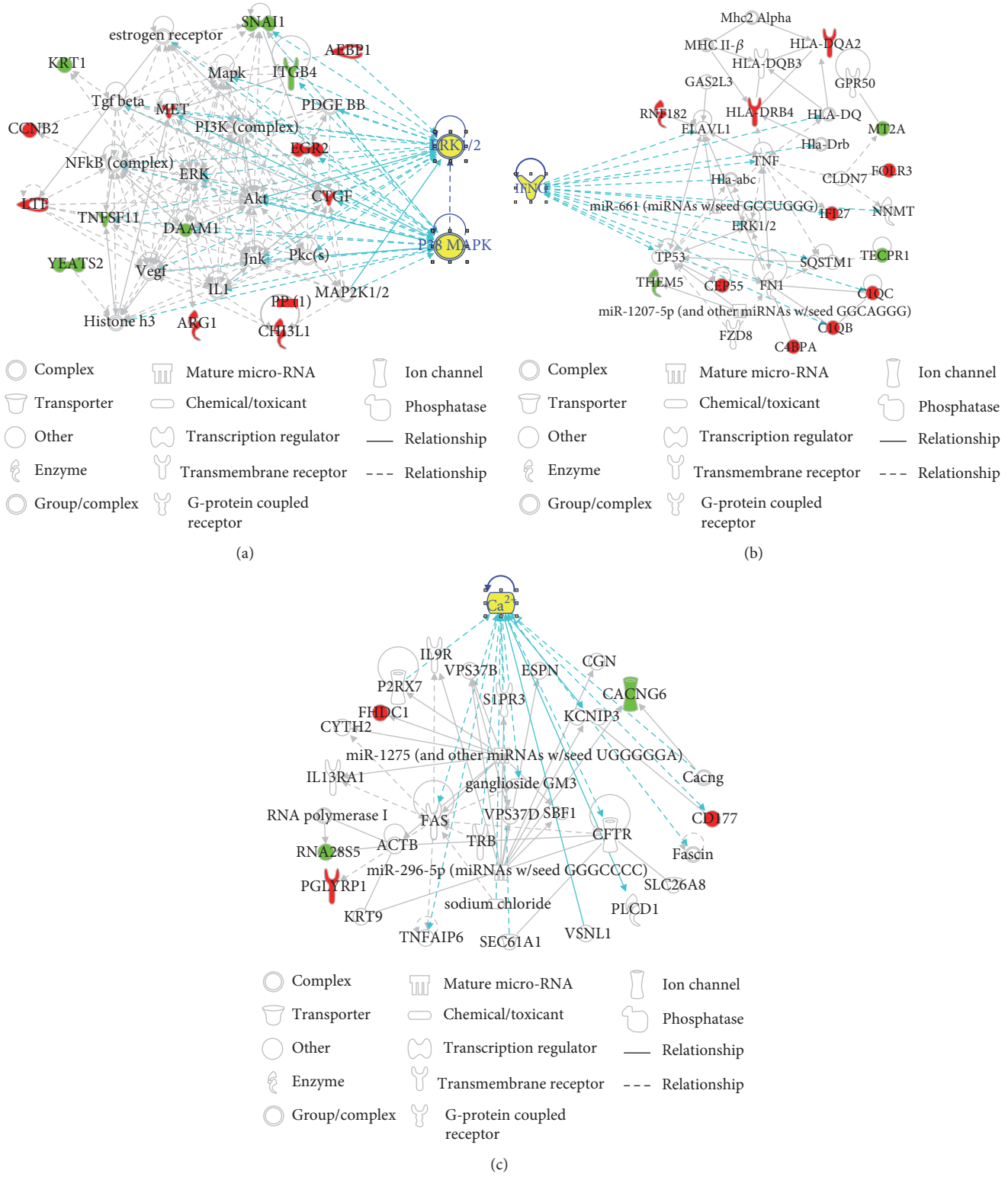
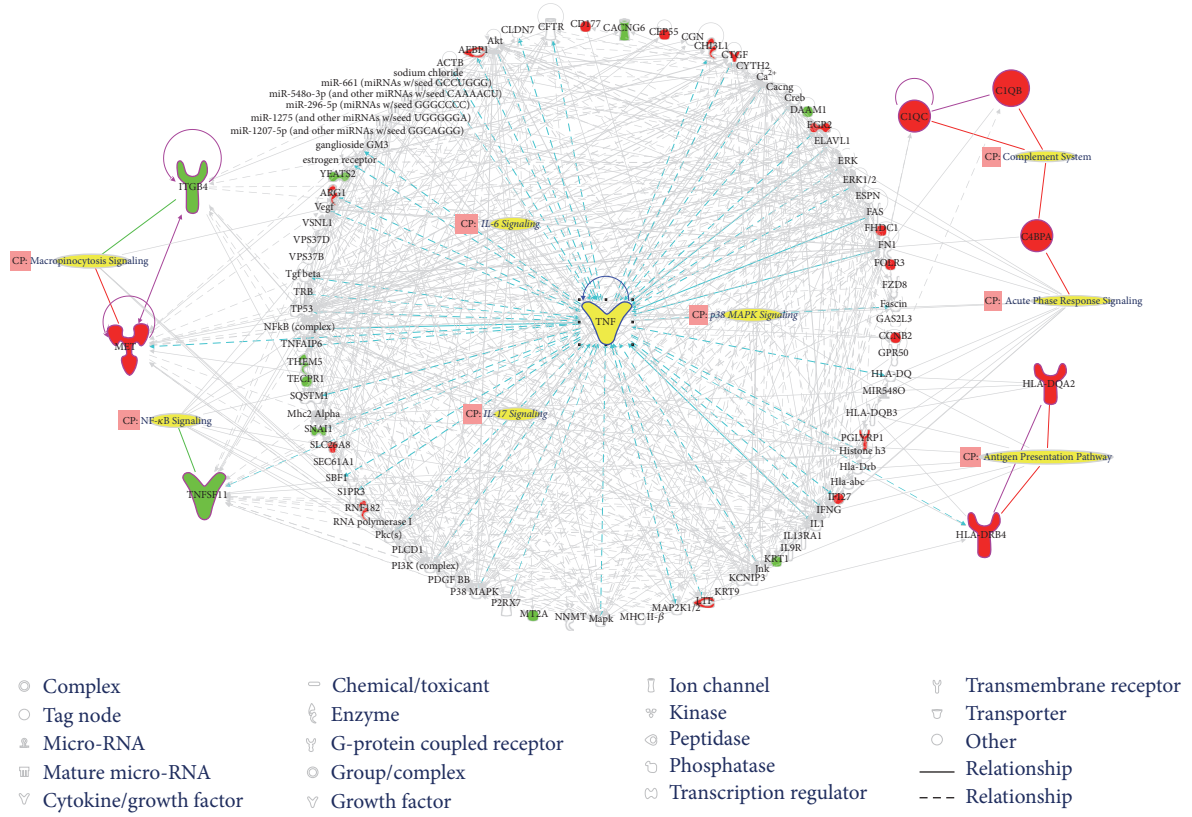


Figure 3: Biomolecular networks related to the shared DEGs. In each network, molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. Red symbols represent upregulated DEGs; green symbols represent downregulated DEGs. Yellow symbols indicate the highly linked molecules from the Ingenuity Knowledge Database. Solid lines between molecules indicate a direct physical relationship between molecules, whereas dash lines represent indirect functional relationships. (a) The first network. (b) The second network. (c) The third network.



Complex
Tag node
Micro-RNA
Mature micro-RNA
Cytokine/growth factor
Chemical/toxicant
Enzyme
G-protein coupled receptor
Group/complex
Growth factor
Ion channel
Kinase
Peptidase
Phosphatase
Transcription regulator
Transmembrane receptor
Transporter
Other
Relationship
Relationship

FIGURE 4: The most significantly relevant pathways and highly linked molecules relevant to the merged bionetwork. In the network, molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. Red symbols represent upregulated DEGs; green symbols represent downregulated DEGs. Yellow symbols indicate the highly linked molecules and signaling pathways from the Ingenuity Knowledge Database. "CP" is an abbreviation of "canonical pathway," which represents signaling pathways related to the merged bionetwork. Solid lines between molecules show a direct physical relationship between molecules, whereas dash lines show indirect functional relationships.

HLA-DRB4, and this upregulation is a common mechanism of PS and RA. Therefore, targeting antigen presentation may also be a new strategy for PS treatment. Macropinocytosis represents a distinct pathway of endocytosis in mammalian cells, and it significantly contributes to antigen presentation by the immune system. A study revealed that the type II collagen in an RA mice model was taken up by DCs and macrophages predominantly via inhibition of micropinocytosis [25]. In addition, the nonapoptotic cell death associated with perturbations of micropinocytosis is one apoptosis mechanism caused by RA [26]. This study showed that micropinocytosis signaling is also involved in PS, which offers a new method to understand the pathogenesis of PS. The acute phase response serves as a core of the innate immune response, and proteins relevant to this response were closely correlated with the development of RA [27]. The results from this study suggest that the pathogenesis of both PS and RA might partially result from the perturbation of the acute phase response. Regarding previous studies, the four signaling pathways discussed above are involved in the metabolism and regulation of cytokines. Specifically, when the complement system is stimulated by certain triggers, proteases in the system cleave specific proteins to release cytokines [28]. Antigen presentation plays an important role in cytokine production in PS and RA [29]. Cytokines, such as IFNγ and IL-17A, regulate macropinocytosis in macrophages [30]. Cytokine levels (IL-6 and IFNγ) mediate the acute phase response [31]. In short, perturbation of these pathways affected particular cytokines, which potentially partially reflects the pathogenesis of PS and RA.

IL-6 is a proinflammatory cytokine that induces activation of Th cells and controls the balance between Treg cells and Th17 cells. In lesional psoriatic skin, IL-6 is markedly elevated, and T lymphocytes encounter high IL-6 levels, thus allowing cutaneous T cells to avoid Treg suppression and increasing the Th17 inflammatory activity [32]. Targeting IL-6 signaling in PS may rebalance Treg/Th17 activity and ameliorate the disease [33]. IL-6 also stimulates the inflammatory and autoimmune processes in RA, and both deregulation of IL-6 production and blockade of IL-6 signaling are effective strategies in treating experimental models of RA [34]. IL-17 is the signature cytokine secreted by Th17 cells. IL-17 is particularly important in PS due to its proinflammatory effects and its involvement in an integrated inflammatory loop with DCs and keratinocytes, contributing to an overproduction of inflammatory cytokines that leads to amplification

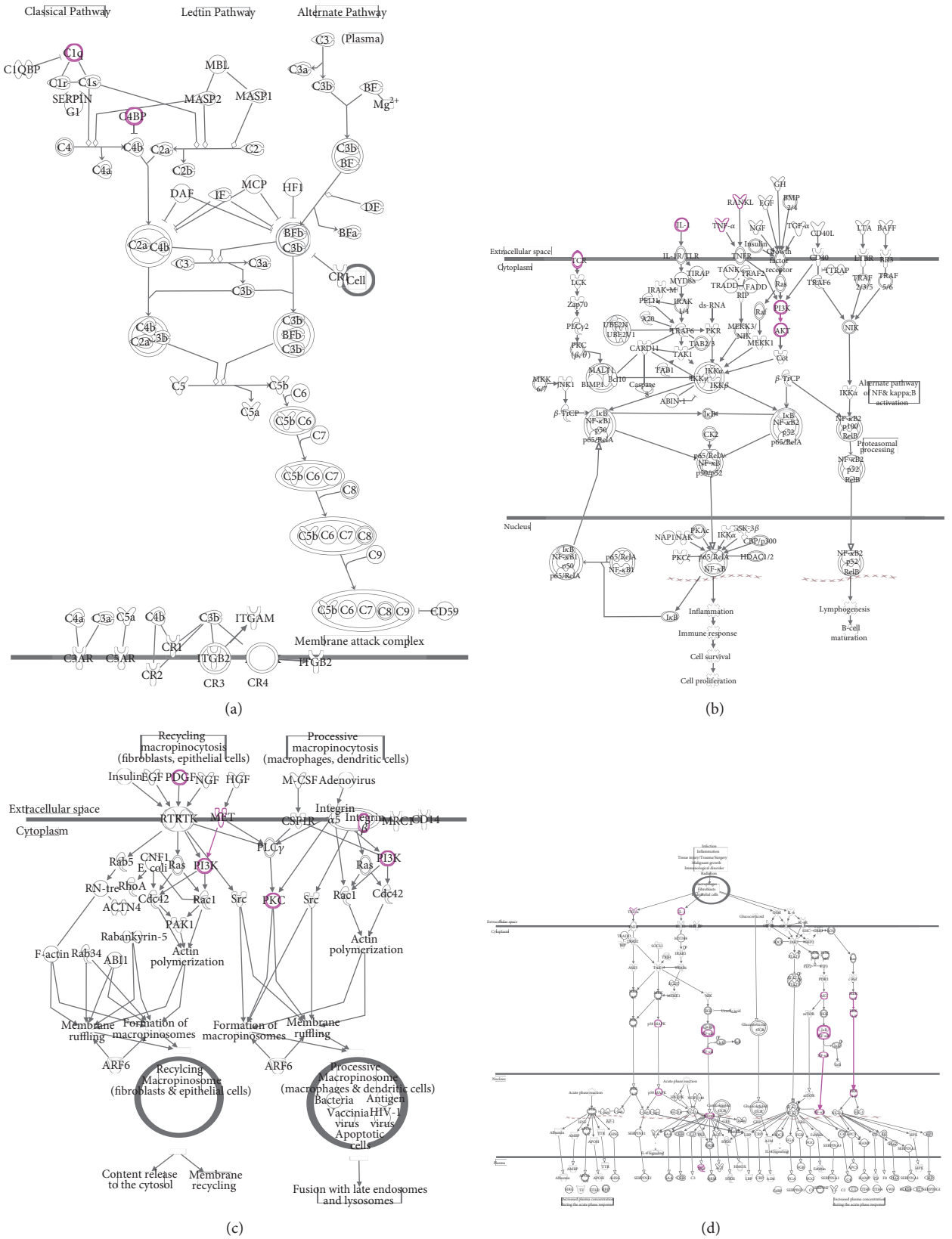
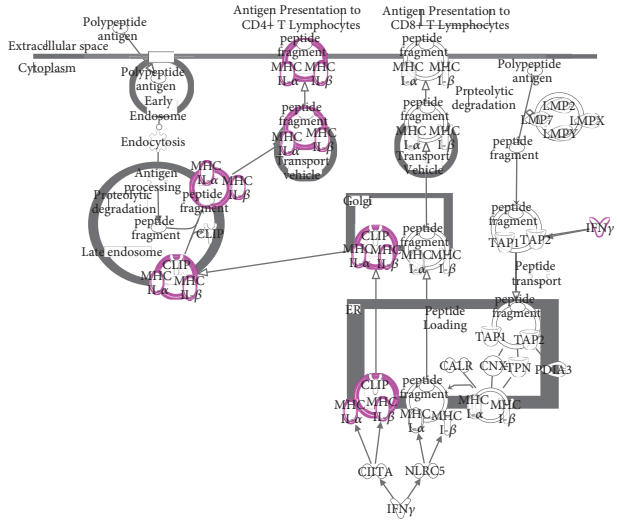


Figure 5: Continued.



(e)

Figure 5: Top five pathways. Purple symbols represent the shared DEGs. (a) Complement system. (b) NF-κB signaling. (c) Macropinocytosis signaling. (d) Acute phase response signaling. (e) Antigen presentation.

of the immune response [35, 36]. A study demonstrated that effective treatment of PS with TNF inhibitors was associated with suppression of IL-17 signaling [37]. Similarly, the therapeutic strategy of IL-17 signaling inhibition was also used to treat RA [38]. TNF, formerly known as TNF-α, is the best-known member of TNF superfamily. As a cytokine, TNF stimulates cell proliferation and cell differentiation and plays a key role in the pathogenesis of PS and RA [39]. A lack of the TNF G allele is associated with reduced PS severity [40]. CSF3 acts as a cytokine and may be produced by the endothelium and by macrophages. A previous study confirmed that CSF3 is a typical IL-17A-regulated gene in the keratinocytes of PS [35]. IFNG encodes the IFNγ protein. IFNγ is a cytokine secreted by Th cells (specifically, Th1 cells) and is an important activator of macrophages. IFNγ has been implicated in the initiation/maintenance of inflammation. A study showed that the pathogenesis of RA was correlated with reduced frequencies of IFNγ producers [41]. This study found that these particular cytokines, such as IL-6, IL-17, TNF, CSF3, and IFNγ, as well as the corresponding signaling pathways are involved in the pathogenesis of PS and RA, which not only verified previous discoveries but also reflected the importance of these cytokines. In brief, an imbalance in these cytokines plays a crucial role in the pathogenesis of PS and RA, and targeting these cytokines is a key strategy for prevention and treatment.

NF-κB is essential for the expression of proinflammatory cytokines and controls a number of essential cellular functions, including the immune response, cell proliferation, and apoptosis. Loss of normal NF-κB signaling regulation is a major contributor to a variety of inflammatory and autoimmune diseases, such as PS and RA [42, 43]. Activator protein-1 (AP-1) is recognized as a regulator of the expression of cytokines, such as CSF3, IL-6, and TNF, and is causally involved in PS and RA [44]. The immediate early gene product Fos is part of the AP-1 transcription factor, and its deregulation is associated with a variety of immunological defects. Selective inhibition of Fos function demonstrated that targeting Fos/AP-1 activity could be an promising new option for arthritis treatment [45]. ERK1/2 and p38 MAPK are important members of the MAPK family, which are responsive to inflammatory cytokines, and they are involved in cell differentiation, apoptosis, and autophagy. ERK1/2 is phosphorylated and activated via cell surface receptors stimulated by cytokines. Emerging data suggest that cytokine expression in response to p38 MAPK and ERK1/2 activation is involved in the etiopathogenesis of PS and that p38 MAPK signaling is an indicator of the loss of keratinocyte cell-cell adhesion in PS [46, 47]. IL-6, NF-κB, and p38 MAPK signaling activation is an important characteristic of the inflammatory response in activated macrophages in RA, and p38 MAPK signaling is involved in the process of RA angiogenesis [48]. In addition, suppressing the expression of TNF-α and IL-6 through inhibiting the activation of NF-κB and ERK1/2 is an important strategy for treating RA [49]. This study indicated that four biomolecules, NF-κB, Fos, p38 MAPK, and ERK1/2, are highly linked molecules or upstream regulators that are closely associated with the shared DEGs in PS and RA and therefore regulated cytokines; these four biomolecules represent novel targets to prevent and treat these two diseases.

5. Conclusions

The common pathogenesis of PS and RA was characterized by a cytokine imbalance. The deregulation of certain key molecules, such as ERK1/2, CSF3, FOS, IFNG, and TNF, as well as the perturbation of signaling pathways, including the complement system, antigen presentation, macropinocytosis signaling, NF-κB signaling, IL-6 signaling, IL-17 signaling,

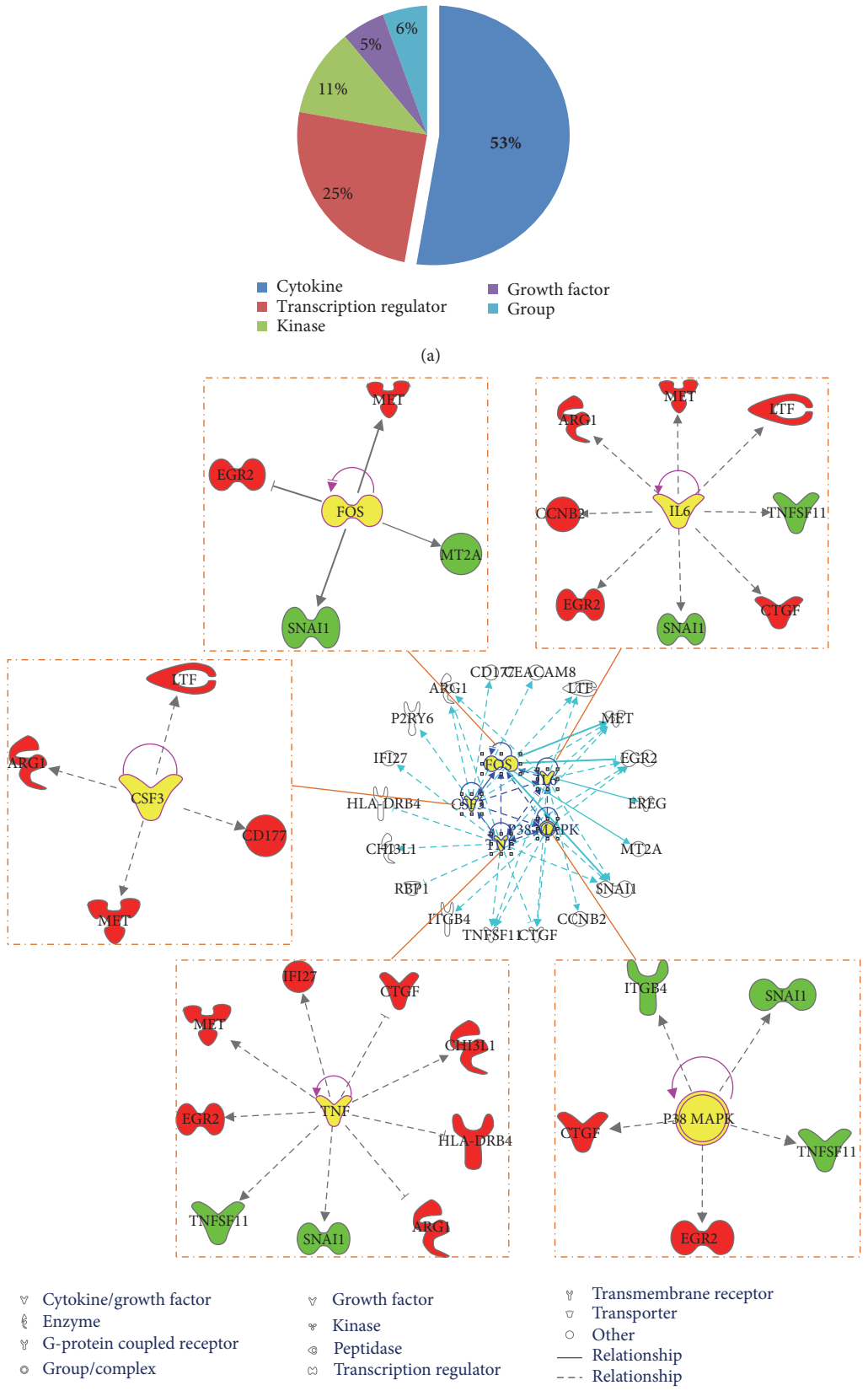


Figure 6: Upstream regulators relevant to the shared to DEGs. (a) The percentage of molecule type of upstream regulator. (b) Top five upstream regulators. Red nodes represent upregulated DEGs; green nodes represent downregulated DEGs. Yellow nodes represent the upstream regulators. Solid lines between molecules indicate a direct physical relationship between molecules, whereas dash lines indicate indirect functional relationships.

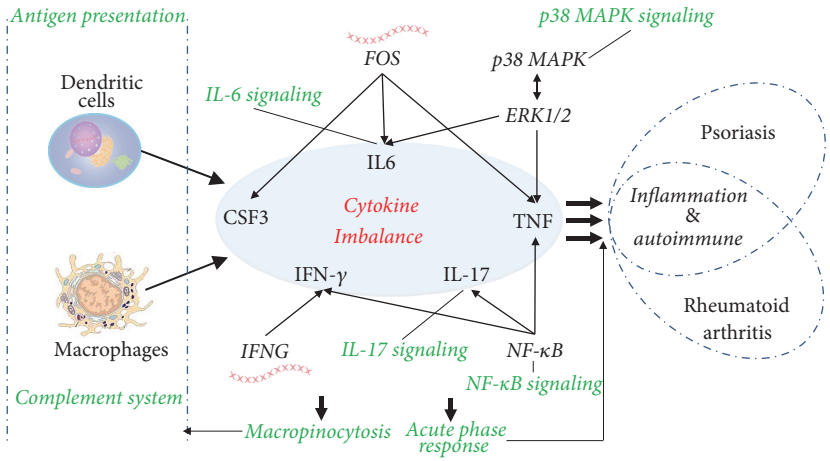


Figure 7: The common molecular mechanisms of PS and RA are characterized by a cytokine imbalance. Arrows represent the relationship between molecules or between molecules and cells. Green terms represent signaling pathways. The molecules in ovals are cytokines.

and p38 MAPK signaling, reflected this type of imbalance. The new findings in this study provided a new molecular understanding of PS and RA and could pave the road for the discovery of new strategies for treating PS and RA.

Abbreviations

PS:	Psoriasis
RA:	Rheumatoid arthritis
PBMCs:	Peripheral blood mononuclear cells
PV:	Psoriasis vulgaris
PASI:	Psoriasis area severe index
BSA:	Body surface area
DAS28:	Disease activity score in 28 joints
BMI:	Body mass index
ESR:	Erythrocyte sedimentation rate
CRP:	C-reactive protein
RF:	Rheumatoid factor
WBC:	White blood cell
HGB:	Hemoglobin
PLT:	Platelets
IL:	Interleukin
Th:	T helper
Treg:	Regulatory T cells
TNF:	Tumor necrosis factor
CSF3:	Colony-stimulating factor 3
IFNγ:	Interferon gamma
IFNG:	Interferon gene
NF-κB:	Nuclear factor-kappa B
MAPKs:	Mitogen-activated protein kinases
ERK1/2:	Extracellular signal-regulated kinase 1/2
NGS:	Next-generation sequencing.

Competing Interests

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

Authors' Contributions

Cheng Xiao, Aiping Lu, and Youwen Zhou conceived and designed the experiments. Cheng Lu, Xuyan Niu, Yanping Bai, and Cheng Xiao performed the experiments. Yong Tan, Xuyan Niu, Chunyan Jiang, and Yang Wang analyzed the data. Aiping Lu and Cheng Xiao contributed reagents/materials/analysis tools. Yong Tan, Qiu Qi, and Cheng Lu wrote the paper. Yong Tan, Qiu Qi, and Cheng Lu contributed equally to this work.

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Research Article

Gαq Regulates the Development of Rheumatoid Arthritis by Modulating Th1 Differentiation

Dashan Wang,[1] Yuan Liu,[2] Yan Li,[2] Yan He,[2] Jiyun Zhang,[3] and Guixiu Shi[2]

[1]*Molecular Biology Research Center, Key Medical Health Laboratory for Laboratory Medicine of Shandong Province, Department of Laboratory Medicine, Shandong Medical College, Linyi, Shandong 276000, China*
[2]*Department of Rheumatology and Clinical Immunology, The First Affiliated Hospital of Xiamen University, Xiamen, Fujian 361003, China*
[3]*Key Medical Health Laboratory for Laboratory Medicine of Shandong Province, Department of Laboratory Medicine, Shandong Medical College, Linyi, Shandong 276000, China*

Correspondence should be addressed to Guixiu Shi; gshi@xmu.edu.cn

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The Gαq-containing G protein, an important member of $G_{q/11}$ class, is ubiquitously expressed in mammalian cells. Gαq has been found to play an important role in immune regulation and development of autoimmune disease such as rheumatoid arthritis (RA). However, how Gαq participates in the pathogenesis of RA is still not fully understood. In the present study, we aimed to find out whether Gαq controls RA via regulation of Th1 differentiation. We observed that the expression of Gαq was negatively correlated with the expression of signature Th1 cytokine (IFN-γ) in RA patients, which suggests a negative role of Gαq in differentiation of Th1 cells. By using Gαq knockout (*Gnaq*−/−) mice, we demonstrated that loss of Gαq led to enhanced Th1 cell differentiation. Gαq negative regulated the differentiation of Th1 cell by modulating the expression of T-bet and the activity of STAT4. Furthermore, we detected the increased ratio of Th1 cells in *Gnaq*−/− bone marrow (BM) chimeras spontaneously developing inflammatory arthritis. In conclusion, results presented in the study demonstrate that loss of Gαq promotes the differentiation of Th1 cells and contributes to the pathogenesis of RA.

1. Introduction

Guanine nucleotide-binding proteins (G proteins) are the most widely used signal transducers in mammalian cells. G proteins transmit signals from ligand activated G protein coupled receptors (GPCRs) to effector proteins and then regulate many biological functions [1]. GPCR ligands include numerous hormones, neurotransmitters, peptides, small proteins, and lipid molecules. Accordingly, biological functions mediated by G proteins and GPCRs are diverse, including behavioral, sensory functions, appetite control, metabolism, development, inflammation, and chemotaxis [2]. The heterotrimeric G proteins are composed of α-subunit that binds and hydrolyzes GTP, as well as β- and γ-subunits that form a functional complex. The α-subunits of G proteins are highly specialized and induce many different downstream signals. Based on the sequence similarity of their α-subunits, G proteins can be classified into four subfamilies: Gi, Gs, Gq/11, and G12/13. Gαq, which is the α-subunit Gq protein, is encoded by *GNAQ* [3]. The Gαq containing G protein initially attracted our attention for its important role in cardiovascular system in 1990s [4]. In recent years, studies have also demonstrated that Gαq are involved in immune regulation and autoimmune disease. Our previous studies reported that Gαq regulated migration of dendritic cells and survival of B cells and T cells [5–7]. More important, *Gnaq*−/− bone marrow (BM) chimeras with Gαq deficiency only in their immune system could spontaneously develop symptoms of arthritis similar to RA [6]. We also reported that the protein and mRNA expression levels of Gαq in peripheral blood lymphocytes (PBLs) of rheumatoid arthritis (RA) patients were significantly lower compared with healthy

Table 1: Demographic and clinical characteristics of the patients with rheumatoid arthritis (RA) and healthy control subjects.

	RA patients ($n = 30$)	Healthy controls ($n = 30$)
Age, mean (range) years	46.1 (33–75)	45.5 (28–64)
Male : female	5 : 25	8 : 22
C-reactive protein (mg/L)	15.9 (1.1–90.6)	—
Rheumatoid factor (IU/mL)	192.0 (6–943)	—
DAS28* mean (range) score	2.99 (1.12–5.07)	—

*DAS28 = 28 joints' disease activity score.

controls [8]. These results indicated a critical role of Gαq in the pathogenesis of RA.

Rheumatoid arthritis (RA) is the most common systemic autoimmune disease characterized by chronic inflammation of joint synovial tissue and subsequent destruction of associated bone, cartilage, and soft tissues [3]. Although the etiology of RA is still not fully understood, T cells are thought to play a central role in joint inflammation and disease progression [9, 10]. Among of them, T helper 1 (Th1) cells have been found to play an important role in RA in several studies. Th1 cells infiltrate the synovium, secret proinflammatory cytokines, and promote macrophage and neutrophil infiltration [11–13]. Our previous study indicated that Gαq is associated with RA. However, how Gαq is involved in the initiation and development of RA is not fully studied. In the current study, we will study the role of Gαq in Th1 cell differentiation and RA. First, we studied the relationship between Gαq and hallmark Th1 cytokine (IFN-γ) in RA patients. Then, we investigated the role of Gαq in Th1 differentiation and inflammatory arthritis by using *Gnaq*−/− mice. We found that Gαq was negatively associated with signature Th1 cytokine (IFN-γ) in RA patients, which suggested that Gαq might be involved in Th1 cells differentiation. In consistent with the result we observed in RA patients, the percentage of Th1 cells was significantly increased in *Gnaq*−/− BM chimeras which spontaneously developed inflammatory arthritis. Moreover, our results showed that the deficiency of Gαq heightened the differentiation of Th1 cells via T-bet and STAT4 by using *Gnaq*−/− mice. Here we show that Gαq might be involved in Th1 cells differentiation and further participate in the pathogenesis of RA.

2. Material and Methods

2.1. Patients. A total of 30 RA patients fulfilling the American College of Rheumatology 1987 revised criteria [14] were recruited from the outpatient clinic of the Department of Rheumatology and Clinical Immunology, The First Affiliated Hospital of Xiamen University. 30 sex-matched and age-matched healthy volunteers were recruited as healthy controls. The study was done after obtaining written informed consent of patients and approval of the Ethics Committee of The First Affiliated Hospital of Xiamen University. The demographic and clinical features of healthy controls and patients with RA were summarized in Table 1.

2.2. Animals. C57BL/6J and *Gnaq*−/− mice ($N > 5$ backcrossed to C57BL/6J) were maintained in a pathogen-free animal facility of Xiamen University and used between 6 and 8 weeks of age. *Gnaq*−/− mice are difficult to study as they are born runted and exhibit motor defects. In order to analyze the role of Gαq in development of arthritis, we generated BM chimeric mice. BM chimeras were generated by irradiating recipient mice with a split dose of 800 Rads and then reconstituting the recipients with 1×10^6 BM cells from C57BL/6 or *Gnaq*−/− donors. All experimental procedures involving mice were approved by the Animal Care and Use Committee of Xiamen University.

2.3. Blood Samples. Peripheral blood samples from RA patients and healthy volunteers were collected into collection tubes containing 0.2 mL sodium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by standard density-gradient centrifugation using Ficoll-Paque Plus (Axis-Shied).

2.4. T Cell Purification and In Vitro Th1 Cells Induction. Spleens from WT and *Gnaq*−/− mice were passed through a fine nylon mesh to obtain single cell suspension. Red blood cells (RBCs) were lysed using ACK lysis buffer. $CD4^+CD62L^+$ cells were isolated using a negative selection step and a positive selection step. First, $CD4^+$ T cells were purified using negative selection with biotinylated Abs against B220, MHC-II, CD8, CD49b, CD11c, and CD11b (eBiosciences); second, $CD4^+CD62L^+$ cells were isolated and then by positive selection using biotinylated Abs against CD62L (eBioscience), followed by streptavidin conjugated magnetic beads (Miltenyi) [15]. Purity, as assessed by flow cytometry, was >92%. Purified naïve $CD4^+$ T cells were stimulated with precoated anti-CD3/CD28 (3 μg/mL) for 5 days, with mouse IL-12 (20 ng/mL), mouse IL-2 (20 ng/mL) (PeproTech), and anti-IL-4 (10 μg/mL) (eBioscience) added to the cultures [16].

2.5. Flow Cytometry and Intracellular Staining. Cells were collected and stimulated with PMA (25 ng/mL) and ionomycin (1 μg/mL) in the presence of 1 μg/mL monesin (Sigma-Aldrich) for 4 hours. Cells were then fixed, permeabilized by using Fixation/Permeabilization Solution Kit (eBioscience) according to the manufacturer's instructions, and stained with FITC-conjugated anti-IFN-γ, PE-conjugated anti-phospho-STAT4, or PE-cy7-conjugated anti-T-bet (eBioscience). In order to stain Th1 cells in mice, single cell suspension was deprived from spleen of WT and *Gnaq*−/− chimeric mice, stimulated with PMA, ionomycin, and monensin for 4 hour. After culture, cells were stained with PE-conjugated anti-CD4, followed by intracellular staining with FITC-conjugated anti-IFN-γ. Cells were analyzed on Cytomic FC500 (Beckman Coulter), and data were analyzed using FlowJo (Tree Star).

2.6. Real-Time PCR Analysis. Total RNA was isolated from PBMCs using TRIzol (Invitrogen). Complementary DNA was synthesized using reverse transcription reagent kits according to manufacturer's instructions (Bio-Rad). The

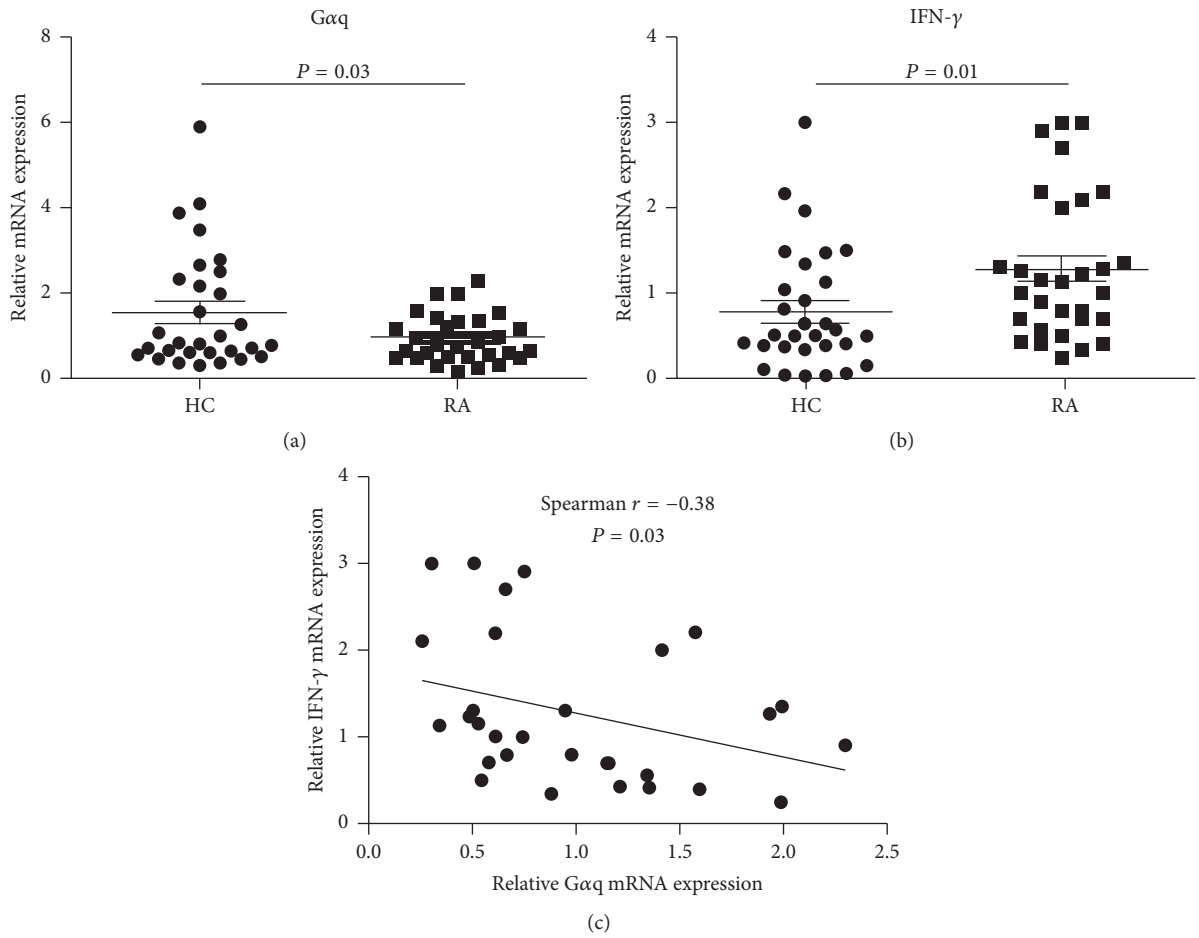

Figure 1: Correlation of mRNA expression level of Gαq and IFN-γ. The mRNA expression of Gαq and IFN-γ was detected by real-time PCR. Relative (a) Gαq and (b) IFN-γ mRNA expression in PBMCs from patients with rheumatoid arthritis (RA; $n = 30$) and healthy controls (HC; $n = 30$). Bars show the mean and standard deviation (SD). P value was determined by Mann–Whitney test. (c) The correlation between Gαq mRNA expression level and IFN-γ mRNA expression level in RA patients ($n = 30$) was determined by using Spearman test.

expression levels of IFN-γ and Gαq were determined by real-time quantitative PCR. A 10 μL SsoFast EvaGreen PCR reaction system was used. It includes 2 μL of cDNA, 2.6 μL ddH2O, 0.2 μL of sense primer, 0.2 μL of antisense primer, and 5 μL SsoFast EvaGreen Supermix (Bio-Rad). The PCR reaction conditions were as follows: 95°C for 1 min, then 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Reactions were performed with iQ™5 real-time PCR Detection Systems (Bio-Rad). Target gene expressions were normalized to GAPDH and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: *GNAQ*, 5′-GTTGATGTGGAGAAGGTGTCTG-3′ and 5′-GTAGGCAGGTAGGCAGGGT-3′; *IFNG*, 5′-GATGACTTCGAAAAGCTGACTAATTATTC-3′ and 5′-GTTCAGCCATCACACTTGGATGAG-3′ *GAPDH*, 5′-GTGAACCATGAGAAGTATGACAAC-3′ and 5′-CATGAGTCCTTCCACGATACC-3′.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). The concentration of mouse IFN-γ was detected using commercially available ELISA kits according to the manufacturer's instructions (BioLegend). Absorbance was measured with an ELISA microplate reader at 450 nm.

2.8. Statistical Analysis. Data were analyzed with Prism 5.01 software (GraphPad Software). Statistical differences between WT and *Gnaq*−/− groups were determined by Student's t-test. Statistical differences between healthy volunteers and RA were determined by Mann–Whitney U test. The correlation between Gαq and IFN-γ was analyzed using Spearman test. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Gαq and IFN-γ Were Negatively Correlated in RA Patients. A paper of our group has reported that expression levels of Gαq were significantly decreased in RA patients and negatively correlated with disease activity [8]. Our previous results also showed that *Gnaq*−/− BM chimeric mice spontaneously developed inflammatory arthritis, indicating that Gαq might be involved in development of RA [6]. Th1 cell is recognized as a main effector cell in RA progression [17, 18];

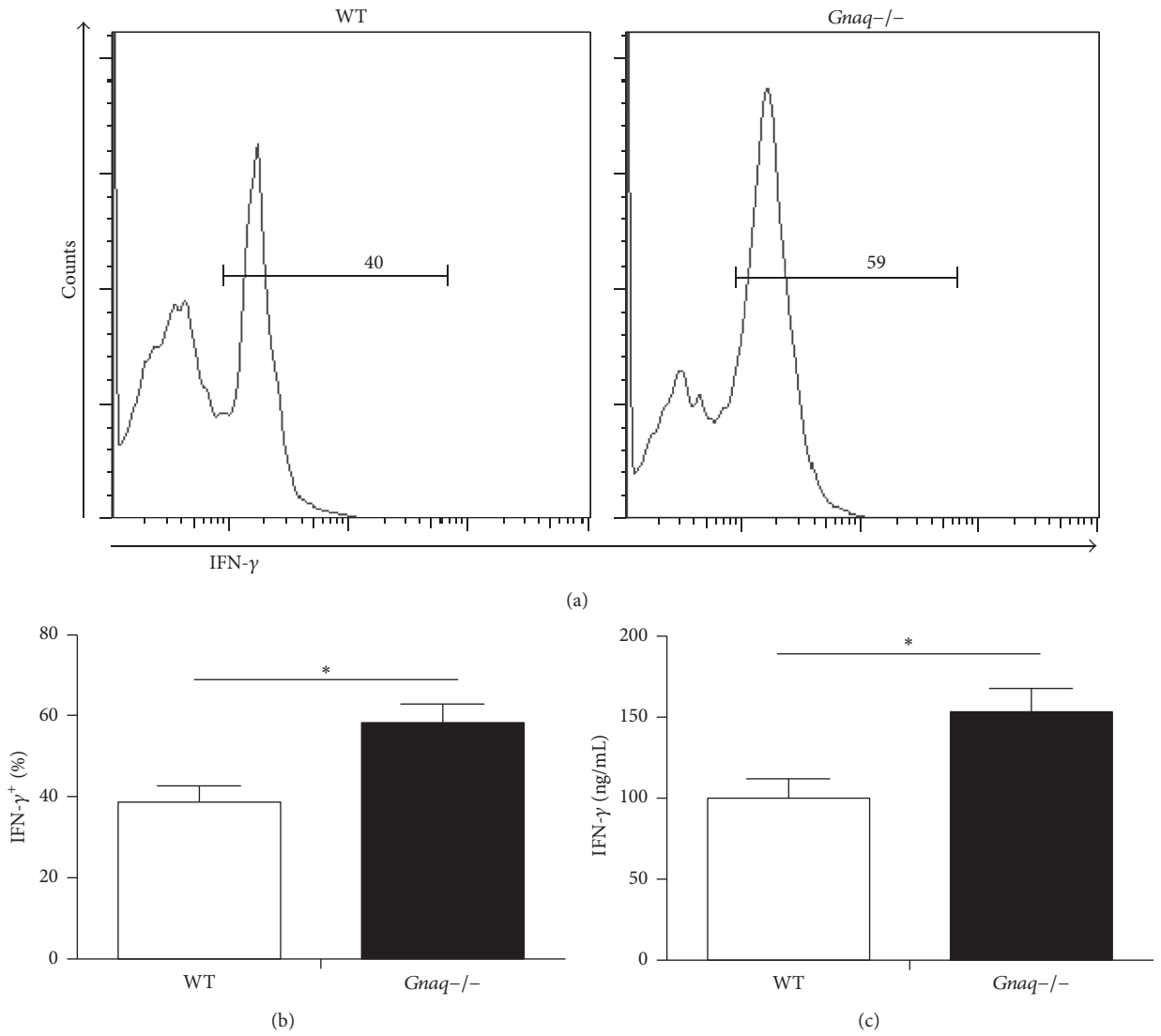

Figure 2: Loss of Gαq enhances the differentiation of Th1 cells. Purified naïve $CD4^+$ T cells from WT and *Gnaq*−/− mice were stimulated with anti-CD3/CD28 (3 μg/mL), in the presence of mouse IL-12 (20 ng/mL), mouse IL-2 (20 ng/mL), and anti-IL-4 (10 μg/mL) for five days. Cells were harvested and analyzed. (a) WT and *Gnaq*−/− $CD4^+$ T cells were stimulated with PMA, ionomycin, and monensin, fixed, permeabilized, and stained with FITC-conjugated anti-IFN-γ, followed by flow cytometry. (b) The percentage of IFN-γ^+ cells was calculated. (c) IFN-γ secretion was detected by ELISA. Cultured $CD4^+$ T cells were harvested, adjusted to same concentration, and stimulated by anti-CD3/CD28 (1 μg/mL) for 24 hours. Supernatants were collected for ELISA assay. All data are presented as mean ± SD; $^*P < 0.05$, $n = 3$. The result is representative of three independent experiments.

thus, whether Gαq can regulate Th1 cell response and further participates in development of RA attracts our interests. We first investigated the association of Gαq and hallmark Th1 cytokine (IFN-γ) in RA patients. We detected Gαq and IFN-γ mRNA expression in PBMCs from 30 RA patients and 30 healthy controls by real-time PCR. Results showed that Gαq mRNA expression was significantly decreased and IFN-γ mRNA expression was significantly increased in RA patients compared to healthy controls (Figures 1(a) and 1(b)). Moreover, we found a negative correlation between expression level of Gαq and IFN-γ (Figure 1(c)). These data demonstrate that Gαq was negatively associated with signature Th1 cytokine (IFN-γ) in RA patients.

3.2. Loss of Gαq Enhanced the Differentiation of Th1 Cells. The result presented above encouraged us to study whether Gαq regulates Th1 cell differentiation. In order to address this question, we used Gαq knockout (*Gnaq*−/−) mice. Naïve $CD4^+$ T cells were purified from spleen of *Gnaq*−/− and WT mice and incubated under Th1 differentiation condition. After 5-day culture, cells were harvested and analyzed by flow cytometry (Figure 2(a)). Intracellular staining showed higher frequency of IFN-γ+ cells in *Gnaq*−/− $CD4^+$ T cell than WT group (Figure 2(b)). We also measured the level of IFN-γ in supernatant by ELISA. Cultured $CD4^+$ T cells were harvested, adjusted to same concentration, and stimulated by anti-CD3/CD28 (1 μg/mL) for 24 hours. Supernatants were

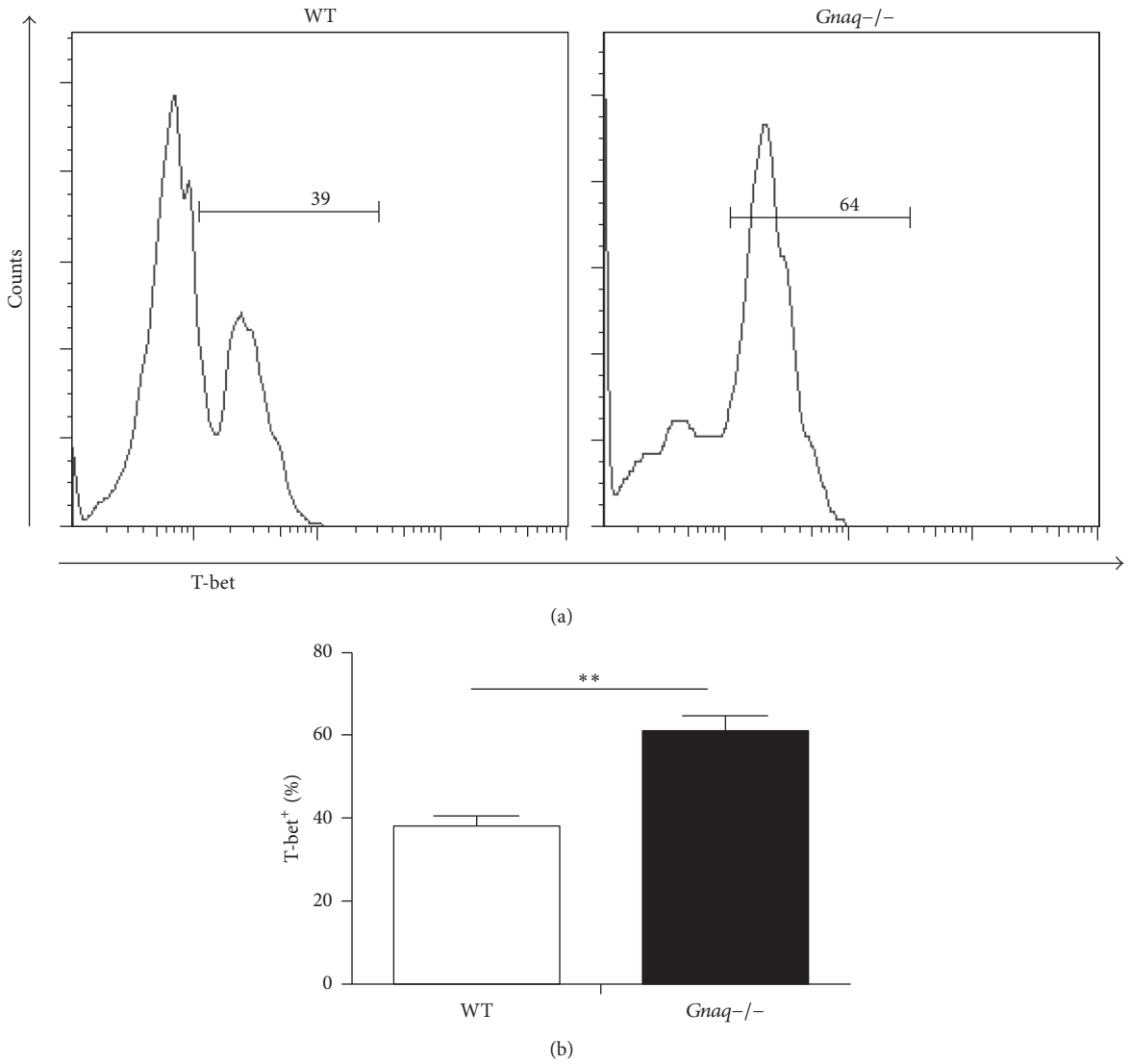

Figure 3: Loss of Gαq enhances the expression of T-bet. (a) Purified naïve $CD4^+$ T cells from WT and *Gnaq*−/− mice were stimulated with anti-CD3/CD28 (3 μg/mL), in the presence of mouse IL-12 (20 ng/mL), mouse IL-2 (20 ng/mL), and anti-IL-4 (10 μg/mL) for five days. Cells were harvested, fixed, permeabilized, and stained with PE-cy7-conjugated anti-T-bet and analyzed by flow cytometry. (b) The percentage of $T\text{-bet}^+$ cells was calculated. All data are presented as mean ± SD; $^{**}P < 0.05$, $n = 3$. The result is representative of three independent experiments.

collected and cytokine concentrations were measured by ELISA assay. Result demonstrated that secretion level of IFN-γ was also much higher in *Gnaq*−/− $CD4^+$ T cell (Figure 2(c)). These results showed that Gαq regulates Th1 differentiation.

3.3. Absence of Gαq Heightened the Expression of T-Bet and p-STAT4 in $CD4^+$ T Cells. Results presented above identified a negative role of Gαq in Th1 differentiation. T-bet, a Th1-specific T box transcription factor that controls the expression of IFN-γ, is a critical regulator for Th1 cell differentiation [19]. To explore underlying mechanism of the regulation of Gαq in Th1 differentiation, we next detected the status of T-bet in WT and *Gnaq*−/− $CD4^+$ T cells under Th1 polarizing condition. After 5 days of induction, cells were harvested and expression of T-bet was analyzed by flow cytometry (Figure 3(a)). Result showed that expression level of T-bet was dramatically increased in *Gnaq*−/− $CD4^+$ T cells compared with WT $CD4^+$ T cells (Figure 3(b)). As STAT4 is also a critical factor in Th1 differentiation, we further measured the phosphorylation of STAT4 by flow cytometry. The level of phospho-STAT4 was obviously higher in *Gnaq*−/− $CD4^+$ T cells than WT controls (Figure 4). Therefore, results demonstrate that Gαq regulates Th1 cell differentiation by modulating T-bet and STAT4 in *Gnaq*−/− mice.

3.4. Percentage of Th1 Cells Was Increased in Gnaq−/− BM Chimeras Spontaneously Developing Arthritis. We have identified a negative correlation between Gαq and hallmark Th1 cytokine (IFN-γ) in RA patients and a negative role of Gαq

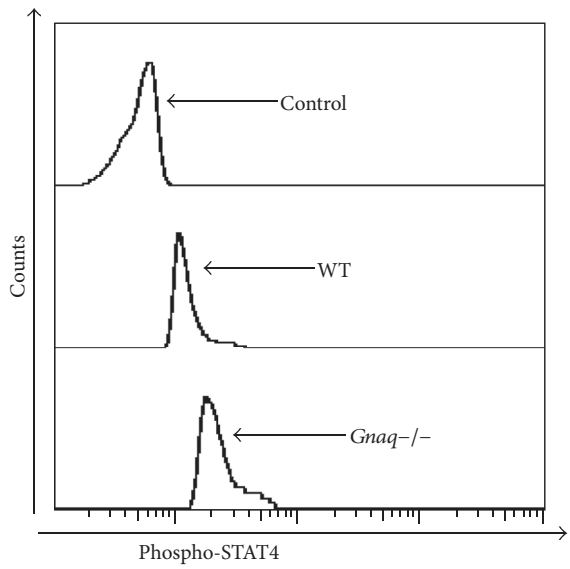

Figure 4: Loss of Gαq enhances the activation of STAT4. Purified naïve $CD4^+$ T cells from WT and *Gnaq*−/− mice were stimulated under Th1 induction condition for 10 minutes. Cells were harvested, fixed, permeabilized, and stained with PE-conjugated anti-p-STAT4 and analyzed by flow cytometry. Data are representative of three independent experiments with similar results.

in Th1 cell differentiation in *Gnaq*−/− mice. Meanwhile, our previous result has demonstrated that *Gnaq*−/− BM chimeric mice can develop symptoms of arthritis similar to RA. Based on these findings, we considered that it is important to determine whether homeostasis of Th1 cell is disturbed in *Gnaq*−/− mice. *Gnaq*−/− mice are difficult when used in the current study as they are born runted and exhibit motor defects. In order to analyze the role of Gαq in development of arthritis, we generated BM chimeric mice. As previous study, *Gnaq*−/− BM chimeras spontaneously developed symptom of inflammatory arthritis 3.5 months after BM reconstruction. We measured the percentage of Th1 cells in the spleen of autoimmune prone *Gnaq*−/− BM chimeras and WT BM chimeras 3.5 months after BM reconstruction by flow cytometry (Figure 5(a)). Single cell suspension was deprived from spleen of WT and *Gnaq*−/− BM chimeras, stimulated with PMA, monensin, and ionomycin for 4 hours. After culture, cells were stained with PE-conjugated anti-CD4, followed by intracellular staining with FITC-conjugated anti-IFN-γ. Results showed that the percentage of Th1 cells was significantly increased in *Gnaq*−/− BM chimeras compared with WT BM chimeras (Figure 5(b)). The spontaneously developed arthritis in *Gnaq*−/− BM chimeras might be partially attributed to increased Th1 response.

4. Discussion

In this study, we explored the role of Gαq in Th1 differentiation and development of RA. We found that expression level of Gαq and hallmark Th1 cytokine (IFN-γ) was negatively related in RA patients and percentage of Th1 cells was significantly increased in *Gnaq*−/− BM chimeras spontaneously developing arthritis. Furthermore, we demonstrated that Gαq negatively regulated Th1 differentiation by modulating T-bet and STAT4 in *Gnaq*−/− mice.

Gαq has been identified as an important factor in immune regulation in several studies. Moreover, we have showed that expression level of Gαq was significantly decreased in RA patients and loss of Gαq in mice leads to autoimmune arthritis [6, 8]. However, the exact mechanism of how Gαq is involved in the pathogenesis of RA has not been fully studied. RA is a chronic autoimmune disease characterized by joint synovial inflammation and destruction of the surrounding tissue [20]. Although it is still unclear how RA is initiated, T cells, particularly $CD4^+$ T helper (Th), are considered to be critical to initiation and maintenance of this disease by secreting proinflammation cytokines that regulate immune reactivity and synovial inflammation [21, 22]. The two $CD4^+$ Th subsets, Th1 cells which secrete IFN-γ as their hallmark cytokine and Th17 cells which secret IL-17, have been recognized as critical factors in the pathogenesis of RA [23]. Although the newly discovered Th17 cells have been proved to be critical in the pathogenesis of RA, the important role of Th1 cannot be dismissed. Numerous studies have demonstrated that Th1 cells play an important role in promoting inflammation in RA [24]. In our study, we found a negative correlation between expression level of Gαq and IFN-γ, which suggests that Gαq might negatively regulate Th1 differentiation in RA patients. A paper of our group reported that *Gnaq*−/− BM chimeras would exhibit manifestations of inflammatory arthritis 3.5 months after reconstitution. Besides, autoantibodies such as anti-nuclear Ab (ANA) and anti-double-stranded (ds) DNA Ab were significantly increased in *Gnaq*−/− BM chimeras [6]. The symptom of inflammatory arthritis in *Gnaq*−/− BM chimeras is similar to RA in humans. What is more, *Gnaq*+/− mice have been used as mice model of autoimmune disease in a recent paper [25]. In line with results we observed in RA patients, the percentage of Th1 cells was significantly increased in spleen of *Gnaq*−/− BM chimeras spontaneously developing inflammatory arthritis. Taken together, these observations indicated that Gαq negatively regulates Th1 differentiation and partially contributed to the pathogenesis of RA.

The induction and maintenance of each $CD4^+$ Th cell are mainly determined by cytokine environment at the time of naïve T cell activation [26]. Th1 cell polarization is usually induced in the presence of IL-12 and IFN-γ, which activate the expression of the master regulator transcription factors, such as T-bet and STAT4 (signal transducer and activator of transcription 4) [27]. T-bet can remodel the *Ifng* gene and promote IFN-γ expression [28]; besides it can upregulate IL-12Rβ2 expression [29], leading to enhanced Th1 cell expansion in response to IL-12. STAT4 is also an essential factor in regulation IL-12 signals and Th1 differentiation; the importance of this molecular in Th1 differentiation is demonstrated by $STAT4^{-/-}$ mice [30]. Both STAT4 and T-bet are needed for maximal IFN-γ production and while loss of either one will lead to disturbed IFN-γ production [31]. In this study, our results showed that expressions of p-STAT4 and T-bet were both upregulated in *Gnaq*−/− $CD4^+$ T cells.

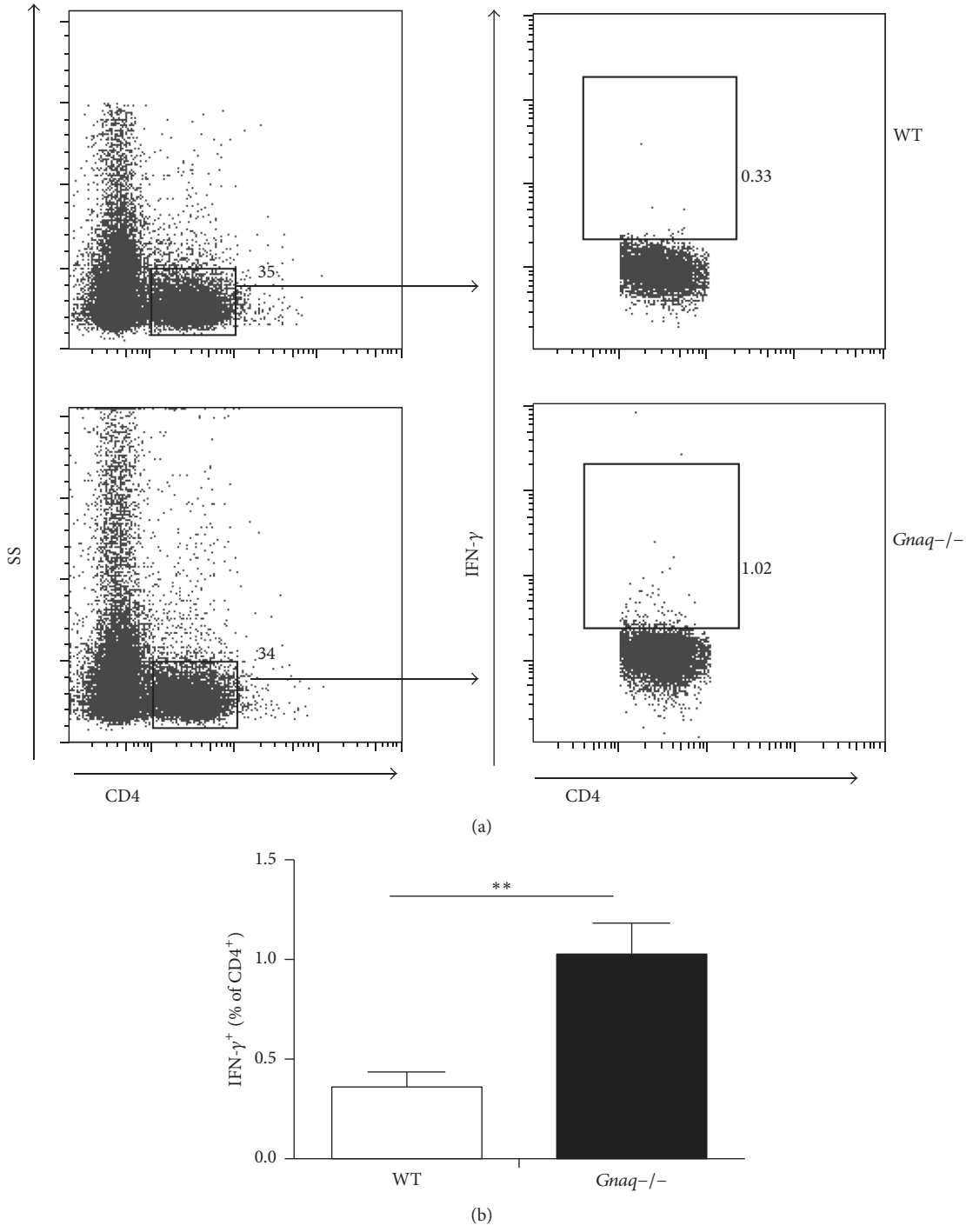

Figure 5: The percentage of Th1 cells is increased in *Gnaq*−/− BM chimeras suffering from inflammatory arthritis. (a) Single cell suspension was deprived from spleen of *Gnaq*−/− BM chimeras suffering from inflammatory arthritis and WT controls, stimulated with PMA, ionomycin, and monensin for 4 hours. After culture, cells were stained with PE-conjugated anti-CD4, followed by intracellular staining with FITC-conjugated anti-IFN-γ, and analyzed by flow cytometry. Gated on $CD4^+$ cells. (b) The percentage of Th1 cells is presented as mean ± SE, $^{**}P < 0.05$, $n = 3$. The result is representative of three independent experiments.

Gαq might regulate Th1 differentiation by modulating T-bet and STAT4. Nuclear factor of activated T cells (NFAT), a Ca^{2+} dependent transcription factor family, has been shown to be important in T cell differentiation. Sustained NFAT signaling promoted $CD4^+$ T cells differentiate to Th1 cells in Th1-skewing conditions [32]. Another study demonstrates that STAT4 enhanced Th1 differentiation IFN-γ expression by upregulating the binding of activator Protein-1 (AP-1) to the

IFN-γ promoter sequence [33]. Interestingly, one previous study reported that Gαq knockdown in T cells significantly increased NFAT and AP-1 activity [34]. These data suggest that Gαq might also regulate Th1 cell differentiation via NFAT/AP-1 signaling pathway.

5. Conclusions

Taken together, we demonstrated that Gαq inhibited the differentiation of Th1 cells and participated in pathogenesis of RA. Gαq might regulate Th1 cell differentiation via modulating activity of STAT-4 and T-bet. This study provides a new insight into the pathogenesis of RA and suggests a novel therapeutic target for autoimmune disease.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Dashan Wang and Yuan Liu contributed equally to this work.

Acknowledgments

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Research Article

Morinda citrifolia (Noni) Fruit Juice Reduces Inflammatory Cytokines Expression and Contributes to the Maintenance of Intestinal Mucosal Integrity in DSS Experimental Colitis

Beatriz Coutinho de Sousa,[1] Juliana Reis Machado,[2] Marcos Vinicius da Silva,[1] Thiago Alvares da Costa,[1] Javier Emilio Lazo-Chica,[1] Thatiane do Prado Degasperi,[1] Virmondes Rodrigues Junior,[1] Helioswilton Sales-Campos,[1] Elizabeth Uber Bucek,[3] and Carlo José Freire Oliveira[1]

[1]*Institute of Natural and Biological Sciences, Federal University of Triângulo Mineiro, 38025-180 Uberaba, MG, Brazil*
[2]*Institute of Tropical Pathology and Public Health, Federal University of Goias, 74605-050 Goiania, GO, Brazil*
[3]*Department of Pharmaceutical Sciences, University of Uberaba, 38050-501 Uberaba, MG, Brazil*

Correspondence should be addressed to Carlo José Freire Oliveira; carlo@icbn.uftm.edu.br

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Morinda citrifolia L. (noni) has been shown to treat different disorders. However, data concerning its role in the treatment of intestinal inflammation still require clarification. In the current study, we investigated the effects of noni fruit juice (NFJ) in the treatment of C57BL/6 mice, which were continuously exposed to dextran sulfate sodium (DSS) for 9 consecutive days. NFJ consumption had no impact on the reduction of the clinical signs of the disease or on weight loss. Nonetheless, when a dilution of 1 : 10 was used, the intestinal architecture of the mice was preserved, accompanied by a reduction in the inflammatory infiltrate. Regardless of the concentration of NFJ, a decrease in both the activity of myeloperoxidase and the key inflammatory cytokines, TNF-α and IFN-γ, was also observed in the intestine. Furthermore, when NFJ was diluted 1 : 10 and 1 : 100, a reduction in the production of nitric oxide and IL-17 was detected in gut homogenates. Overall, the treatment with NFJ was effective in different aspects associated with disease progression and worsening. These results may point to noni fruit as an important source of anti-inflammatory molecules with a great potential to inhibit the progression of inflammatory diseases, such as inflammatory bowel disease.

1. Introduction

The potential anti-inflammatory activities of several natural compounds in the downregulation of key players in the development of inflammation have been explored in different scenarios including the modulation of cytokines, transcription factors, enzymes, and the production of protein and non-protein inflammatory mediators. These activities include the modulation of cytokines (e.g., IL-6, TNF-α, IFN-γ, IL-17, and IL-12), transcription factors, enzymes (e.g., myeloperoxidase-MPO and cyclooxygenase COX-1 and COX-2), and also the production of nitric oxide (NO) [1–3]. Due to their importance in controlling inflammation, therapies targeting such molecules have been suggested as possible aids in the prevention and/or treatment of inflammatory diseases, such as rheumatoid arthritis [4], dermatitis [5], and inflammatory bowel disease (IBD) [6]. IBDs are chronic inflammatory diseases of the gastrointestinal tract, which are clinically present as one of the two disorders, Crohn's disease (CD) or ulcerative colitis (UC) [7, 8]. These pathologies are of special interest since they affect millions of people worldwide, and current therapies are still not fully effective in controlling disease progression or preventing the occurrence of side effects [6].

Morinda citrifolia L. (noni) belongs to the Rubiaceae family, and it is a source of natural molecules that has been

used as a medicinal plant by the Polynesians for more than 2,000 years [9]. So far, several bioactive compounds have been isolated from noni fruits, including fatty acids, flavonoids, polysaccharides, and sterols [10–13]. The anti-inflammatory potential of noni fruit compounds has been demonstrated in an experimental model of *Helicobacter pylori* infection in which ethanol and ethyl acetate extracts were used. These extracts were able to reduce both neutrophil chemotaxis and production of inducible nitric oxide (iNOS) and COX-2 [14]. Accordingly, C57BL/6 mice orally treated with noni fruit juice at 500 mg kg^{-1} day^{-1} for 60 days showed reduced inflammatory infiltrate and cytokine expression for IL-12, TNF-α, TGF-β, and IL-10, in the footpad infected with *Leishmania amazonensis* [15]. It is important to mention that cytokines such as IL-12, IL-6, TNF-α, IFN-γ, IL-17, and IL-23 are associated with the development and worsening of IBD, so they have been approached differently in order to treat this inflammatory disorder [7]. Furthermore, the anti-inflammatory potential of *Morinda citrifolia* leaf extract was shown by the reduction of TNF-α, IL-1β, and NO levels in macrophages after stimulation with lipopolysaccharide (LPS) [16]. Even though the role of noni fruit compounds in controlling inflammatory players is of special relevance, their effects in the development of intestinal inflammation are still poorly explored.

Therefore, this study showed the effects of noni fruit juice on cytokine interplay and intestinal architecture in a murine model of dextran sulfate sodium-induced colitis as underlying mechanisms for its immunomodulatory activity.

2. Materials and Methods

2.1. Collection and Botanical Identification of the Plant. The fruits used in this study were obtained from the monoculture of 150 noni plants on *Fazenda Boa Vontade*, a farm in the municipality of Araguari, Triângulo Mineiro/MG, Brazil, at coordinates 18°43′47.23″S, 48°6′49.50″O (data from Google Earth, 2013). All the specimens were prepared according to conventional herborization techniques [17] and deposited in the herbarium of the Federal University of Uberlândia (HUFU Herbarium) under the registration number HUFU-67210, as *Morinda citrifolia* L. (Rubiaceae).

2.2. Juice Extraction Process. *Morinda citrifolia* (noni) juice was prepared in the Laboratory of Pharmacognosy of University of Uberaba, in Uberaba, Minas Gerais, Brazil. *M. citrifolia* fruit was manually and randomly collected from 150 plants, washed in ozonated water, and kept at room temperature for 3–5 days. The fruits were mechanically depulped using a fruit depulper and, after seed removal, the resulting pulp was centrifuged at 4,000 rpm under refrigeration until the supernatants were clear, and it was then considered 100% (v/v) juice and stored at −70°C until further use.

2.3. Animal Studies. Male C57BL/6 mice aged 6–8 weeks and weighing 20–25 g were housed in specific pathogen-free and standard-controlled environmental conditions at constant temperature (25°C) on a 12-hour light/dark cycle, with *ad libitum* access to food and water, in the animal housing facility of the Federal University of Triângulo Mineiro (UFTM), Brazil. All animal studies were performed in accordance with the Institutional Animal Care and Use Committee of UFTM under protocol 275. The experiments were performed with 8 mice/groups, as follows: *saline*, healthy control mice treated with saline; DSS 2.5%, mice exposed to dextran sulfate sodium (DSS); DSS 2.5% + *pure noni*, mice exposed to DSS and treated with pure noni fruit juice; DSS 2.5% + *noni* 1 : 10, mice exposed to DSS and treated with a 1 : 10 dilution of noni fruit juice; and DSS 2.5% + *noni* 1 : 100, mice exposed to DSS and treated with a 1 : 100 dilution of noni fruit juice. A volume of 100 μl per mouse was administered orally for 9 consecutive days.

2.4. DSS-Induced Colitis and Clinical Assessment. Colitis was induced by 2.5% DSS (MP Biomedicals, Illkirch, France, Molecular weight: 36,000–50,000 kDa) continuously added to the drinking water for 9 consecutive days for sample collection. In addition to recording daily food and water intake, body weight changes and clinical signs of disease were also assessed every day so as to obtain a clinical disease score for every mouse. Each sign presented by the animals corresponded to one point, and the sum of points for each mouse defined a clinical score. Clinical scores were determined as previously described herein [18].

2.5. Euthanasia and Sample Collection. The mice were euthanized on day 9, and the colon was removed for further analysis. The colon samples were divided into smaller sections that were immersed into PBS/10% formaldehyde for paraffin embedding or were immediately frozen in liquid nitrogen for quantification of myeloperoxidase (MPO) or nitric oxide (NO) activity by enzymatic assays. Moreover, one intestinal section from each mouse was collected in a solution containing protease inhibitors (Complete®, Roche Pharmaceuticals, Mannheim, Germany) for cytokine quantification by enzyme-linked immunosorbent assay (ELISA).

2.6. Myeloperoxidase (MPO) and Nitric Oxide (NO). Briefly, for MPO assay, the sections were homogenized and erythrocytes were lysed. The pellet obtained after centrifugation was resuspended, followed by three freeze-and-thaw cycles. After centrifugation, the supernatant was placed in 96-well plates and revealed with tetramethylbenzidine (TMB) substrate (BD OptEIA™, San Diego, CA) at 37°C. The reaction was stopped and readings were performed in a spectrophotometer at 450 nm. MPO activity was determined as previously described [19]. Results were normalized to the dry weight of each intestinal section and expressed as optical density per gram of tissue (nm/g of tissue).

For NO measurement, the nitrite accumulated in intestinal homogenates was measured as an indicator of NO production using Griess reaction [20]. Then, 100 μL of tissue homogenate was mixed with 100 μL of Griess reagent, which is composed by equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthylethylenediamine-HCl, and incubated at room temperature for 10 min. The absorbance was measured at 540 nm in a 96-well plate reader (Perkin Elmer Cetus, CA, USA). The amount

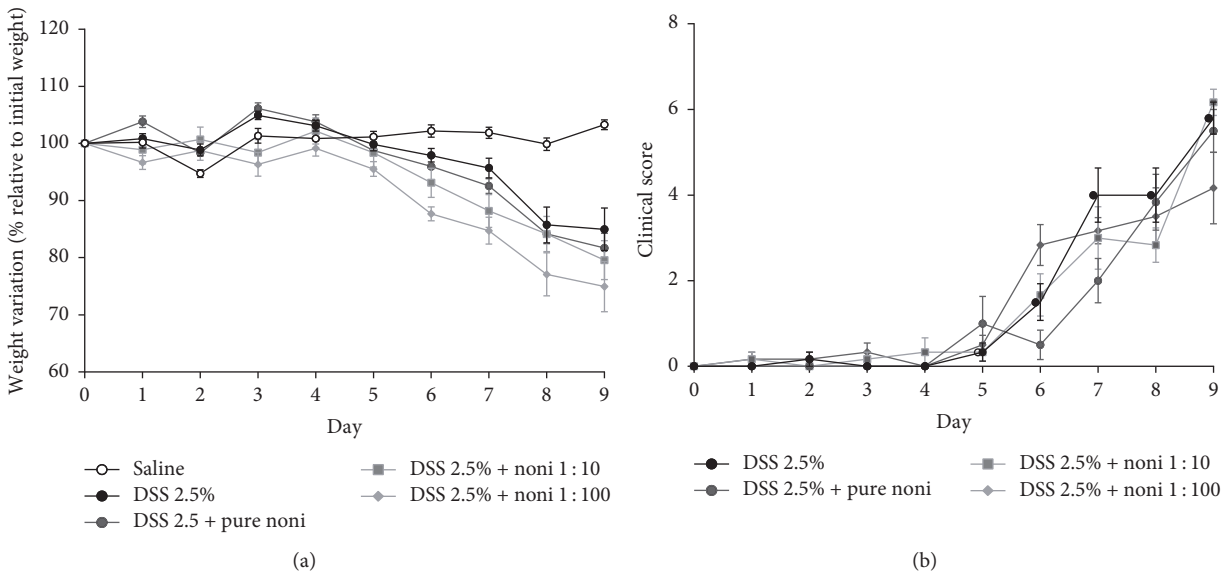


Figure 1: Treatment with noni fruit juice and disease outcome. C57BL/6 mice were exposed to dextran sulfate sodium (DSS) 2.5% and treated daily with noni fruit juice. On day 9, mice were euthanized to obtain intestinal sections. (a) Percentage of weight change. (b) Clinical disease score. *Saline*, healthy control mice treated with saline; DSS 2.5%, mice exposed to DSS; DSS 2.5% + *pure noni*, mice exposed to DSS and treated with the pure noni fruit juice; DSS 2.5% + *noni* 1 : 10, mice exposed to DSS and treated with a 1 : 10 dilution of the fruit juice; and DSS 2.5% + *noni* 1 : 100, mice exposed to DSS and treated with a 1 : 100 dilution of the fruit juice.

of nitrite in the samples was calculated using linear regression analysis of the absorbance of the serial dilution of sodium nitrite standard curve. Results were normalized to the dry weight of each intestinal section and expressed as picogram per milliliter per gram of tissue (pg/ml/g).

2.7. Cytokine Quantification by ELISA. Cytokines IL-10, IL-17, IFN-γ, TNF-α, IL-12, IL-4, and IL-23 were quantified in tissue homogenates by ELISA according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Results were normalized to the dry weight of each intestinal section and expressed as nanogram per milliliter per gram of tissue (ng/mL/g of tissue).

2.8. Histology and Histopathological Analysis. In order to assess the microscopic damage, the intestinal sections were cut longitudinally, washed with PBS, fixed in 10% buffered formalin for 24 h, and then processed for paraffin embedding followed by microtome sectioning. Tissue sections (5 μm) were obtained and stained with hematoxylin and eosin (H&E). For histopathological analysis, the mucosa, submucosa, muscle layers, and serosa were evaluated. These intestinal sections were also assessed for the presence of edema, inflammatory infiltrate, and epithelial abnormalities.

Images were captured using a digital video camera (Evolution MP 5.0 color Media Cybernetics, Silver Spring, MD, USA), with a 10x objective, coupled to a light microscope (Nikon Eclipse 50i, Melville, NY, USA). Morphometry was performed using Image-Pro Insight (Media Cybernetics). The inflammatory infiltrate was measured based on the damaged area containing inflammatory infiltrate divided by the total area of tissue visualized in the acquired image and expressed as a percentage (%). A trained pathologist who was blinded to treatment performed the histopathological analysis.

2.9. Data Analysis and Statistics. Normal distribution and homogeneous variance were tested for all of the variables. When the distribution was considered normal and the variance was homogeneous, parametric tests were used: unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test. In cases of non-Gaussian distribution of data, the following nonparametric tests were used: Mann–Whitney test or Kruskal-Wallis test accompanied by Dunn's post hoc test. The results were expressed as mean ± SD. The differences observed were considered significant when $p < 0.05$ (5%). Statistical analysis was performed using GraphPad Prism, version 5.0 (La Jolla, CA, USA).

3. Results

3.1. Treatment with Noni Fruit Juice and Disease Outcome. First, in order to assess whether noni fruit juice was able to prevent weight loss and the outcome of DSS-induced colitis, the mice were exposed to DSS for 9 days and then treated with the fruit juice, as described in *Materials and Methods*. The noni fruit juice group did not seem to reduce weight loss when compared to their control counterparts (DSS 2.5%) (Figure 1(a)). Furthermore, regardless of the concentration used, there was no effect on the presentation of clinical signs of disease in the mice treated with noni fruit juice in relation to the untreated mice (Figure 1(b)).

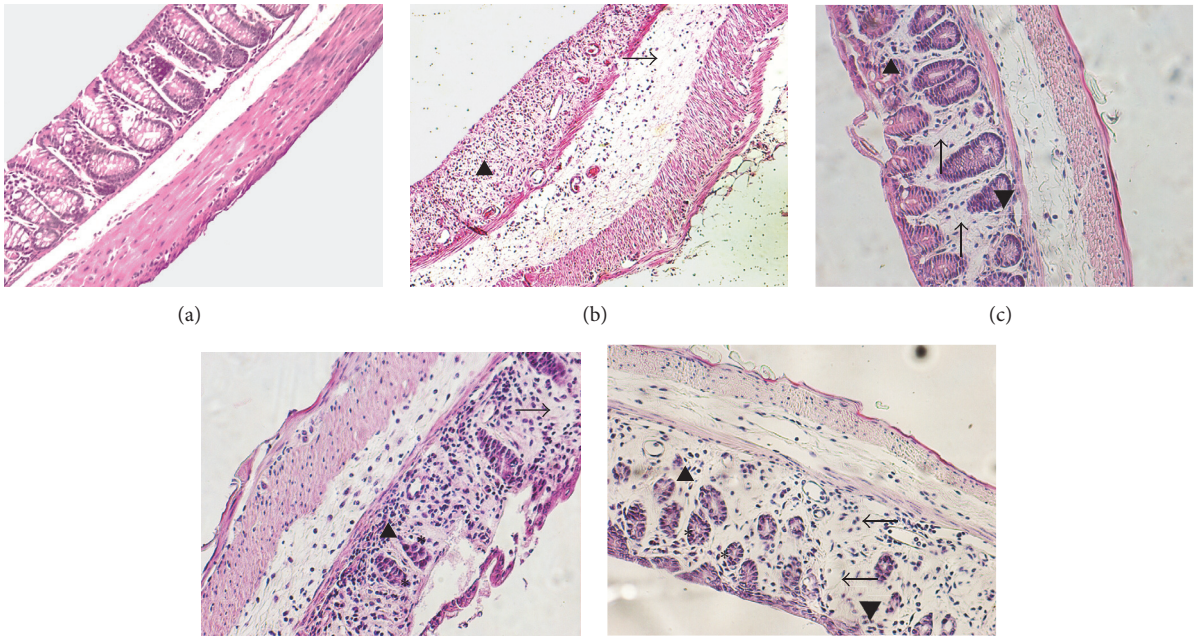


Figure 2: Noni fruit juice consumption preserves intestinal architecture in a dose-dependent manner. C57BL/6 mice were exposed to dextran sulfate sodium (DSS) 2.5% and treated daily with noni fruit juice. The colon was collected on day 9 for histopathological analysis. (a) Healthy mice without colitis; (b) mice exposed to DSS: lamina propria with moderate edema and mononuclear cell infiltration (arrowhead), submucosa with moderate mononuclear infiltrate and severe edema (arrow); (c) mice exposed to DSS and treated with pure noni fruit juice: lamina propria with moderate edema (arrow) and mononuclear infiltrate (arrowhead); (d) mice exposed to DSS and treated with noni fruit juice diluted 1 : 10: crypts with preserved irregularities (asterisk); lamina propria with moderate edema (arrow) and mononuclear infiltrate (arrowhead); (e) mice exposed to DSS and treated with noni fruit juice diluted 1 : 100: regular and atrophic crypts (asterisk); lamina propria with moderate edema (arrow) and mononuclear infiltrate (arrowhead).

Table 1: Histopathological scores of mice exposed to DSS in different conditions.

	DSS 2.5%	DSS 2.5% + pure noni	DSS 2.5% + noni 1 : 10	DSS 2.5% + noni 1 : 100
Lamina propria edema	Moderate	Moderate	Mild	Moderate
Submucosal edema	Severe	Moderate	Moderate	Moderate
Mucosal mononuclear infiltrate	Moderate	Mild	Mild	Moderate
Submucosal mononuclear infiltrate	Moderate	Mild	Mild	Mild
Intestinal crypts	Absent	Continuous	Irregular	Irregular

3.2. Noni Fruit Juice Consumption Inhibits Inflammation and Preserves Intestinal Architecture in a Dose-Dependent Manner. After that, since no macroscopic effects were observed after treatment with noni fruit juice, we aimed to determine if the consumption of fruit juice had any microscopic effect on intestinal architecture. It was observed, in a dose-dependent manner, that the group treated with noni fruit juice was able to preserve their intestinal architecture. Healthy mice (Figure 2(a)) had the epithelium surface preserved, rectilinear crypts, composed by habitual number of goblet cells. In the lamina propria, the usual mononuclear infiltrate was visualized. The submucosa, muscular, and serous muscles had normal architecture. Mice exposed to DSS had erosions on the surface of intestinal epithelium and absence of crypts (Table 1). The lamina propria showed moderate edema and mononuclear cell infiltration (Figure 2(b), arrowhead), the submucosa had moderate mononuclear infiltrate and severe edema (Figure 2(b), arrow), and the muscular layer was slightly thickened, with colitis activity. In mice exposed to DSS and treated with pure noni juice, crypt irregularities were scarce and mild (Table 1), and the lamina propria had moderate edema (Figure 2(c), arrow) and mononuclear infiltrate (Figure 2(c), arrowhead); however, only a mild mononuclear infiltrate and moderate edema were detected in the submucosal layer, and the muscular layer was slightly thickened. On the other hand, in mice treated with noni fruit juice diluted 1 : 10, crypts had their irregularities preserved (Figure 2(d), asterisk), the lamina propria showed both moderate edema (Figure 2(d), arrow) and mononuclear infiltrate (Figure 2(d), arrowhead), and the submucosa only had a mild mononuclear infiltrate and moderate edema (Table 1). In the mice treated with noni fruit juice diluted 1 : 100, crypts were irregular and atrophic (Figure 2(e), asterisk), the lamina propria had moderate edema and mononuclear infiltrate, and

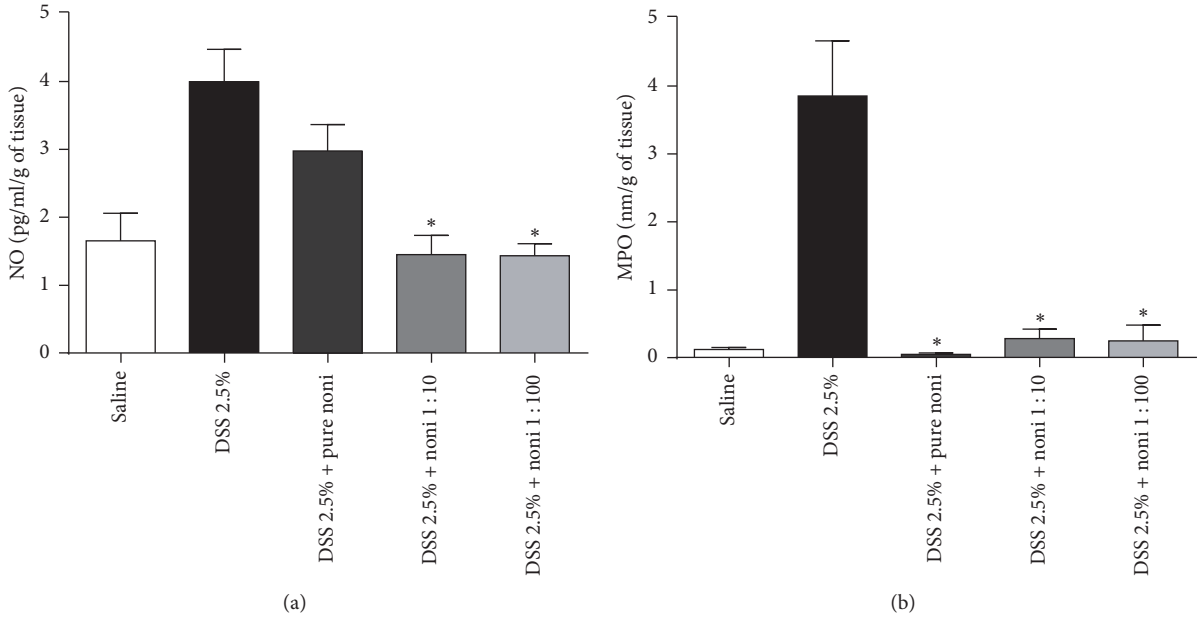


Figure 3: Noni fruit juice consumption inhibits inflammation by reducing nitric oxide and myeloperoxidase activities. C57BL/6 mice were exposed to dextran sulfate sodium (DSS) 2.5% and treated daily with noni fruit juice. On day 9, the mice were euthanized in order to obtain intestinal sections. Quantification of intestinal production of nitric oxide (a) and myeloperoxidase (MPO) activity, expressed as picogram per milliliter per gram of tissue (pg/ml/g) and as optic density per gram of tissue (nm/g of tissue), respectively. Data are represented as mean ± SEM. $^{*}P < 0.05$.

the submucosal layer showed a mild mononuclear infiltrate and moderate edema (Table 1). In general, the architecture of intestinal crypts was preserved in the mice treated with noni fruit juice, and it was more preserved in mice that received noni juice at 1 : 10 and 1 : 100 dilutions in comparison with those treated with pure noni or DSS.

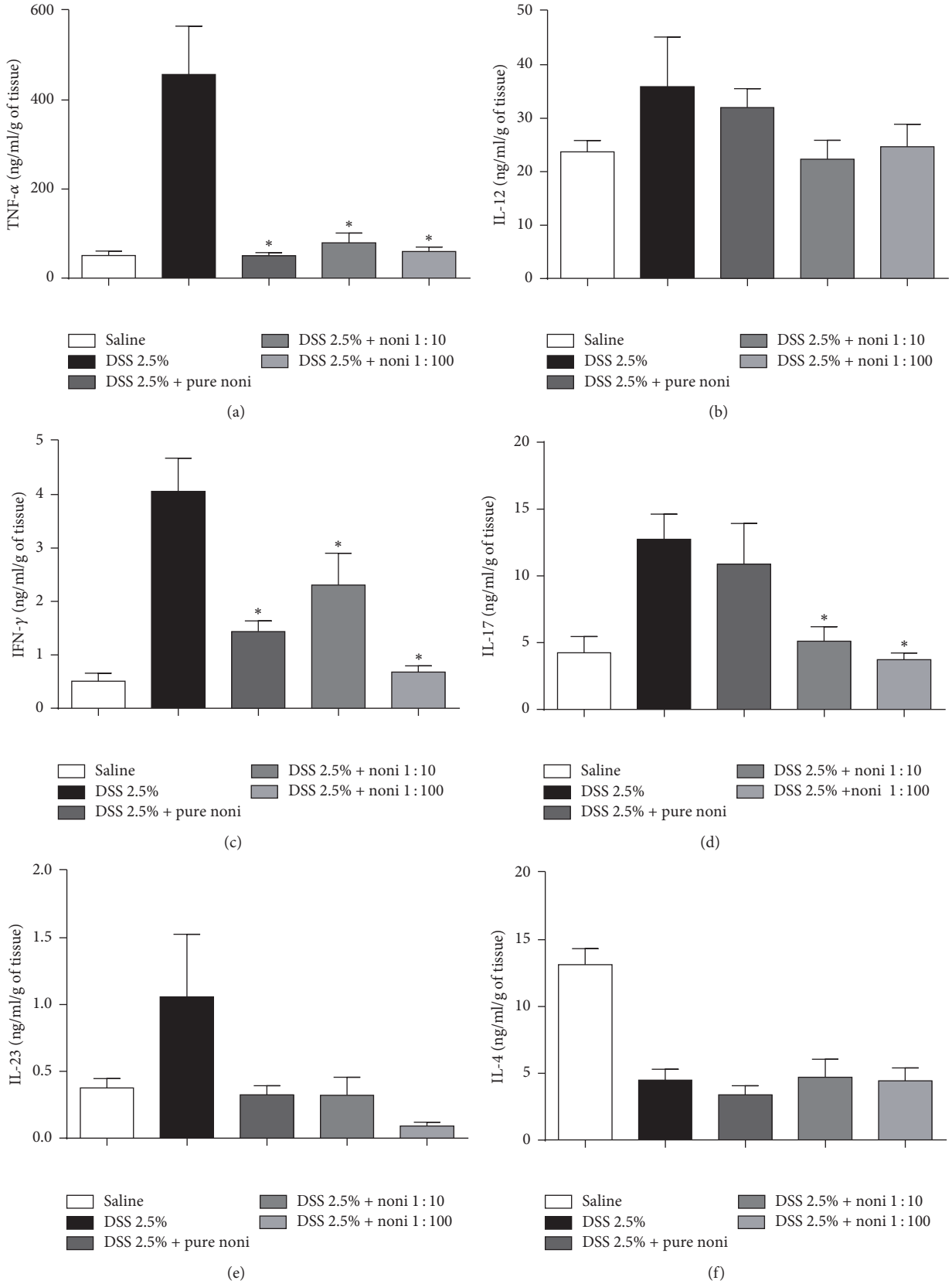
The production of NO was reduced in the mice treated with noni fruit juice diluted 1 : 10 and 1 : 100 in relation to those that received pure noni or DSS (Figure 3(a)). Moreover, regardless of the concentration used, mice treated with noni fruit juice had reduced activity of MPO (Figure 3(b)) in comparison with DSS-exposed mice. Even though weight loss and disease outcome were not affected by noni fruit consumption, the improved intestinal architecture and the reduced activity of enzymes responsible for inflammation were associated with fruit juice consumption in a dose-dependent manner.

3.3. Noni Fruit Juice Consumption Reduces Key Inflammatory Cytokines in the Intestine in a Dose-Dependent Manner. Finally, we aimed to evaluate whether the improvement in intestinal architecture could also be associated with the modulation of key cytokines associated with disease worsening/protection. Mice treated with noni fruit juice, regardless of the concentration used, showed reduced production of inflammatory cytokines TNF-α and IFN-γ (Figures 4(a) and 4(c), resp.). A reduction in IL-17, another key cytokine associated with disease worsening, was observed only in mice treated with 1 : 10 and 1 : 100 dilutions (Figure 4(e)). Nevertheless, there were no differences in the production of IL-12 (Figure 4(b)), IL-4 (Figure 4(d)), IL-23 (Figure 4(f)), and IL-10 (Figure 4(g)). Taken together, these results suggest that improved intestinal architecture might also be associated with the local reduction of key inflammatory cytokines.

4. Discussion

The results presented herein demonstrate that noni fruit juice can reduce key inflammatory cytokines involved in the development of intestinal inflammation. Furthermore, treatment using fruit juice was also shown to be able to improve intestinal architecture, mainly when the dilution 1 : 10 was used. However, at least apparently, no effects were detected on the presentation of clinical signs of disease.

The beneficial properties of treatment using noni fruit juice in colitis control were partially attributed to an improvement in intestinal architecture along with a reduction in inflammatory infiltrate. This scenario was followed by a decrease in the activity of NO (only when noni fruit juice was diluted 1 : 10 and 1 : 100) and MPO (at any concentration). NO is a strong proinflammatory mediator mainly derived from inducible nitric oxide synthase (iNOS) after stimulation with bacterial endotoxins and inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ, in different cell types, including macrophages, neutrophils, endothelial cells, and smooth muscle cells [21, 22]. Overexpression of iNOS, especially at mucosal sites, such as gastrointestinal tract, is reported to be associated with the development of inflammatory diseases, including IBD [23]. In this context, the production of high NO levels by infiltrating cells, such as macrophages, neutrophils, and lymphocytes, as well as by colon epithelial cells, was described to be directly associated with local tissue



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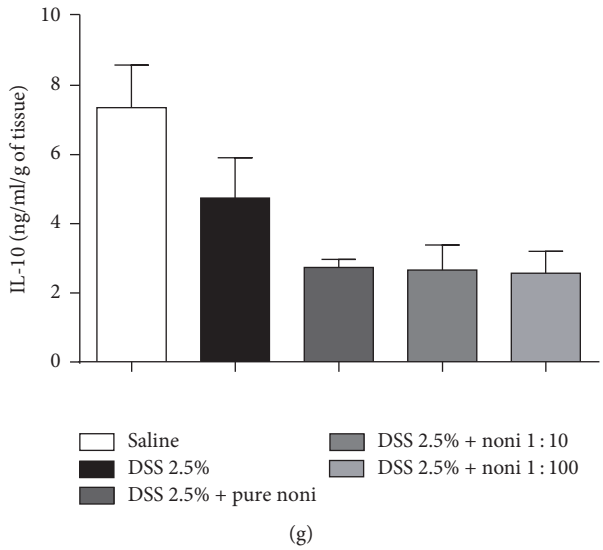

Figure 4: Noni fruit juice consumption reduces key inflammatory cytokines in the intestine in a dose-dependent manner. C57BL/6 mice were exposed to dextran sulfate sodium (DSS) 2.5% and treated daily with noni fruit juice. Enzyme-linked immunosorbent assay (ELISA) was performed in gut homogenates. (a) TNF-α, (b) IL-12, (c) IFN-γ, (d) IL-17, (e) IL-23, (f) IL-4, and (g) IL-10. Results were expressed as nanograms of cytokine per milliliter per gram of tissue (ng/ml/g of tissue), normalized by tissue dry weight. Data are represented as mean ± SEM. $^{*}P < 0.05$.

damage and disease worsening in IBD [24]. This observation was reinforced by the fact that the overexpression of iNOS induced by the inflammatory cytokines IL-1β, TNF-α, IFN-γ, IL-6, IL-17, and IL-23 was found in the plasma, in lamina propria mononuclear cells, and in colon epithelial cells of IBD patients and mice with intestinal inflammation [25–28], thus reinforcing the role of iNOS derivatives such as NO, and inflammatory cytokines, in the disease worsening and outcome. Therefore, it seems reasonable to believe that therapies aiming to modulate such aspects may represent an important tool to constrain inflammation and disease progression. In this context, monotropein isolated from the roots of *Morinda officinalis*, a member of the family Rubiaceae like *M. citrifolia*, reduced the in vitro production of NO in murine macrophages after stimulation with LPS in a dose-dependent manner [29]. A positive correlation between NO and the severity of IBD was proposed in clinical studies describing the occurrence of high levels of nitrite/nitrate in the plasma, urine, and lumen of IBD patients [27, 30, 31]. Shin et al. also demonstrated the activity of monotropein when mice were exposed to 4% DSS for 9 consecutive days. In this case, the treatment was able to reduce the activity of the inflammatory players COX-2 and MPO [29]. The study of MPO activity is a critical marker of neutrophil infiltration in the intestinal mucosa [32]. Indeed, studies using different protocols to induce colitis have demonstrated a positive correlation between the determination of MPO activity and disease severity [33–36]. Although the expressions of COX-2 and iNOS were not determined in our study, these results suggest that different members of Rubiaceae family can modulate inflammation in a similar way, thus inhibiting the progression and severity of DSS-induced colitis, using analogous mechanisms. The capability to modulate key inflammatory components by therapies aiming to control the activity of different cell types, such as macrophages, besides reducing the production of cytokines and NO, such as that observed in our study when NFJ was used, represents a promising approach to constrain the progression of inflammation in IBD. The control of inflammation by these mechanisms may explain the maintenance of intestinal architecture observed in our study when the dilutions 1 : 10 and 1 : 100 were used.

The complex and heterogeneous mechanisms associated with the development of intestinal inflammation include host genetics, environmental triggers, and disorders both in microbiota composition and in immune balance [7]. The severity of bowel inflammation is associated with increased production of proinflammatory cytokines (e.g., TNF-α, IFN-γ, IL-6, IL-17, and IL-23), which makes therapies aiming to reduce the levels of these cytokines relevant. In our study, treatment with noni fruit juice, in a dose-dependent manner, was able to reduce the production of TNF-α, IFN-γ, and IL-17 in the intestine.

TNF-α is one of the central players in the development of intestinal inflammation [37], and it is increased in the intestinal mucosa of patients with IBD [38]. This cytokine also has a pivotal role in the production of NO, and it increases the production of metalloproteinase, which contributes to the loss of epithelial integrity [39] and to disease worsening. The effects of TNF-α are mediated by two receptors, TNF receptor-1 (TNFR-1) and TNF receptor-2 (TNFR-2). The former can be expressed in either immune or nonimmune cells, resulting in the activation of NF-κB, cytotoxicity, and production of inflammatory cytokines [40]. In our study, treatment with noni fruit juice considerably reduced the production of TNF-α in the intestine, regardless of the dilution used. This modulation could be, at least,

partly attributed to the inhibition of NF-κB by ascorbic acid and flavonoid glycoside, which were isolated from fermented noni fruit juice [41]. However, it is possible to speculate that these molecules might reduce the levels of TNF-α in the intestine by downregulating TNFR-1. Indeed, due to the importance of this cytokine in the development and aggravation of IBD, therapies aiming at targeting TNF-α to prevent the development of intestinal inflammation could be useful. The administration of monoclonal antibodies against IL-6 and TNF-α was able to reduce disease severity and attenuate intestinal inflammation in DSS-induced colitis [42]. Nevertheless, a substantial number of patients lose responsiveness especially due to production of antidrug antibodies and accelerated drug clearance [43], and that reinforces the need for new therapeutic approaches aiming to better control IBD progression and to reduce side effects.

IFN-γ is a proinflammatory cytokine produced by a broad range of cells, including T helper cells ($CD4^+$T cells) and cytotoxic T cells ($CD8^+$T cells), natural killer cells, and group 1 innate lymphoid cells [44]. A higher frequency of $CD4^+$T cells and $CD8^+$T cells producing IFN-γ was shown in patients with IBD in comparison with their control counterparts [44]. Both IFN-γ and TNF-α are increased in the mucosa of IBD patients and act synergistically contributing to the development and maintenance of inflammation that culminates in barrier breakdown [45]. These cytokines were shown to disrupt intercellular junction proteins under inflammatory conditions, which are not observed in noninflamed areas of unhealthy tissue [46]. Some of the mechanisms underlying these disorders include epithelial apoptosis [47] and reduced transcription of tight-junction proteins [48]. Therefore, therapies aiming to reduce this cytokine might be helpful in controlling inflammation and improving disease outcome. In this context, a short-term glucocorticoid treatment of mice exposed to DSS for six days was able to reduce the frequency of IFN-γ-producing $CD4^+$ cells in the spleen and to decrease the expression of this cytokine and IL-1β in the intestine [49]. This reduction in the activity of IFN-γ was followed by an improvement in the clinical outcome and restoration of immune balance [49]. Additionally, the beneficial effects associated with the reduction of inflammatory cytokines such as IL-1β and IFN-γ were further elucidated in a murine model of colitis. In this case, colitis was induced by intracolonic administration of dinitrobenzene sulfonic acid (DNBS), and mice were treated using a nonpsychotropic cannabinoid, known as cannabigerol [50]. Even though the effects of noni fruit juice treatment on apoptosis and intestinal permeability were not investigated in this study, the improvement in intestinal architecture and the reduction in inflammatory infiltrate could be partly attributed to the reduction of IFN-γ.

IL-17-producing cells are involved in the pathogenesis of numerous inflammatory and autoimmune diseases, and they have been shown to mediate disease pathogenesis in IBD [51]. The role of Th17 cells in the pathogenesis of IBD was primarily attributed to a mutation in the *IL-23R* gene, which was identified in IBD patients and which regulates the production of Th17-related cytokines [52]. Indeed, an increased production of IL-17 by lamina propria cells in both UC and CD had already been shown [53]. Furthermore, cytokines produced by Th17 cells, such as IL-17 and IL-21, were found to upregulate the expression of TNF-α, IL-1β, IL-6, and IL-8 and the recruitment of neutrophils [54], which may contribute to IBD worsening. In addition to the classic role of Th17 lymphocytes in IBD pathogenesis, group 3 innate lymphoid cells have been also implicated in IL-17 production and disease pathogenesis in both experimental models and human subjects [55, 56]. In our study, reduced levels of IL-17 were detected in the colon of mice treated with noni fruit juice, but only when the 1 : 10 and 1 : 100 dilutions were used. Although we did not identify whether the reduction in IL-17 in the intestine was more associated with innate or adaptive immunity, or both, we cannot underestimate the importance of therapies aiming to produce these cytokines in order to inhibit inflammation in colitis.

Overall, our data showed that the treatment with noni fruit juice plays an important role in inhibiting inflammation during the development of experimental colitis. One of the key aspects regarding the use of fruit juice as a therapeutic option concerns its immune modulatory effect, which has a consequent impact on the improvement of intestinal architecture. Nonetheless, further studies must be performed in order to elucidate the molecules underlying the reduction of inflammatory cytokines in this model.

Competing Interests

The authors declare no conflict of interests.

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Research Article

Hip Osteonecrosis Is Associated with Increased Plasma IL-33 Level

Jinhui Ma,[1] Wanshou Guo,[1,2] Zirong Li,[1,2] Bailiang Wang,[1,2] Shirui Li,[3] and Peng Wang[3]

[1]*Department of Bone and Joint Surgery, Peking University China-Japan Friendship School of Clinical Medicine, Beijing 100029, China*
[2]*Department of Bone and Joint Surgery, Center for Osteonecrosis and Joint Preserving & Reconstruction, China-Japan Friendship Hospital, Beijing 100029, China*
[3]*Department of Endocrinology, China-Japan Friendship Hospital, Beijing 100029, China*

Correspondence should be addressed to Bailiang Wang; wang_orthopaedic@126.com

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The recently discovered IL-33 as an IL-1 cytokine family member has been proved to be specifically released from osteonecrotic bones. We aimed to investigate the potential role of IL-33 in the development of osteonecrosis of femoral head (ONFH). Forty patients diagnosed with ONFH and forty age-, sex-, and body mass index- (BMI-) matched healthy subjects were included in this prospective study between March 2016 and September 2016. A commercially available ELISA kit was used to test the level of plasma IL-33. The IL-33 levels were compared among different ARCO stages, CJFH types, and etiology groups. Plasma IL-33 levels were significantly higher in the ONFH patients than that in the control subjects. The levels of IL-33 did not differ significantly among the ONFH patients with different ARCO stages. The IL-33 levels of patients with CJFH type L3 were significantly higher than that of patients with types L1 and L2. No significant differences were observed in IL-33 levels between steroid-induced, alcohol-induced, and idiopathic patients. Our findings seem to indicate that IL-33 effects may be detrimental during ONFH, which appeared to be associated with the prognosis of ONFH. The IL-33 deserves particular attention in the pathogenesis of ONFH.

1. Introduction

Nontraumatic osteonecrosis of the femoral head (ONFH), also known as avascular necrosis, is a refractory and progressive disease that commonly affects young patients and has a poorly understood etiology and pathogenesis [1]. Without effective treatment, ONFH can progress and might eventually lead to femoral head collapse and degenerative changes to the hip joint [2, 3]. Nontraumatic ONFH has been associated with corticosteroid usage, chronic alcohol consumption, infection, hyperbaric events, storage disorders, marrow infiltrating diseases, coagulation defects, immoderately low or high temperatures, and some autoimmune diseases [4, 5].

The pathogenesis of nontraumatic ONFH is not completely clear, but it may be attributed to vascular injury, altered lipid metabolism/fat emboli, cell and bone death, mechanical stress, disruptive immune system, and defective bone repair [4, 6]. Previous study has shown that femoral head osteonecrosis may be caused by disruption of the immune system via lipopolysaccharide- (LPS-) activated toll-like receptor 4 (TLR4) signalling [6]. In addition, some defective repair processes including delayed new bone formation, excessive bone resorption, and tissue fibrosis instead of new bone formation have been observed after ONFH, which are suggestive of pathologic bone remodeling during the repair of the necrotic bone [7, 8]. Meanwhile, it has been shown that the immune system is substantially involved in the regulation of bone homeostasis and that chronic inflammation in particular can disturb this balance [9]. Therefore, abnormal immune responses may contribute to the pathogenesis of ONFH by impacting bone remodeling.

Cytokines are a large group of proteins, peptides, and glycoproteins secreted by cells, which could regulate immune responses and immune response cell function by combining the corresponding receptors [10]. Cytokines with diverse biological functions including growth, differentiation, and

activation are also known to play an important role in regulating the balance between osteoclasts and osteoblasts and determining the rate of bone remodeling [10]. IL-33, the most recently discovered member of the IL-1 family of cytokines, was initially recognized as an orphan receptor associated with inflammatory and autoimmune diseases [11]. Early studies revealed that it is expressed in intestine of patients with Crohn's disease, blood vessels of inflamed tonsils, and synovium of patients with RA and that this expression appeared to be associated with the severity of the inflammatory condition [12]. And IL-33 is now believed to be constitutively expressed in human bone and particularly released by cells undergoing necrosis from the osteoblasts, adipocytes, and osteocytes rather than through active secretion [13–16]. Recent studies suggest that IL-33 may act as a proinflammatory cytokine in asthma, septic shock, fibroproliferative diseases, collagen vascular diseases, pleural malignancy, and cardiovascular diseases [17]. During ONFH, IL-33 may also play a role by impacting bone remodeling both directly and indirectly after it is released from osteonecrotic bones [18]. However, it is still unknown whether IL-33 increases with the repair of necrotic bone or whether IL-33 acts as a positive or a negative effect after ONFH.

The purpose of this study was to investigate the plasma level of IL-33 in the patients with ONFH. We hypothesized that after ONFH, necrotic bone stimulates the inflammatory cytokine, IL-33, and expression and that IL-33 is an important player in the development and prognosis of ONFH. An evaluation of the role of IL-33 would reveal potential mechanisms responsible for disruptive immune responses after ONFH, which might provide a novel nonsurgical therapeutic approach for ONFH.

2. Materials and Methods

2.1. Study Population. This was a prospective clinical control study. Forty patients (40 consecutive hips) diagnosed with unilateral nontraumatic osteonecrosis of femoral head (ONFH) between March 2016 and September 2016 were included in this study. The patients were diagnosed with ONFH based on clinical history, physical examination, and radiological evaluations (X-ray and MRI) by orthopedic surgeons in our department. The inclusion criterion was a diagnosis of unilateral nontraumatic ONFH. Patients presenting the following criteria were excluded: history of trauma, active infection of the affected hip, inflammatory diseases, cardiovascular diseases, immunodeficiency, HIV infection, diabetes mellitus, renal disease, or previous surgery on the hip with ONFH. Forty age-, sex-, and body mass index- (BMI-) matched healthy subjects were simultaneously recruited as the controls. The study was approved by China-Japan Friendship Hospital (CJFH), and the methods were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects or their guardians.

The included patients were evaluated both clinically and radiologically using the Harris hip score (HHS) [19], CJFH type (Figure 1) [20], and the ARCO classification system [21]. The characteristics of the included ONFH patients are presented in Table 1. According to ARCO stage, 4 patients had stage II disease, 20 patients had stage III disease, and 16 patients had stage IV disease. CJFH types were as follows: L1, 12 patients; L2, 18 patients; L3, 10 patients; M, 0 patients; and C, 0 patients. The mean patients' age was 49.2 ± 12.4 years (range, 18–69 years), of which 31 were male and 9 were female. The average body mass index (BMI) was 24.2 ± 3.2 kg/m^2 (range, 18.7–31.2 kg/m^2). The mean HHS for all patients was 62.6 ± 14.6 score (range, 38–95 score). ONFH was idiopathic in 11 patients, secondary to steroid use in 14 patients, and associated with alcohol use in 15 patients. The patients were divided into two groups according to whether the lateral pillar of the femoral head (LPFH) was preserved [22]: LPFH and non-LPFH groups. The LPFH group consisted of 8 patients with the preservation of the lateral pillar of the femoral head (including CJFH type L1). The non-LPFH group consisted of 32 patients without the preservation of the lateral pillar of the femoral head (including CJFH type L2 and L3). Also, the patients were grouped into two categories according to whether the femoral head collapsed or not. The precollapse group consisted of 4 patients with stage ARCO II, and the postcollapse group consisted of 36 patients with stage ARCO III and IV. After dividing the patients into the different groups, the IL-33 levels were compared within each of the groups.

2.2. Staging and Typing. The stages by ARCO classification system were stage II in 4 patients, stage III 20 patients, and stage IV in 16 patients. All subjects with ONFH underwent an MRI or CT evaluation according to CJFH type [20] (Figure 1) based on three pillars (Figure 2) [23]. According to the involvement of necrosis in the three pillars on a mid-coronal section on MRI or CT, ONFH location was divided into three types (M, C, and L), and the intact degree of the lateral pillar was divided into subtypes (L1, L2, and L3). Using this type to predict the prognosis of the patients with ONFH and the efficacy of joint-preserving surgery for ONFH [20], the CJFH types were type L1 in 12 hips, type L2 in 18 hips, and type L3 in 10 hips.

2.3. IL-33 Measurements. Blood samples were collected in sterile anticoagulation tubes from the included patients and the healthy controls, and the samples were centrifuged at 1600 rpm × 6 min to obtain plasma. The plasma was immediately frozen and stored at −80°C for analysis later. The plasma samples were tested for IL-33 levels using a commercially available ELISA kits (RayBiotech. Inc., Atlanta, USA) by following the user manual. The sensitivity of the kit is less than 2 pg/mL (range 2–500 pg/mL). The IL-33 levels were measured by two experienced independent investigators using the same instrumentation who were unaware of the study design in order to enhance measurement accuracy. All samples were duplicated during measurements. The two investigators had the same professional qualifications and were trained before the initial measurement. The data extraction and quality assessment were independently performed by two of the authors (WS. G and BL. W). If there were any disagreements, all of the authors discussed until consensus can be reached.

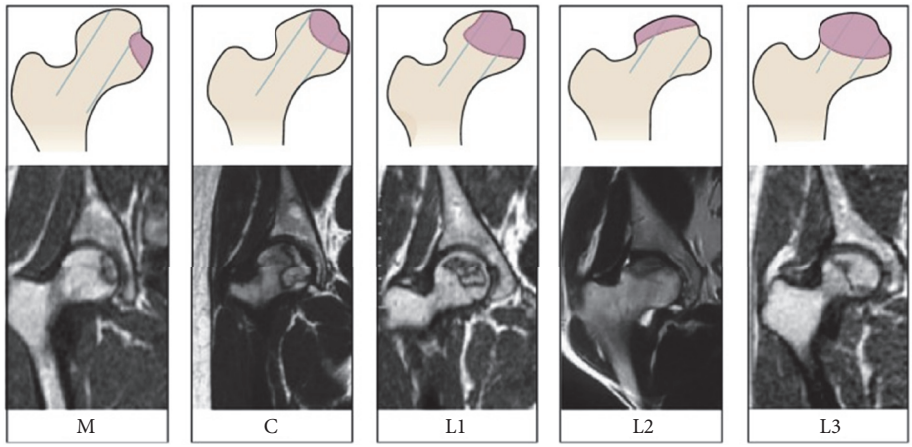

Figure 1: Schematic diagram and magnetic resonance image of China-Japan Friendship Hospital (CJFH) classification for osteonecrosis of the femoral head based on three pillars [19]. Type M: necrosis involves the medial pillar. Type C: necrosis involves the medial and central pillars. Type L1: necrosis involves the three pillars but the partial lateral pillar was preserved. Type L2: necrosis involves the entire lateral pillar and part of the central pillar. Type L3: necrosis involves the three pillars including the cortical bone and marrow.

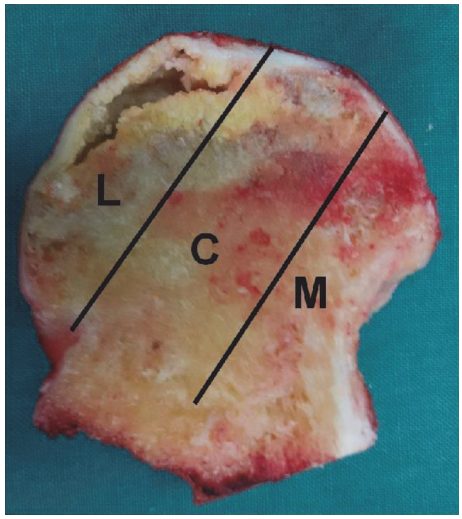

Figure 2: Image of coronal section of the femoral head showing three pillars of the femoral head: lateral (30%), central (40%), and medial (30%) [21].

Table 1: The demographics of patients with ONFH.

Demographic	Number/mean (SD)
Patients (M/F)	40
Male	31
Female	9
Mean age, year	49.2 (12.4)
Mean BMI, kg/m^2	24.2 (3.2)
Mean HHS, score	62.6 (14.6)
Mean length of disease history, month	38.9 (54.1)
Etiology	
Idiopathic	11
Corticosteroids	14
Alcohol	15
ARCO stage	
Stage II	4
Stage III	20
Stage IV	16
CJFH classification	
L1	12
L2	18
L3	10

2.4. Statistical Analysis. The data were analyzed using SPSS version 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Quantitative variables are reported as mean ± standard deviation (SD). Nonpaired *t*-tests were used to compare the IL-33 levels between different groups. One-way analysis of variance was used to compare IL-33 levels among different stage, type, and etiology groups. For statistically significant differences, groups were compared using the least significant difference (LSD) test. Pearson's correlation test was used to identify the correlation between the IL-33 levels and length of disease history. All tests were two-tailed at the 5% level of significance.

3. Results

Demographic data were comparable between the two groups (Table 2). Plasma IL-33 levels were significantly higher in the patients with ONFH (11.48 ± 8.34 pg/mL) than that in the control subjects (5.30 ± 4.36 pg/mL) ($P < 0.001$). The mean plasma IL-33 levels among the ONFH patients in the different etiologies, ARCO stages, and CJFH types are shown in Table 3. Despite the different ONFH etiologies (steroid use, excessive alcohol intake, or idiopathic origin), there were no significant differences between the cases with respect to the IL-33 levels ($P = 0.260$).

Table 2: Demographic data of ONFH group and control group.

	ONFH group ($n = 40$)	Control group ($n = 40$)	P value
Age (years)	49.2 ± 12.4	49.6 ± 16.0	0.895
Gender (male/female)	31/9	30/10	0.793
Height (cm)	168.3 ± 6.8	165.3 ± 9.0	0.099
Weight (kg)	69.0 ± 12.4	65.7 ± 9.7	0.192
BMI (kg/m^2)	24.2 ± 3.2	24.0 ± 2.7	0.743

Table 3: Plasma IL-33 levels between different groups.

	Group	IL-33 level (pg/mL)	P value
Etiology	Corticosteroids	9.07 ± 3.27	$P = 0.260$
	Alcohol	14.14 ± 11.17	
	Idiopathic	10.94 ± 7.96	
ARCO stage	II	7.54 ± 5.13	$P = 0.129$
	III	14.11 ± 10.70	
	IV	9.19 ± 3.38	
Collapse	Precollapse	7.54 ± 5.13	$P = 0.324$
	Postcollapse	11.92 ± 8.56	
CJFH type	L1	7.27 ± 4.16	$P = 0.010$
	L2	10.86 ± 0.86	
	L3	17.67 ± 14.55	
Lateral pillar	LPFH	7.27 ± 4.16	$P = 0.034$
	Non-LPFH	13.29 ± 9.06	

The levels of plasma IL-33 were 7.54 ± 5.13 pg/mL, 14.11 ± 10.70 pg/mL, and 9.19 ± 3.38 pg/mL in the ONFH patients with ARCO stage II, stage III, and stage IV, respectively. The levels of IL-33 did not differ significantly among the ONFH patients with different ARCO stages ($P = 0.129$). Although the postcollapse group showed a tendency of higher IL-33 level than the precollapse group, no significant differences were observed between the two groups ($P = 0.324$).

The levels of plasma IL-33 were 7.27 ± 4.16 pg/mL, 10.86 ± 0.86 pg/mL, and 17.67 ± 14.55 pg/mL in the ONFH patients with CJFH types L1, L2, and L3, respectively. The IL-33 levels of CJFH type L3 patients were significantly higher than that of CJFH types L1 ($P = 0.003$) and L2 ($P = 0.028$) patients, while the IL-33 levels of patients with CJFH type L2 were not significantly different with that of patients with CJFH type L1 ($P = 0.210$) (Figure 3). Also, the IL-33 levels were significantly higher in the non-LPFH group than that in the LPFH group ($P = 0.034$) (Figure 4).

The results of Pearson's correlation test between the IL-33 levels and length of disease history for the ONFH patients showed that the IL-33 level (11.48 ± 8.34 pg/mL) did not significantly correlate with the length of disease history (38.9 ± 54.1 months) ($r = -0.136$, $P = 0.403$).

4. Discussion

In the present study, our findings indicated that plasma IL-33 levels were significantly higher in the ONFH patients than that in the control subjects. And the IL-33 levels of patients with CJFH type L3 were significantly higher than that of patients with types L1 and L2. However, the levels of IL-33 did not differ significantly among the ONFH patients with different ARCO stages. No significant differences were observed in IL-33 levels between steroid-induced, alcohol-induced, and idiopathic patients.

The balance between osteoclasts and osteoblasts which determines the rate of bone remodeling could be disturbed by several molecular pathways. A variety of studies in vitro and in vivo have been conducted to investigate the effect of cytokines on osteoblasts and osteoclasts and their ultimate effect on bone resorption and formation [10, 24]. IL-33, as a proinflammatory cytokine, has been proved to be specifically released from osteonecrotic bones [18]. And our results showed that the elevated IL-33 levels that were observed in

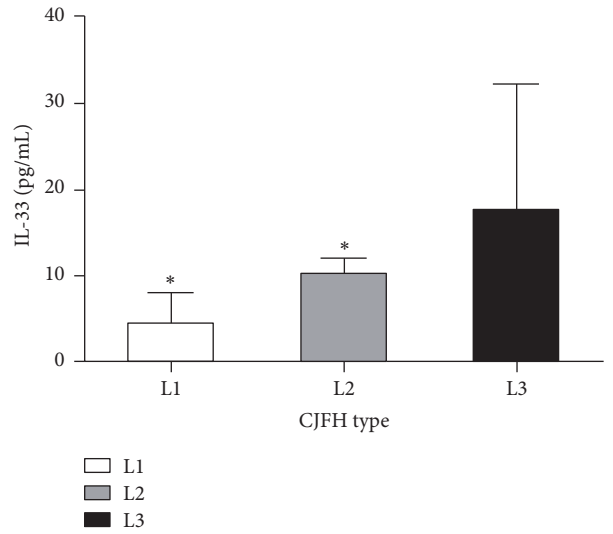

Figure 3: The IL-33 levels of CJFH type L3 patients were significantly higher than that of CJFH types L1 ($P = 0.003$) and L2 ($P = 0.028$) patients. $^*P < 0.05$ compared with patients with type L3.

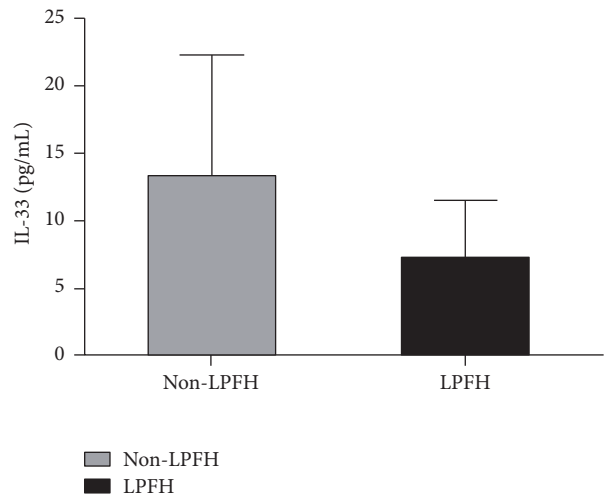

Figure 4: The IL-33 levels were significantly higher in the non-LPFH group than that in the LPFH group ($P = 0.034$).

the patients with ONFH also suggested this finding. During ONFH, IL-33 could modulate bone remodeling in both direct and indirect way after it is released from osteonecrotic bones [18]. IL-33 exerts its direct effects on osteoclastogenesis through the toll-like/IL-1 receptor ST2 [11]. The stimulatory effect of IL-33 on osteoclastogenesis may be in part direct. And Mun et al. [25] pointed that IL-33 stimulated formation of functional osteoclasts from human CD14(+) monocytes, enhanced expression of osteoclast differentiation factors including TNF-α receptor-associated factor 6 (TRAF6), nuclear factor of activated T cells cytoplasmic 1, c-Fos, c-Src, cathepsin K, and calcitonin receptor and eventually induced bone resorption. The property of IL-33 that regulates the inflammatory response and vascularization through modulating the recruitment and behavior of inflammatory cells may explain the indirect effect on bone remodeling during ONFH [18]. In addition, IL-33 may stimulate osteoclasts differentiation and activation and disturb necrotic bone repair by elevating vascular permeability [25, 26]. As a result, the normal balance of bone metabolism was disturbed and osteonecrosis of femoral head would progress.

Our study showed that the ONFH patients with CJFH type L3 hips had a higher IL-33 level than those with type L1 or L2. Also, the IL-33 levels were significantly higher in the patients without LPFH than that with LPFH. Previous studies [20, 22] in our institution have proven that the ONFH patients with CJFH type L3 hips involving all the three pillars of the femoral head had poor prognosis. Furthermore, the efficacy of joint-preserving surgery for patients with ONFH involving the lateral pillar was unsatisfactory [22]. In the patients with ONFH undergoing bone grafting through a window at the femoral head-neck junction, those with necrotic lesions involving the lateral pillar showed high surgical failure rates [27]. This indirectly illustrated that the increased IL-33 appeared to be related to the prognosis of ONFH and might act as a predictive factor for the efficacy of joint-preserving surgery in the treatment of ONFH.

Excessive corticosteroid administration is thought to be the primary risk factor in nontraumatic osteonecrosis [28]. Until now, the underlying molecular mechanisms of steroid-induced ONFH are still unknown [24]. Some researchers thought that the pathogenesis of corticosteroid-induced osteonecrosis may be due to reduced blood flow by numerous mechanisms, including marrow adipocytic hypertrophy leading to sinusoidal compression, venous stasis and, eventually, obstruction of the arteries and arterial occlusion by fat emboli and lipid-loaded fibrin-platelet thrombi [5]. Previous studies have shown that inflammatory cytokines are increased during the development of steroid-induced osteonecrosis, such as IL-1, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, and TNF-α [10]. Interestingly, we did not find that the increased plasma IL-33 level was related to risk factors of ONFH in our study, and there were no significant differences between steroid-induced, alcohol-induced, and idiopathic patients. Corticosteroid-induced osteonecrosis, which possesses the characteristic of decreasing bone formation, promoting osteoclastic resorption, impairing bone cell survival, and strengthening adipocytic differentiation, may be largely attributed to osteoblast and osteocyte apoptosis, rather than necrosis [24, 29, 30]. It is extremely important to note that IL-33 is not released during apoptosis. On the contrary, IL-33 is mainly retained inside apoptotic bodies during apoptosis, therefore preventing its accidental release [18]. In theory, the level of IL-33 should be decreased in steroid-induced ONFH. And low IL-33 levels in the patients with steroid-induced ONFH were observed in the present study.

The present study has some limitations. Firstly, the sample size is relatively small, and the analysis was not stratified for age and sex. Secondly, our observations were limited to inpatients, with no long-term follow-up. This marker may reflect the progression profile of osteonecrosis more precisely if it is based on the results from larger and more long-term clinical observations. Thirdly, the sensitivity and specificity of plasma IL-33 level have not been assessed. Fourth, we did not analyze the relationship between the necrotic area and the levels of plasma IL-33 in ONFH. Fifth, the controls were only matched in age, sex, and BMI, but not in other factors, such as alcohol intake. Last, the ONFH patients with stage ARCO I were not included in the study because most of them would not seek treatment in the absence of symptoms. Moreover, further studies are needed to clarify the effects of IL-33 on the natural progression outcomes and the joint-preserving surgery outcomes of patients with ONFH.

In conclusion, our findings seem to indicate that IL-33 effects may be detrimental during ONFH, which appeared to be associated with the prognosis of ONFH. The application of cytokines in the prognosis and therapy of ONFH shows promise; our findings may promote the development of novel therapies for the treatment of ONFH targeting IL-33. However, further experimental and clinical studies are still needed to investigate the mechanisms of IL-33 involved in the pathogenesis of ONFH and to determine the therapeutic potential of IL-33 blockade to improve bone repair after ONFH.

Disclosure

The first author is Jinhui Ma.

Competing Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

General and Specific Genetic Polymorphism of Cytokines-Related Gene in AITD

Chen Xiaoheng,[1] Mei Yizhou,[2] He Bei,[3] Li Huilong,[1] Wang Xin,[1] Hu Rui,[1] Li Lu,[1] and Ding Zhiguo[1]

[1]*Department of General Surgery, Dongzhimen Hospital of Beijing University of Chinese Medicine, Beijing, China*
[2]*State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, Beijing, China*
[3]*Department of Medical Service, The Third Clinical Medical College, Beijing University of Chinese Medicine, Beijing, China*

Correspondence should be addressed to Ding Zhiguo; dingzhiguo_1@163.com

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Autoimmune thyroid disease (AITD) shows the highest incidence among organ-specific autoimmune diseases and is the most common thyroid disease in humans, including Graves' disease (GD) and Hashimoto's thyroiditis (HT). The susceptibility to autoimmune diseases is affected by increased autoantibody levels, susceptibility gene polymorphisms, environmental factors, and psychological factors, but the pathogenesis remains unclear. Various cytokines and related genes encoding them play important roles in the development and progression of AITD. CD152, an expression product of the CTLA-4 gene, downregulates T cell activation. The A/A genotype polymorphism in the CT60 locus may reduce the production of thyroid autoantibodies. The C1858T polymorphism of the PTNP22 gene reduces the expression of its encoded LYP, which increases the risk of GD and HT. GD is an organ-specific autoimmune disease involving increased secretion of thyroid hormone, whereas HT may be associated with the destruction of thyroid gland tissue and hypothyroidism. These two diseases exhibit similar pathogenesis but opposite trends in the clinical manifestations. In this review, we focus on the structure and function of these cytokines and related genes in AITD, as well as the association of polymorphisms with susceptibility to GD and HT, and attempt to describe their differences in pathogenesis and clinical manifestations.

1. Introduction

Autoimmune thyroid disease (AITD) accounts for 90% of all thyroid diseases, mainly including Graves' disease (GD) and Hashimoto's thyroiditis (HT). Both diseases show similar pathological features and pathogenesis: (1) thyroid symmetric hyperplasia and thyroid lymphocyte infiltration; (2) varying number of thyroid antibodies detected in the serum; (3) family inheritance; (4) occurring in the same thyroid at the same time; and (5) GD and HT in the same patient which can be exhibited as phase transformation [1].

However, there are some differences in the pathology, clinical manifestations, and clinical outcomes of GD and HT. GD is the main cause of hyperthyroidism, accounting for more than 85% of all types of hyperthyroidism; hyperthyroidism syndrome and different degrees of goiter and exophthalmos are the most common clinical features [2]. Low thyroid parenchyma lymphocyte infiltration and simultaneously present thyrotropin receptor antibodies induce thyroid follicular cell proliferation, which eventually develops into hyperthyroidism. In HT onset, occult, early clinical symptoms are not typical because of the slow pace of development. As the disease progresses, approximately 25% of HT patients will suffer from hypothyroidism or transient hyperthyroidism, accompanied by diffuse enlargement of the thyroid. In patients with HT thyroid tissue, thyroid parenchymal lymphocyte infiltration is more serious than GD, which may cause thyroid follicular damage and ultimately become hypothyroidism [3].

AITD is a complex organ-specific autoimmune disease with risk factors that mainly include genetic factors, environmental factors, and autoimmune regulation disorders. AITD has a genetic background, and smoking, infection stress, iodine content in food, drug effects, radiation exposure,

mental stress, and other factors can induce thyroid autoantibodies and autoantibodies, a process involving a variety of cytokines [4]. Under normal physiological conditions, many cytokines and immune cells precisely regulate the dynamic balance of the body's immune function. Once the body's immune balance becomes abnormal, AITD will occur [5]. Abnormal expression of a variety of cytokines such as HLA, CD152, LYP, FcRL3, CD40, and their genes will inhibit autoimmune tolerance [6].

2. Cytokines and Cytokine-Related Genes in AITD

2.1. HLA-II. HLA class II molecules are cell surface receptors that bind to antigenic peptides and present them to T cells. HLA on the short arm of chromosome 6 6p21.3 has a full length of approximately 4000 kb and consists of a group of closely linked genes, including more than 100 loci from a total of 554 alleles. HLA is currently known as the most complex and polymorphic gene in the human genome. Genetic and environmental factors play an important role in autoimmunity and increase the risk of HLA-susceptible alleles in some circumstances. HLA-II gene polymorphisms determine the diversity of HLA-II molecules. More than 70 diseases have been associated with HLA polymorphisms, and many autoimmune-related diseases are associated with HLA-II genes.

Polymorphisms in the HLA-II gene are very important for regulating immune activity. These polymorphisms determine the specificity of binding to an antigen and initiation of the immune response, as well as affecting the differentiation of T cells in the thymus. HLA also controls the secretion of cytokines and modulates the immune response by cytokine genes on haplotypes. Susceptibility alleles on HLA may also lead to GD/HT by preferentially regulating the Th2/Th1 pathways, respectively [7–10].

There are two hypotheses regarding the genetic predisposition mechanism of HLA and AITD. The structure and function of HLA itself are associated with disease development, such as the molecular modeling hypothesis, linkage disequilibrium hypothesis, autoantigen presentation hypothesis, and T cell receptor pool selection hypothesis. Another hypothesis is that other genes linked to HLA are associated with AITD. McLachlan [11] found that HLA molecules that bind to specific antigenic peptides misinterpret thyroid tissue antigens as T lymphocytes and CD4+/CD8+ T lymphocytes in the presence of environmental factors, bacteria, viruses, iodine, and stress (such as trauma). This immune response occurs through the activation of T and B lymphocytes to produce cytokines and autoantibodies, which may cause AITD. Sanjeevi et al. [12] suggested that the distal domain of the extracellular domain of HLA-II is an antigen-binding groove containing an important amino acid associated with the disease. Any changes in the amino acid at these sites can alter the nature of the interaction between HLA antigen and the T cell receptor to control the immune response to foreign and autoantigens. Barlow et al. [13] suggested that, for AITD to occur, a number of requirements must be met. Clinical symptoms of the disease are observed when environmental and genetic factors are encountered and exceed a certain theoretical threshold.

2.2. CTLA-4 Gene and CD152. Since Yanagawa et al. [14] first described the relationship between CTLA-4 and AITD in 1995, the correlation between CTLA-4 and the incidence of GD has been verified in a number of ethnic groups [15–18].

One of the expression products of the CTLA-4 gene is CD152, which was originally proposed by Brunet et al. [19] in 1987 and is mainly expressed on activated CD4+ CD8+ T cells. CTLA-4 is typically located in the cytoplasm and is rapidly expressed on the surface of the cell membrane 48–72 h after T cell stimulation [20]. The mechanism by which CTLA-4 downregulates the activation of T cells is as follows: CD152 expression on T cells increases in the late stage of immune activation and competes with CD28 for binding to B7. In the early stage of CD152 activation, CTLA-4 binds to the intracellular domain and mediates the negative signaling that inhibits T cell activation. CTLA-4 plays an important role in the development of a variety of autoimmune diseases. Its activation was shown to inhibit autoimmune diseases such as systemic lupus erythematosus, autoimmune glomerulonephritis, and type 1 diabetes in a variety of experimental animals [21].

Incorrect handling of CTLA-4 in the endoplasmic reticulum leads to inefficient glycosylation and reduced CTLA-4 protein expression on T cells. The reduction of CTLA-4 on T cells and variations in the CTLA-4 gene resulted in decreased CD152 expression and function, which may be related to the occurrence of AITD [22–24]. In a study on adolescent HT patients, Kouki et al. [25–29] found that CD152 expression was decreased in the HT group compared to that in the normal control group, indicating that peripheral blood T cells contained CD152 expression defects. This may abnormally activate T cells and lead to increases in thyroid autoantibodies, eventually leading to AITD. In a study by Bossowski [30, 31], CD152 expression on peripheral blood T cells increased in GD patients, which may be related to defective CD152 function caused by CTLA-4 gene polymorphisms. The increased expression of CTLA-4 on T cells is sufficiently effective to suppress the abnormal activation of T cells.

The association between CTLA-4 gene polymorphisms and GD has been demonstrated in studies worldwide. Transitions of threonine (Thr)/alanine (Ala) in the 17-codon 49-site A/G of the exon 1 leader of the CTLA-4 gene lead to errors in the handling of CTLA-4 in the endoplasmic reticulum, leading to inefficient glycosylation reaction and reduced expression of CTLA-4 protein on the T cell surface [30, 31]. The 49G allele decreased the inhibitory function of CTLA-4 on T cells. In GD patients, the AT repeat polymorphism can reduce the inhibitory effect of CTLA-4; as the length of the AT repeat sequence increases, T cell inhibitory function of CTLA-4 decreases. Genealogy scans were performed on a number of American Caucasian families with AITD, revealing thyroid autoantibodies for the individual susceptibility locus at 2q33. Compared to the control group, these families with the G (49) single-nucleotide polymorphism (SNP) of the CTLA-4 A/G genotype showed significantly increased thyroid autoantibody production [28] ($P = 0.02$).

2.3. PTPN22 Gene and LYP. The protein tyrosine nonreceptor type 22 (PTPN22) gene is located at 1P13.3-13.1, contains 16 exons, and encodes a 110 kDa lymphoid protein tyrosine phosphatase (LYP), a potent inhibitor of T cell activity.

LYP dephosphorylates the phosphorylated Lck, Fyn, and Zap-70 tyrosine kinases of the Src family to inactivate them by interacting with the C-terminus of Src tyrosine kinase (Csk). LYP interrupts T cell activation of signaling pathways, thereby downregulating T cell signaling [32]. LYP also suppresses T cell signaling by binding to Csk and interacts with Grb2, playing a negative regulatory role in T cell signaling [33].

LYP inhibits excessive immune responses and thus plays an extremely important role in autoimmune diseases. There are two views to illustrate this mechanism. One theory is functional acquisition. The T cell surface receptor (TCR) signal of both PTPN22 C1858T polymorphism carriers and patients was found to be weakened, but the PTPN22 expression product containing a polymorphism had stronger inhibitory effect on T cells than wild type.

In addition, the B cell surface receptors (BCR) signal is also decreased, suggesting that B cell function is also affected by gene polymorphisms. Changes in the BCR threshold may also damage B cell tolerance, and coupled with the presence of autoreactive B cells, this may lead to autoimmune diseases [34, 35]. Another paradox is the lack of gene function, which was also observed in knockout mice. The expression of LYP in PTPN22 C1858T polymorphism mice, adhesion to lymphocyte surface receptor, and immunosuppressive effect were decreased, enhancing the immune function of effector T cells, B cells, and dendritic cells [36]. The 1858 locus C→T missense mutation changes codon 620 in the LYP protein from tryptophan to tryptophan. This alters LYP function, significantly reduces the affinity to Csk, reduces the inhibition of the T cell activation signaling pathway, and may induce autoimmune diseases [37]. A recent study found that the PTPN22 1858 C/T polymorphism was associated with AITD [38], RA, SLE, and T1DM [39].

2.4. FcRL3. FcRL3, also known as crystallizable fragment receptor (FcR) homolog (FcRH3), is a membrane glycoprotein. The FCRL3 gene is one of five genes in the FCRL family and has obvious structural homology with the other four genes. FCRL expression is regulated by B cell differentiation, and germinal center B cells can express high levels of FCRL3 [40]. Its functional structure lies in its cytoplasmic domain, the presence of immune receptor tyrosine-activated motif (ITAM), and immune receptor tyrosine inhibitory motif (ITIM) [41]. In vitro studies have shown that tyrosine kinase syk and ZAP70 can be recruited to the ITAM region, and tyrosine phosphatases SHP-1 and SHP-2 are recruited to the ITIM region to enhance enzymatic activity and fine-tune the T cell activation signal [42].

Previous studies found that FCRL3 promoter polymorphisms are closely related to AITD and other autoimmune diseases such as SLE and RA. This polymorphism affects the expression levels of autoantibodies. The −169C susceptibility allele can bind more tightly to NF-κB compared to −169T. This can significantly enhance the transcription efficiency of the gene [40]. Transfection studies revealed that the −169C allele was significantly more potent than the −169T allele, suggesting that transcriptional activity was enhanced [40]. The mechanism may increase the affinity of FCRL3 isoforms for NF-κB, and NF-κB enhances FCRL3 expression. Highly expressed FCRL3 promotes the production of autoantibodies. This interaction produces additional FCRL3 and autoantibodies. The FCRL3 promoter polymorphism and NF-κB promoter polymorphism interact synergistically to promote the expression of FCRL3 and production of autoantibodies [43].

2.5. CD40. The CD40 gene is located on chromosome 20q11.2-20q13.2. CD40 is mainly expressed on the surface of antigen-presenting cells and can specifically bind to CD40L on the surface of target cells. Antigen-presenting cells present antigens to T cells to form MHC-TCR complexes. Transient upregulation of CD40L expression on the T cell surface and the synergism of CD28/B7 amplify the T cell-dependent immune response [44].

Both the membrane-bound form and free sCD40L can bind to CD40, which binds to monocyte-macrophages and secretes interleukin- (IL-) 12 from dendritic cells, while activating T cells to become helper T cells 2 (Th2), shifting the Th1/Th2 balance to the Th2 pathway. Monocyte-macrophage and dendritic cells can be induced to secrete IL-12 [45, 46], and activated T cells can be differentiated into Th2. In addition, CD40 has an important role in the differentiation and development of various stages of B cells [47]. CD40 and CD40L can also inhibit T cell function, and this negative regulation may lead to autoimmunity [48].

Immunohistochemistry and flow cytometry were used to confirm the increased expression of CD40 in thyroid follicular cells from GD patients in vivo and in vitro [49]. The level of CD40 and CD40L in the serum of patients with GD hyperthyroidism was higher than that of healthy subjects by enzyme-linked immunosorbent assay [50]. These studies showed that CD40 and AITD are closely related. CD40 in GD patients' thyroid tissue, particularly in epithelial cells, follicular cells, and fibroblasts, showed increased expression to different degrees [49]. The autoantigens of GD patients are processed by thyroid follicular epithelial cells and presented to the infiltrating T cells of the thyroid tissue to induce their activation, and CD40/CD40L upregulate the immune response during this process [51]. After T cell activation, the expression of CD40/CD40L increases on the cell surface, leading to a variety of autoimmune diseases, including GD [52, 53]. The interaction of CD40/CD40L molecules activates CD40 antigen-presenting cells, expressing FasL on the cell surface and inducing the activation of Fas+ T cells [54].

3. Genetic Polymorphism of Cytokine-Related Genes Associated with Both GD and HT

3.1. CTLA-4 Polymorphism. Polymorphisms in the promoter −318C/T, exon 1 + 49A/G in the 3′-untranslated region of exons, and CT60 in the CTLA4 gene have been confirmed to be associated with organ-specific autoimmune diseases [25, 55, 56]. The SNPs of exon 1 + 49 A/G are highly related to

autoimmune endocrine diseases and are involved in a variety of autoimmune diseases including GD and HT [14, 57]. The TG gene at the 8q24 locus of the CTLA-4 gene is strongly associated with AITD. Previous studies demonstrated that amino acid substitutions occur in the SNPs of exon 33 and exons 10–12, which result in autoimmune disease in the thyroid tissue [58]. The CT60 polymorphism is one of the most closely correlated polymorphisms with autoimmune thyroid disease [59]. Studies have suggested that GD and HT are associated with CT60 [15].

By evaluating 67 newly diagnosed GD patients to identify their genotypes and autoantibodies, Zaletel et al. [60] found that for patients carrying the G genotype compared to the A/A genotype, TPOAb, the TgAb-positive rate was significantly higher. For the G allele, the median value of TPOAb was significantly higher than the other groups, confirming that the CTLA-4 exon 1G allele can produce higher levels of TPOAb and TgAb, supporting that the CTLA-4 gene plays an important role in thyroid autoantibody production. A study of HT showed that the A/A genotype produced less TPOAb and TgAb than the AG, GG genotype. These results support that the polymorphism of the CLTA-4 gene is closely related to the production of thyroid autoantibodies [61].

3.2. PTPN22 Polymorphism. A study conducted in 2004 showed that the PTPN22 C1858T polymorphism was associated with T1MD [62]. Subsequently, a variety of autoimmune diseases associated with T1MD, such as SLE, RA, HT, juvenile rheumatoid arthritis, vitiligo, and neutrophil-positive Wegener granulomatosis, were found. T alleles were not detected (or only a small number was detected) in other autoimmune diseases such as psoriasis, multiple sclerosis, or primary biliary cirrhosis [63–70]. These studies suggest that the PTPN22 C1858T polymorphism is a common pathogenic gene involved in autoimmune diseases.

Velaga et al. [38] evaluated a UK population and found that the PTPN22 1858T allele is the main susceptibility allele for GD. In a Polish population, PTPN22 1858T was associated with GD and negatively correlated with age at onset. The TT genotype resulted in an onset age of 20.8 years, CT genotype of 35 years, and CC genotype of at least 42 years (mean age) [71]. Tryptophan variants of the PTPN22 gene are very rare in the Asian population [72]. The association shows a significant population difference, possibly because of ancestral effects and/or the lack of susceptible variants in the population. The 1858T allele frequency decreased from the north to south in Europe, and, in central Africa and Asia, the allele was nearly nonexistent in the healthy population [73–75].

4. Specific Genetic Polymorphism of Cytokine-Related Gene Associated with GD

4.1. HLA Gene Polymorphism. In recent years, as the number of studies on HLA haplotypes has increased, it was shown that genes constituting the HLA gene cluster are highly linked. Disease-specific susceptibility genes associated with AITD are not single but are a combination of several genes. In the haplotype, each susceptible gene can independently express its genetic characteristics in order to exert its function. A series of different haplotypes are formed through the strong linkage disequilibrium in HLA genes.

In the 1990s, with the application of HLA genotyping methods, researchers carried out a more detailed analysis of susceptibility genes and found that HLA and GD were related mainly in the DQ site. In a study on Caucasians, Yanagawa et al. [76] used the PCR-sequence-specific oligonucleotide method to analyze the DQ DR allele of GD patients in the US and found that the HLA-DQA1*0501 gene frequency was significantly higher in GD patients and positively correlated with the incidence of GD. A study on GD patients carried out in Germany and Canada also confirmed these results. In a study on Asians, Inoue et al. [77] found that Japanese patients with GD did not show a significant correlation with HLA, and Tamai [78, 79] found no significant correlation with DQ allele and GD in a Japanese population.

4.2. FcRL3 Gene Polymorphism. In a correlation analysis of GD and the FCRL3 SNP, rs7528684 was first identified by Kochi et al. [40] in a Japanese population. The SNP was located at position −169 of the FCRL3 gene promoter ($P = 7.4 \times 10^{-5}$) and was subsequently confirmed by Cappelli et al. [80] in 1059 GD patients ($P = 0.024$). Subsequently, the FCRL3-related rs3761759 SNP (tag rs7528684) detected in GD patients was also found in GD patients of Caucasian ethnicity in a study by Simmonds et al. [81]. Regression analysis showed that rs3761759 association was secondary to the FCRL3 rs11264798 and rs10489678 SNPs, and thus, FCRL3 rs11264798 and rs10489678 may affect the role of FCRL3 in the occurrence of GD. Therefore, FCRL3 SNPs can lead to GD, but the variation location for the etiological variant remains to be verified [81].

4.3. CD40 Gene Polymorphism. CD40 plays an important role in the development and progression of various autoimmune diseases. A study of GD and HT patients in a Chinese population found that the CD40 C/T-1 polymorphism was associated with GD risk [82]. The CD40 C/T-1 polymorphism was found significantly associated with GD in a meta-analysis of 14 studies (4214 cases and 3851 controls) and 4 studies (623 cases and 774 controls) investigating the association between CD40 C/T-1 gene polymorphism and autoimmune thyroid disease risk. Carriers of the C/C and C/T genotype had a higher risk of GD than those with the T/T genotype. However, these genotypes were not found to be associated with HT, suggesting that this is a unique feature of GD [83].

5. Specific Genetic Polymorphism of Cytokine-Related Gene Associated with HT

5.1. HLA Gene Polymorphism. Early serological analysis found that DR3, DR4, and DR5 molecules correlated with the occurrence of HT in the Caucasian population [84]. Different gene signatures associated with HT may reflect the heterogeneity of different patients. In recent years, molecular biology techniques have developed rapidly. Badenhoop et al.

first used RFLP method for the analysis of HT in Caucasian patients from the UK and Canada and found that DQW7 DQB1*0301 frequency increased. They concluded that DQW7 might be an immunogenetic marker on susceptible HLA haplotypes [85]. Shi et al. [86] found that the frequency of DQA1*0301 DQB1*0201 in Canadian patients with HT was significantly increased by PCR-sequence-specific oligonucleotide, and the susceptibility of HT might be through DQA1*0301/DR4 DQB1*0201/DR3.

6. Conclusion and Prospects

The pathogenesis of AITD is not completely clear. In the natural course of AITD, cytokines play a critical role, particularly in breaking autoimmune tolerance, autoantigen presentation, T and B lymphocyte activation, autoantibody production, and inhibition of autoimmunity. Therefore, changes in the expression of these cytokines are important pathogenic factors of AITD.

Although the mechanism is still not entirely clear, the occurrence of GD and HT has the same cytokine-related genes abnormalities. These aberrantly expressed genes may indicate a common occurrence in AITD. The PT1822 C1858T polymorphism was found to increase the risk of GD and HT [38]. Because C1858T can produce LYP, one of the AITD-related cytokines, expression decreases and the effect of inhibition of T lymphocytes is weakened, leading to autoimmune diseases. Therefore, PTPN22 may be a key gene in the breaking of immune tolerance. As inhibitory factors, CTLA-4 gene polymorphism [25, 55, 56, 87] and A/A genotype [60] expression may reduce the production of TPOAb and TgAb, leading to the onset of AITD. These cytokine-associated gene polymorphisms directly affect cytokine expression levels to break immune tolerance, causing specific organ damage. In future studies, the role of PTPN22 in the process of autoimmune tolerance breaking during the initial phase of autoimmune disease as well as the negative immunoregulatory effect of the CTLA-4 gene should be determined to reveal the common mechanisms of these diseases.

GD and HT are the same type of diseases, but their clinical manifestations and the final outcomes are very different. This suggests that GD and HT also have their own specific pathogenic mechanisms in addition to the cooccurring mechanisms of AITD. Although both GD and HT exhibit autoimmune tolerance breaking and autoantigen presentation, the expressions of various cytokines such as HLA-II molecules, CD152, FcRL3 molecules, and CD40 are different and are closely related to HLA-II, CTLA-4e, FcRL3, and CD40 gene polymorphisms. FcRL3 promoter polymorphism and CD40/CD40L high expression will lead to increased autoantibodies. This will only lead to the occurrence of GD while the aberrant expression of other genes will only induce HT. This apparent difference in cytokines and cytokine-related genes may explain the differences in the pathogenesis of GD and HT during disease progression and their underlying clinical manifestations. Additional studies on the differential genes and cytokines may reveal the pathogenesis of GD and HT, providing a foundation for the development of specific treatments.

Disclosure

Mei Yizhou is common first author and Ding Zhiguo is corresponding author.

Competing Interests

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

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Research Article

Serum Cytokine Profiles in Children with Crohn's Disease

Ekaterina Vasilyeva,[1] Sayar Abdulkhakov,[2,3] Georgi Cherepnev,[2,3,4] Ekaterina Martynova,[2] Irina Mayanskaya,[1] Alina Valeeva,[5] Rustam Abdulkhakov,[5] Dilyara Safina,[5] Svetlana Khaiboullina,[2,6] and Albert Rizvanov[2]

[1] *Volga Region Federal Medical Research Center of the Ministry of Public Health, Nizhny Novgorod, Russia*
[2] *Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia*
[3] *Kazan Federal University Clinic, Kazan, Russia*
[4] *Department of Clinical Laboratory Diagnostics, Kazan State Medical Academy, Kazan, Russia*
[5] *Kazan State Medical University, Kazan, Russia*
[6] *Department of Microbiology and Immunology, University of Nevada Reno, Reno, NV, USA*

Correspondence should be addressed to Svetlana Khaiboullina; skhaiboullina@medicine.nevada.edu and Albert Rizvanov; albert.rizvanov@kpfu.ru

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Crohn's disease (CD) is a chronic inflammatory bowel disease that can be diagnosed at any age. There are two major patient groups based on diagnosis of this disease, before or after the age of 20 (juvenile/adolescent or adult), with disease progression in adults usually milder than in juvenile CD patients. Immune mechanisms have been suggested to play an important role in CD pathogenesis, with cytokines governing the development of the immune response. Upregulation of inflammatory cytokines in serum of juvenile and adult CD patients has been documented; still little is known about age-dependent differences in serum cytokine profiles of CD patients. We applied multiplex technology to analyze serum levels of 12 cytokines in juveniles and adults. We show that during the acute stage of the disease all CD patients have high serum levels of CXCL10, which remains upregulated during remission. Increased serum levels of TNF-α and IL-6 during the acute stage was characteristic of juvenile CD patients, whereas adult CD patients had upregulated levels of GM-CSF and IFN-γ. Taken together, these results demonstrate age-dependent differences in cytokine profiles, which may affect the pathogenesis of CD in patients at different ages of disease onset.

1. Introduction

Crohn's disease (CD) is a form of inflammatory bowel disease (IBD) affecting the entire gastrointestinal (GI) tract [1–4]. CD is characterized by transmural inflammation of GI wall in a noncontiguous pattern anywhere from the mouth to the anus [5, 6]. Little is known about the etiology. It is believed that, in genetically predisposed individuals, interaction between local microbiota and the intestinal mucosa can cause chronic inflammation and ulceration [7–9]. A key pathological feature of CD is a granulomatous inflammatory response, characterized by the presence of epithelioid and multinucleated giant cells as well as macrophage infiltration [10, 11]. It is believed that the onset and development of the inflammatory reaction depend on persistence of an inciting agent, resulting in a chronic inflammatory milieu and perpetuating a complex immune reaction subsequent to the local granulomatous response. Persistent inflammation and immune activation are believed to be the leading causes of tissue necrosis and fibrosis.

Clinically CD is characterized by reoccurring episodes that are typical of IBD [12]. Newly diagnosed CD usually presents with diarrhea, abdominal pain, fever, fatigue, stomatitis, and weight loss [12]. In more advanced cases, strictures and fistulas may develop, changing the clinical presentation to include severe abdominal pain, distension, bloating, vomiting, perianal fistulae, and abscesses. CD can be diagnosed at any age; however disease progression and clinical presentation frequently differ in young and adult patients [13]. CD in children and adolescents tends to have a more severe clinical

manifestation often with development of strictures or fistulae [1, 3, 14]. In contrast, later onset of CD is often characterized by a milder course with a lower frequency of complications [13, 14]. Furthermore, it has been shown that the childhood-onset CD is more likely to require immunosuppressive therapy and has a higher frequency of surgery as compared to CD diagnosed in adulthood [4, 13]. Additionally, juvenile and adolescent onset CD has more frequent involvement of the small bowel, while adult CD is generally more likely to be colon-associated [15–19].

An altered immune response, together with genetic and environmental factors, may govern the timing of CD onset. There is a higher frequency of CD family history in patients diagnosed before the age of 20 relative to those diagnosed later in life, suggesting that genetic predisposition plays a role in early onset [13, 20]. Furthermore, immune mechanisms may be important in the pathogenesis of CD. For example, it has been shown in adult CD that higher levels of serum antibody reactivity towards increasing amount of microbiota in small intestine correlate with a greater frequency of complications [21, 22]. A similar observation has been made in a pediatric cohort [23, 24]. Furthermore, CD patients have been shown to have increased numbers of circulating Th17 lymphocytes and upregulation of IL17 transcriptional activity in intestinal mucosa [25]. This indicates that the progression of CD is characterized by an exacerbated Th17 response, which may explain the continuing nature of the disease.

Immune mechanisms are believed to play a role in the pathogenesis of adult and juvenile CD, with cytokines being essential to establish and maintain the immune response. However, little is known about differences in serum cytokine profiles between juvenile and adult CD cases. Therefore, we sought to determine whether the cytokine profiles differ. Potentially this information could suggest biomarkers related to the age of onset. We have found that all CD patients have upregulated serum CXCL10 levels, regardless of the age of diagnosis and the stage of the disease. Also, we have demonstrated upregulation of IL-1b and IL-6 in juvenile CD, with no changes detected in levels of GM-CSF and IFN-γ. In contrast, the adult CD serum profile was characterized by upregulation of GM-CSF and IFN-γ, as well as IL-6 and IL-1b, when compared to controls. It has been shown that GM-CSF and IFN-γ promote a Th1 immune response, while IL-6 is essential for activation of Th17 lymphocytes [26–28]. Therefore, this suggests that the mechanisms of pathogenesis differ in adult and juvenile CD patients.

2. Materials and Methods

2.1. Subjects. Clinical surplus serum samples from 64 patients diagnosed with CD were utilized in this study; 12 juvenile patients (6 male, 6 female; average age 14.0 ± 2.1) hospitalized in the Volga Region Federal Medical Research Center of the Ministry of Public Health (Nizhny Novgorod, Russia) and 52 adult patients (26 male, 26 female, average age 35.1 ± 13) hospitalized in the Department of Gastroenterology of Republican Clinical Hospital (Kazan, Russia). Diagnosis of CD was established in all patients based on clinical presentation and was verified by upper GI endoscopy.

Serum samples were collected from patients during both acute stage and remission. Additionally, a small number of samples (6) were collected from juvenile patients during their period of clinical improvement. Age and gender matched control samples were collected from 10 juvenile (5 male, 5 female) as well as from 23 adult (13 male, 10 female) controls. All serum samples were stored at -80°C until use. The study was approved by the Ethics Committee of Kazan State Medical University (protocol 23) and the Ethics Committee of Volga Region Federal Medical Research Center of the Ministry of Public Health Nizhny Novgorod (protocol 4). Informed consent was obtained from each study subject and legal guardian.

2.2. Cytokine Analysis. Serum cytokine levels were analyzed using single-plex sets for IL-1b, IL-2, IL-5, IL-4, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, IFN-γ, CXCL10, and TNF-α (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Serum aliquots (50 μL) were used for analysis, with a minimum of 50 beads acquired per analyte. Median fluorescence intensities were measured using a Luminex 200 analyzer. Data collected was analyzed with MasterPlex CT control software and MasterPlex QT analysis software (Hitachi Software, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by the manufacturer.

2.3. Statistical Analysis. Statistical analysis was performed using the STATISTICA 7.0 Software Package (StatSoft, Tulsa, OK, USA). Data are presented as the median (25th–75th percentile range) for continuous variables. Differences between independent study groups were tested by nonparametric methods. The Kruskal-Wallis ANOVA by Ranks test was used for multiple independent samples. Pair comparisons were made by the Mann–Whitney U test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Serum Cytokine Activation in Juvenile Crohn's Disease Patients. Levels of 12 cytokines were analyzed in juvenile CD serum collected during both the acute stage and remission. Acute serum was characterized by upregulation of IL-1b, IL-6, CXCL10, and TNF-α levels compared to controls (Table 1). In contrast, no changes in IL-2, IL-5, IL-4, IL-10, IL-12p40, GM-CSF, and IFN-γ were found in acute juvenile patients compared to controls. Median values of IL-8 in acute serum were upregulated (1415.30 [997.1–2654.4] versus 2777.00 [611.0–7382.5]); however, differences were not statistically significant from controls.

During remission, serum levels of CXCL10 ($P < 0.05$) and IL-1b ($P < 0.05$) remained significantly upregulated in juvenile CD patients compared to controls (Table 1). The median value of IL-1b during remission was higher than that in acute patients (832.75 [481.9–1016.0] versus 550.00 [192.0–784.3]), suggesting an upward trend in cytokine production ($P = 0.067$). IL-6 and TNF-α serum levels were significantly higher during the acute stage but then declined in remission and remained comparable to controls. Similarly,

Table 1: Serum cytokine activation in juvenile CD (median [25th–75th percentile]).

Cytokine (pg/mL)	Controls ($n = 10$)	Acute patients ($n = 25$)	Remission patients ($n = 12$)
IL-1b	107,7 [16,4–340,8]	550,0 [192,0–784,3]*	832,75 [481,9–1 016,0]*
IL-2	2,3 [1,5–3.0]	4,3 [3,2–5,4]	5,5 [4,1–7,7]
IL-5	9,0 [3,5–11,8]	6,4 [3,4–16,0]	8,0 [4,5–15,6]
IL-6	23,5 [17,2–27,3]	51,8 [22,8–67,3]*	20,3 [12,9–25,3]**
IL-8	1 415,3 [997,1–2 625,4]	2 777,0 [611,0–7 382,5]	4 194,0 [411,9–7 220,9]
IL-12	203,2 [120,3–280,0]	217,5 [137,5–519,5]	225,8 [69,8–286,1]
GM-CSF	30,4 [13,5–38,0]	32,0 [10,2–45,3]	35,3 [21,6–50,0]
IFN-γ	2,5 [1,8–3,2]	3,8 [3,2–4,8]	2,8 [2,2–4,3]
CXCL10	384,9 [265,3–435,4]	3 660,0 [2 809,5–4 457,5]*	3 685,5 [2 663,5–5 124,0]*
TNF-α	4,6 [3,3–6,2]	8,5 [5,3–12,0]*	5,0 [3,4–8,6]
IL-4	8,5 [6,3–9,1]	8,7 [7,4–11,7]	7,1 [6,2–8,4]
IL-10	23,9 [17,6–32,4]	22,0 [16,0–60,0]	11,8 [5,9–20,3]**

* $P < 0.05$ versus controls (Mann–Whitney test).
** $P < 0.05$ versus acute patients (Mann–Whitney test).

Table 2: Changes in the peripheral lymphocyte population in juvenal CD.

Lymphocytes (10^9/L)	Control ($n = 10$)	Acute patients ($n = 25$)	Remission patients ($n = 12$)
CD3+	1.91 ± 0.5	1.34 ± 0.4	1.22 ± 0.3
CD8+	0.73 ± 0.2	0.52 ± 0.2	0.56 ± 0.1
CD4+	1.93 ± 0.3	0.82 ± 0.1	0.74 ± 0.2
CD16+CD56+	0.12 ± 0.1	0.26 ± 0.08*	0.14 ± 0.02**
CD19+	0.71 ± 0.3	0.34 ± 0.2	0.25 ± 0.2

* $P < 0.05$ between patients and control.
** $P < 0.02$ between acute and remission CD.

in acute stage patients, median values of IL-8 were upregulated during remission (1415.30 [997.1–2654.4] versus 4 194.0 [411.9–7 220.9]); however, differences were not statistically significant from controls.

3.2. Analysis of Lymphocyte Profile in Juvenile CD Subgroups (Acute Stage and Remission). Several leukocyte surface markers were examined including CD3 (lymphocytes), CD4 (T-helper (Th) lymphocytes), CD8 (cytotoxic T lymphocytes (CTL)), CD16 (natural killer (NK) cells), CD19 (B lymphocytes), and CD56 (NK cells) (Table 2). Increased numbers of CD16+CD56+ leukocytes were characteristic for acute stage juvenile CD compared to remission (0.26 ± 0.08 versus $0.14 \pm 0.02 \times 10^9$/L; $P < 0.02$) (Table 2). When the CD4/CD8 ratio was analyzed, two groups of acute stage patients could be identified having a ratio higher or lower than 2.0. Since cytokines play a central role in differentiation and proliferation of leukocyte CD4 and CD8 subsets, we analyzed serum cytokines in the CD4/CD8 subgroups of juvenile CD patients (Table 3). Acute stage patients with a CD4/CD8 ratio above 2.0 were characterized by upregulated serum levels of IL-1b, CXCL10, TNF-α, and IL-10. Additionally, high CD4/CD8 ratio patients had a greater IL-8 median value compared to those with a CD4/CD8 ratio below 2.0 (4161.50 [2956.0–8372.8] versus 1760.00 [257.4–6014.1]); however, these differences were not significant. Interestingly, CXCL10 levels were significantly higher in juvenile CD patients with a CD4/CD8 ratio below 2.0 than those with a CD4/CD8 ratio above 2.0.

3.3. Serum Cytokine Profile of Adult CD Patients. Serum cytokine profiles in adult CD patients depended on the stage (acute or remission) of the disease. For example, a subset of serum cytokines, including IL-1b, IL-6, GM-CSF, IFN-γ, and CXCL10, were upregulated in the acute stage of CD relative to controls (Table 4) and remained upregulated as in remission. However, serum levels of TNF-α and IL-10, unchanged in acute stage the disease, were downregulated during remission (Table 4). It should be noted that the IL-12 median value was 20-fold higher in adult CD patients in either acute stage or remission than controls, though not statistically significant. Although these differences were not significant, there was a strong trend towards upregulation in adult serum, both during the acute stage and the remission. Interestingly, in contrast, the serum level of IL-12 in juveniles in both the acute stage and the remission did not differ from controls. Further, in serum of acute stage, adults with CD both GM-CSF and IFN-γ were upregulated, whereas these cytokines remained unchanged in acute juvenile CD patients. This strongly suggests that several cytokines, including GM-CSF, IFN-γ, and IL-12, play a role in the pathogenesis of CD in adult patients.

4. Discussion

CD is a chronic inflammatory bowel disease affecting the integrity and function of the GI tract. The disease has a bimodal age distribution, with 20–25% of cases diagnosed early in life (childhood and adolescence), while 70% of cases are diagnosed in adulthood. CD incidence continues to increase especially in industrialized countries [29, 30]. Clinical symptoms of CD include abdominal pain, diarrhea,

Table 3: Serum cytokine activation in CD4/CD8 ratio subgroups of juvenile CD (median [25th–75th percentile]).

Cytokine (pg/mL)	Controls ($n = 10$)	Acute patients, CD4/CD8 < 2 ($n = 18$)	Acute patients, CD4/CD8 > 2 ($n = 7$)
IL-1b	107,7 [16,4–340,8]	392,7 [131,6–807,7]	567,5 [444,5–650,0]*
IL-2	2,3 [1,5–3.0]	4,4 [3,4–6,2]*	3,2 [2,8–3,9]
IL-5	9,0 [3,5–11,8]	5,9 [2,7–13,6]	15,3 [4,7–22,2]
IL-6	23,5 [17,2–27,3]	54,3 [20,32–66,7]	27,8 [25,2–99,1]
IL-8	1 415,3 [997,1–2 625,4]	1760,0 [257,4–6014,1]	4161,5 [2956,0–8372,8]
IL-12	203,2 [120,3–280,0]	204,5 [144,1–426,5]	257,0 [171,8–885,3]
GM-CSF	30,4 [13,5–38,0]	33,0 [13,5–45,5]	29,5 [9,5–33,5]
IFN-γ	2,5 [1,8–3,2]	4,15 [3,2–5,2]*	3,4 [2,5–4,4]
CXCL10	384,9 [265,3–435,4]	4089,5 [2835,8–4597,8]*	2925,0 [2448,5–3426,0]*,**
TNF-α	4,6 [3,3–6,2]	7,4 [5,3–9,4]*	12,0 [11,5–13,0]*,**
IL-4	8,5 [6,3–9,1]	8,9 [7,4–11,3]	8,7 [7,6–11,3]
IL-10	23,9 [17,6–32,4]	16,8 [14,3–28,0]	60,0 [32,3–94,5]*,**

* $P < 0.05$ versus controls (Mann–Whitney test).
** $P < 0.05$ versus acute patients with CD4/CD8 ratio < 2.0 (Mann–Whitney test).

Table 4: Serum cytokine activation in adult CD (median [25th–75th percentile]).

Cytokine (pg/mL)	Controls ($n = 7$)	Acute patients ($n = 36$)	Remission patients ($n = 16$)
IL-1b	17.4 [11.3–26.1]	47.1 [32.2–87.2]*	53.2 [31.0–98.0]*
IL-2	110.0 [92.0–141.0]	102.0 [89.0–131.7]	93.0 [52.5–112.5]
IL-4	45.3 [41.6–77.2]	41.0 [34.8–47.8]	56.8 [45.4–68.9]
IL-5	15.2 [11.2–17.2]	12.8 [11.2–16.4]	14.2 [11.2–22.4]
IL-6	120.3 [110.2–128.3]	177.4 [130.6–296.7]*	307.8 [174.0–636.2]*
IL-8	207.5 [114.4–289.4]	293.3 [163.5–593.9]	236.3 [153.2–625.5]
IL-10	238.4 [166.4–273.1]	258.3 [196.0–291.3]	167.3 [153.5–180.7]**
IL-12	18.0 [18.0–415.2]	475.7 [18.0–1076.5]	487.9 [18.0–1 127.1]
CXCL10	6 910.5 [4 729.3–10 178.0]	21 111.7 [17 153.1–27 546.8]*	20 009.8 [18 836.5–25 123.4]*
GM-CSF	80.2 [45.0–113.7]	199.2 [70.0–470.0]*	120.7 [90.9–494.4]
IFN-γ	2 967.0 [2 344.5–3 675.6]	6 750.4 [4 806.5–11 050.0]*	6 846.2 [4 054.3–11 456.4]*
TNF-α	179.2 [143.6–196.4]	193.0 [179.0–211.3]	113.3 [76.2–171.0]**

* $P < 0.05$ versus controls (Mann–Whitney test).
** $P < 0.05$ versus acute patients (Mann–Whitney test).

weight loss, and fever. In addition to the common GI symptoms, juvenile CD is often associated with failure to thrive that can include retarded growth, malnutrition, pubertal delay, and bone demineralization [31, 32].

Activation of innate and acquired immune responses are characteristic of CD. Studies using animal models have shown that CD pathogenesis is closely associated with a Th1 cytokine profile, while activation of the Th2 cytokines is more prevalent in the pathogenesis of ulcerative colitis [33, 34]. Activation of T cell-mediated immunity is linked to the development of epithelioid granulomas, which are pathognomonic to CD [35].

The serum cytokine profile of CD patients is also indicative of activation of a Th1 and a Th17 type immune response. For example, upregulation of IL-17A, IL-17F, IL-21, IL-22, IL-26, and CCL20 has been demonstrated in CD cases pointing to activation of Th17 type immunity [36]. Increased serum levels of TNF-α are a hallmark of CD suggesting a fundamental role for this cytokine in disease pathogenesis [37–39]. The role of cytokines in CD was supported by Prehn et al. who demonstrated enhanced production of IFN-γ, the key cytokine for activation of a Th1 type immune response, by mucosal T lymphocytes upon stimulation with TNF-α [40]. Therefore, these data suggest that cytokines play an important role in activating and maintaining the Th1 and Th17 type immune response, which is indicative of CD.

Although the role of cytokines in CD pathogenesis is well established, little is known about age differences in cytokine activation in CD patients. Juvenile CD is usually characterized by severe clinical manifestations, often including development of strictures or fistulae requiring surgical intervention, whereas adults frequently have a milder clinical presentation with a lower likelihood of developing complications [4, 13]. Therefore, we sought to determine whether the

cytokine profiles in juvenile and adult CD differed. Serum levels of 12 cytokines were evaluated during both the acute stage and the remission in juvenile and adult CD patients. An interesting observation was that there was upregulation of CXCL10 cytokine in all patients regardless of age and stage of the disease. Although increased CXCL10 serum levels and enhanced expression of CXCL10 in inflammatory bowel tissue has previously been shown in adult CD, here we present the first evidence for upregulation of this chemokine in the serum from juvenile CD cases. CXCL10 is a chemokine known to exclusively recruit activated T lymphocytes and NK cells [41–43]. Additionally, it has been demonstrated that CXCL10 targets Th1 lymphocytes [42, 43]. The essential role of CXCL10 in the gut lymphocyte infiltration and inflammation has been established by Grip and Janciauskiene [44]. These authors demonstrated that reduced plasma levels of CXCL10 correlate with decreased level of the prominent inflammation marker, C reactive protein. In another study, Hyun et al. demonstrated that a reduction in CXCL10 serum levels impairs activation and reduces recruitment of Th1 lymphocytes into the gut mucosa and lymphoid tissue [45]. Therefore, our data support the notion that upregulation of CXCL10 can facilitate Th1 lymphocyte infiltration and perpetuate epithelial inflammation in the gut. Furthermore, increased serum levels of CXCL10 were found in all patients regardless of the stage of the disease, suggesting chronic Th1 lymphocyte activation, which is ongoing even during clinical remission.

An interesting observation regarding serum IL-8 levels in juvenile and adult CD patients was that, although not significant, the serum level of IL-8 was higher (1.9 and 2.9 times) in acute and remission juvenile CD compared to controls. In contrast, serum IL8 levels in adult CD remained similar to age-matched controls. IL-8 is a pleotropic cytokine, known as the prototype neutrophil attractant [46], which also activates neutrophils by upregulating phagocytosis and respiratory burst [47]. Since inflamed tissue is often infiltrated by activated neutrophils [48], this suggests a contribution of IL-8 to both local and systemic inflammation. Although high circulating IL-8 is characteristic of inflammation, a lack of change in serum IL-8 in some cases of gastroduodenitis has been documented [49]. Further investigation demonstrated that although IL-8 levels in serum were unchanged, there were increased levels of IL-8 transcripts within intestinal tissue [50, 51], suggesting local production of IL-8. Therefore, this suggests that lack of changes in IL-8 in serum may not always reflect ongoing in situ inflammation. Therefore, we propose that in juvenile CD that high serum IL-8 may reflect systemic inflammation, whereas, in adult CD, the lack of changes in circulating IL-8 level indicates that inflammation could be restricted to bowel tissue.

Another finding was that the serum level of TNF-α was significantly upregulated in juvenile CD compared to controls, while it remained unchanged in adult cases. These data suggest that juvenile CD, relative to adult, is characterized by a stronger inflammatory milieu in the gut. Additionally, increased serum IL-6 was detected all CD cases, regardless of patient's age, relative to controls. IL-6 is a B cell differentiating factor [52] that, when combined with TGFβ, promotes the differentiation of Th17 lymphocytes [53]. Moreover, IL-6 is considered an essential cytokine for Th17 differentiation of naive T cells [54]. Th17 lymphocytes are believed to be pathogenic and are often associated with the severe tissue damage and development of autoimmunity [55]. The mechanisms of Th17-associated tissue damage are linked to a breach in the integrity of the intestinal epithelium due to increased production of inflammatory cytokines and matrix metalloproteinases, as well as inhibition of proliferation of human gut epithelial cells [56, 57]. Interestingly, juvenile CD is characterized by upregulation of the IL-6, while levels of IFN-γ were unaffected. However, the opposite pattern of cytokine activation was found in adult CD, where only IFN-γ levels were significantly upregulated. IFN-γ is secreted by CTL and often used as a marker of the Th1 type immune response [58]. The Th1 (IL12-IFN-γ) and Th17 (IL-6-IL-17) pathways seem to be mutually exclusive, with IFN-γ and IL-17 acting as reciprocal inhibitory cytokines [59]. However, a subpopulation of Th17 lymphocytes producing IL-17 and IFN-γ was identified in CD gut tissue by Annunziato et al. [60]. The authors suggested that IL-17/IFN-γ positive Th17 are important for CD pathogenesis, as these cells were able to support B cell proliferation, had low cytotoxic activity, and were poorly responsive to autologous regulatory T cells. Therefore, we hypothesize that the Th17 response is characteristic for juvenile and adult CD. However, upregulation of IL-17 and IFN-γ in adult CD suggests that there are more complex mechanisms of pathogenesis relative to juvenile CD. We propose that the Th17 (IL-17/IFN-γ), or a combination of Th17 and Th1 immune responses, play a leading role in pathogenesis of adult CD. Studies using tissue biopsies from juvenile and adult CD will help to better understand the role of these Th subsets in CD pathogenesis.

Another finding was upregulation of GM-CSF in adult CD patients. It has been suggested that GM-CSF can alter the course of intestinal inflammation. For example, Däbritz et al. have shown that GM-CSF activated macrophages protect mice from T cell-induced colitis [61]. These authors suggested that GM-CSF-activated macrophages facilitate differentiation of Th2 lymphocytes by upregulating production of IL-4, IL-10, and IL-13 in lamina propria resident leukocytes, which may decrease local inflammation, improve bacterial clearance, and promote healing. In another study, Gathungu et al. have shown that the presence of anti-GM-CSF antibody can serve as marker for the severity of CD [62]. Further, these authors demonstrated that elevated anti-GM-CSF antibody levels were associated with intestinal stricture, penetration, and resection in CD patients. A similar observation was published by Gathungu et al., where CD patients with elevated anti-GM-CSF antibody exhibited increased bowel permeability as compared to those with lower levels of this antibody [62]. It has been suggested that GM-CSF neutralization promotes ileac disease by modulating expression of CCL25 by intestinal epithelium and CCR9 by T lymphocytes via Nod2-dependent and -independent pathways [62]. Therefore, increased GM-CSF levels in adult CD may contribute to inhibition of bowel inflammation and prevention of tissue damage.

Adult CD was characterized by upregulation of serum GM-CSF and IFN-γ, a combination with strong anti-inflammatory potential. For example, it has been shown that treatment of an LPS challenge with GM-CSF and IFN-γ inhibited an inflammatory response by lowering neutrophil counts and increasing the number of suppressive neutrophils (CD16 (bright)/CD62L (dim)) [63] in healthy volunteers [64]. The GM-CSF/IFN-γ combination may have a profound effect on leukocyte polarization. Both cytokines play a key role in developing M1 type macrophages, which are associated with Th1 type immune response [65]. M1 macrophages exhibit both strong microbiocidal activity and cell proliferation inhibitory capacity [66]. Therefore, increased serum GM-CSF and IFN-γ may reflect activation of Th1 type immune response in adult CD.

5. Conclusion

Our data suggest that juvenile and adult CD are characterized by unique serum cytokine profiles, which may reflect age-dependent difference in disease pathogenesis.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Extra-Articular Symptoms in Constellation with Selected Serum Cytokines and Disease Activity in Spondyloarthritis

Hanna Przepiera-Będzak,[1] Katarzyna Fischer,[2] and Marek Brzosko[1]

[1] *Department of Rheumatology, Internal Medicine and Geriatrics, Pomeranian Medical University in Szczecin, Unii Lubelskiej 1, 71-252 Szczecin, Poland*
[2] *Independent Laboratory of Rheumatic Diagnostics, Pomeranian Medical University in Szczecin, Unii Lubelskiej 1, 71-252 Szczecin, Poland*

Correspondence should be addressed to Hanna Przepiera-Będzak; hannapb@pum.edu.pl

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Objectives. In this study, we assessed the extra-articular symptoms in constellation with selected serum cytokines and disease activity in spondyloarthritis (SpA). *Patients and Methods.* We studied 287 SpA patients: 131 had AS, 110 had PsA, and 46 had SAPHO. We assessed extra-articular symptoms in all cases. In 191 SpA patients, we measured serum interleukin-6 (IL-6), interleukin-18 (IL-18), interleukin-23 (IL-23), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). *Results.* Patients with acute anterior uveitis (AAU) had higher VAS ($P = 0.0008$), BADSDAI ($P = 0.0001$), ASDAS-ESR ($P = 0.04$), CRP ($P = 0.006$), IL-6 ($P = 0.02$), and IL-18 ($P = 0.03$) levels. Patients with inflammatory bowel disease (IBD) had higher VAS ($P = 0.03$), CRP ($P = 0.0009$), and IL-6 ($P = 0.0003$) levels. Patients with skin psoriasis had lower VAS ($P = 0.001$) and BASDAI ($P = 0.00007$) levels. Patients with psoriatic onycholysis had lower VAS ($P = 0.006$), BASDAI ($P = 0.00001$), and CRP ($P = 0.02$) and higher IL-23 ($P = 0.04$) levels. Patients with PPP had lower BASDAI ($P = 0.04$) and higher ET-1 ($P = 0.001$) levels. *Conclusions.* SpA patients with increased serum IL-18 and decreased serum ET-1 had an increased risk of extra-articular symptoms. In SpA patients, increased disease activity was associated with an increased risk of AAU and IBD and a decreased risk of skin psoriasis, psoriatic onycholysis, and PPP.

1. Introduction

Ankylosing spondylitis (AS), psoriatic arthritis (PsA), and SAPHO syndrome (SAPHO) are seronegative spondyloarthropathies (SpA) which are connected with the presence of HLA-B27 antigen [1–3]. In the course of the diseases different extra-articular clinical symptoms could appear, such as acute anterior uveitis AAU, inflammatory bowel disease (IBD), psoriasis, psoriatic onychopathy, and palmoplantar pustulosis (PPP) [1–3]. The presence of different extra-articular symptoms in the course of SpA has an influence on the course of SpA and therapeutic decisions [4–9].

Several proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-18 (IL-18), interleukin-23 (IL-23) are considered to play a role in pathogenesis of SpA [10–14]. Also cytokines involved in angiogenesis such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and proinflammatory peptide endothelin-1 (ET-1) are considered to be involve in inflammatory process in SpA [15–17].

There are data that development of different extra-articular symptoms is connected with elevated levels of different markers of inflammatory process [4, 6, 13]. Additionally vascular abnormalities and endothelial dysfunction are considered to play a role in the pathogenesis of AAU, IBD, and psoriasis [18–20].

2. Objectives

The aim of our study was to assess extra-articular symptoms in constellation with selected serum cytokines and disease activity in SpA. We selected cytokines that play a role in the

Table 1: The clinical characteristics and the percentage of extra-articular symptoms of spondyloarthritis patients.

	Total (n = 287) $N \pm$ SD N (%)	AS (n = 131) $N \pm$ SD N (%)	PsA (n = 110) $N \pm$ SD N (%)	SAPHO (n = 46) $N \pm$ SD N (%)
%	100	45,6	38,3	16,0
Age (age)	49.2 ± 12.8	47.3 ± 13.2	50.7 ± 12.5	51.0 ± 12.1
Sex	130 F, 157 M	28 F, 103 M	62 F, 48 M	40 F, 6 M
Disease duration (years)	8.7 ± 8.2	13.3 ± 9.2	5.8 ± 4.9	2.9 ± 2.6
AAU, n (%)	35 (12.1)	34 (26.0)	1 (0.9)	0 (0)
IBD (any), n (%)	10 (3.5)	10 (7.6)	0 (0)	0 (0)
Amyloidosis, n (%)	3 (1,1)	2 (1,5)	1 (0.9)	0 (0)
Psoriasis, n (%)	93 (32.4)	2 (1.5)	91 (82.7)	0 (0)
Psoriatic onycholysis, n (%)	73 (25.4)	0 (0)	73 (66.4)	0 (0)
Only skin psoriasis without nail involvement, n (%)	62 (21.6)	2 (1.5)	60 (54.5)	0 (0)
Only psoriatic onycholysis without skin involvement, n (%)	31 (10.8)	0 (0)	31 (28.2)	0 (0)
Palmoplantar pustulosis, n (%)	42 (14.6)	0 (0)	0 (0)	42 (91.3)
Acne, n (%)	3 (1.1)	0 (0)	0 (0)	3 (6.5)
Primary biliary cirrhosis, n (%)	1 (0.3)	1 (0.8)	0 (0)	0 (0)
HLA B-27 (positive/done)	105/165	88/91	13/51	4/23

AAU: acute anterior uveitis, AS: ankylosing spondylitis, IBD: inflammatory bowel disease, N: number of patients, PsA: psoriatic arthritis, SAPHO: synovitis acne pustulosis hyperostosis osteitis syndrome, and SD: standard deviation.

pathogenesis of SpA and are involved in angiogenesis and endothelial function.

3. Materials and Methods

This study was approved by the Ethics Committee of the Pomeranian Medical University in Szczecin (KB-0012/106/10; 27SEP2010). Informed consent was obtained from all patients. We studied 287 SpA patients: 131 had AS, 110 had PsA, and 46 had SAPHO. All patients were Caucasian. The following data were recorded: age, sex, disease duration, and extra-articular symptoms: acute anterior uveitis (AAU), inflammatory bowel disease (IBD), skin psoriasis, psoriatic onychopathy, and positivity for HLA B27. The diagnosis of AS was made according to modified New York criteria [1]. The diagnosis of PsA was made according to the Caspar classification criteria [2]. The diagnosis of SAPHO syndrome was made according to the Kahn criteria [3]. In the PsA group, skin changes were assessed according to the Psoriasis Area and Severity Index (PASI) [21]. The patient's pain due to the disease at the time of examination was assessed using a visual analogue scale (VAS).

We also assessed the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). This index has a possible score of 0–10, with a higher score indicating greater disease activity. We regarded patients as active if the BASDAI score was > 4 [22].

The Ankylosing Spondylitis Disease Activity Score (ASDAS) was assessed using ESR. The ASDAS-ESR was calculated, in AS patients and PsA patients with axial joint involvement, using online calculator available at the Assessment of Spondyloarthritis International Society website.

Disease activity score calculation, in PsA patients, were made use of free online Disease Activity Score 28 (DAS28) calculator.

In the first 191 SpA patients, 81 had AS, 76 had PsA, and 34 had SAPHO; we studied serum levels of selected cytokines: IL-6, IL-23, IL-18, endothelin-1(ET-1), VEGF, and EGF. Additionally, we studied CRP and ESR. The controls were 30 healthy volunteers. The methods of assessment of these cytokines were presented in our previous paper [13].

Data distributions were assessed using the Kolmogorov–Smirnov test. Data are described as mean ± standard deviation and median (Q1 and Q3). The R values of correlations were determined and corresponding P values <0.05 were considered significant. The groups were compared using Student's t-test, Mann–Whitney U test, and Kruskal-Wallis test. To assess parameters Pearson's chi-squared test (χ^2), logistic regression analysis, and step-wise analysis were performed. The level of significance was set at $P < 0.05$. The statistical analysis was performed using STATISTICA version 8.0, StatSoft, Inc., Tulsa, United States.

4. Results

The clinical characteristics of SpA patients and the prevalence of extra-articular symptoms are presented in Table 1. Data presenting serum levels of selected cytokines and markers

Table 2: Comparison of serum levels of selected cytokines and markers of disease activity in spondyloarthritis patients with and without acute anterior uveitis and inflammatory bowel disease.

	AAU present	AAU absent	P	IBD present	IBD absent	P
Age (years)	44.8 ± 12.1	48.9	0.06	42.6 ± 11.4	48.3 ± 13.1	0.1
Disease duration (years)	10.0 (5.0, 19.0)	5.0 (2.0, 9.0)	0.0002	10.0 (5.0, 13.5)	5.0 (2.0, 10.)	0.06
HLA-B27	94.4 ± 23.5	47.1	0.0001	100	62	0.04
VAS pain (mm)	63.7 ± 28.3	49.4 ± 23.8	0.0008	67.5 ± 28.7	51.4 ± 24.8	0.03
BASDAI	5.8 ± 2.8	3.9 ± 2.7	0.0001	5.3	4.2	0.1
ASDAS-ESR	2.7 (2.2, 3.4)	2.3 (1.8, 3.0)	0.04	3.1 (2.2, 4.1)	2.4 (1.8, 3.2)	0.06
DAS28	0.0	4.24	<0.0001	0.0	4.24	<0.0001
CRP (mg/L)	10.5 (2.6, 21.1 0)	5.0 (2.0, 11.4)	0.006	19.0 (7.8, 31.1)	5.5 (2.1, 12.3)	0.0009
ESR (mm/h)	14.0 (5.0, 35.)	13.5 (6.0, 24.5)	0.4	25.5	14.0	0.05
IL-6 (pg/mL)	4.5 (1.5, 10.1)	2.9 (1.5, 6.5)	0.02	3.3 (1.5, 6.4)	2.9 (1.5, 20.7)	0.0003
IL-18 (pg/mL)	323.5 (228.7, 454.5)	271.8 (203.5, 346.2)	0.03	333.8 (237.5, 285.2)	271.8 (207.3, 373.9)	0.4
IL-23(pg/mL)	0.0 (0.0, 2.8)	0.0 (0.0, 1.8)	0.2	0.0 (0.0, 0.3)	0.0 (0.0, 2.0)	0.2
VEGF (pg/mL)	406.2 (270.0, 880.0)	343.1 (223.2, 545.7)	0.05	341.3 (206.3, 505.0)	347.4 (225.9, 562.2)	0.3
EGF (pg/mL)	115.0 (62.0, 182.0)	104.0 (68.0, 168.0)	0.5	81.0 (32.0, 141.0)	104.0 (68.0, 182.0)	0.2
ET-1 (pg/mL)	1.1 ± 0.4	1.3 ± 0.6	0.07	1.3 ± 0.5	1.3 ± 0.5	0.3

Data are presented as mean ± standard deviation, median (Q1 and Q3). AAU: acute anterior uveitis, ASDAS-ESR: Ankylosing Spondylitis Disease Activity Score, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, CRP: C-reactive protein, DAS28: Disease Activity Score 28, EGF: epidermal growth factor, ESR: erythrocyte sedimentation rate, ET-1: endothelin-1, IBD: inflammatory bowel disease, IL-6: interleukin-6, IL-18: interleukin-18, IL-23: interleukin-23, VAS pain: visual analogue scale of patient's pain, and VEGF: vascular endothelial growth factor.

of disease activity in subgroup of 191 SpA patients and 30 controls are presented in our previous paper by Przepiera-Będzak et al. [13].

4.1. Acute Anterior Uveitis. AAU was observed in 12.1% of SpA patients, in 26.0% of AS, and in 0.9% of PsA. No one patient with SAPHO had AAU (Table 1). The prevalence of AAU was higher in AS than in PsA ($P < 0.000001$) and in AS than in SAPHO ($P = 0.0001$). We compared SpA patients with AAU to those without AAU. SpA patients with AAU had longer disease duration ($P = 0.0002$) and higher prevalence of HLA-B27 antigen ($P = 0.0001$). SpA patients with AAU had higher disease activity as assessed using the VAS ($P = 0.0008$), BADSDAI ($P = 0.0001$), ASDAS-ESR ($P = 0.04$), and CRP ($P = 0.006$). Additionally, they had lower disease activity as assessed using the DAS28 ($P < 0.0001$) (Table 2).

In SpA patients increased CRP ($P = 0.01$) and BASDAI ($P = 0.005$) were associated with increased risk of AAU (Table 4). SpA patients with AAU compared to those without AAU had higher serum IL-6 ($P = 0.02$) and IL-18 ($P = 0.03$) levels (Table 2). SpA patients with AAU compared to healthy controls had higher serum IL-6 levels ($P = 0.02$). SpA patients with AAU compared to healthy controls had higher serum IL-18 levels ($P = 0.00009$). In SpA patients as compared to healthy controls, increased serum levels of IL-6 ($P = 0.02$), IL-23 ($P = 0.03$), and Il-18 ($P = 0.0006$) were associated with increased risk of AAU (Table 5). In SpA patients as compared to healthy controls, decreased serum levels of ET-1 ($P = 0.0007$) were associated with increased risk of AAU (Table 5).

4.2. Inflammatory Bowel Disease. IBD was present in 3.5% SpA patients and in 7.6% AS patients. No one patient with PsA or SAPHO had IBD (Table 1). The prevalence of IBD was higher in AS than in PsA patients ($P = 0.004$). We compared SpA patients with IBD to those without IBD. SpA patients with IBD had higher prevalence of HLA-B27 antigen ($P = 0.04$) and higher disease activity as assessed using the VAS ($P = 0.03$), CRP ($P = 0.0009$), and IL-6 ($P = 0.0003$). Additionally, they had lower disease activity as assessed using the DAS28 ($P < 0.0001$) (Table 2). SpA patients with IBD compared to healthy controls had higher serum IL-6 levels

Table 3: Comparison of serum levels of selected cytokines and markers of disease activity in spondyloarthritis patients with and without skin psoriasis, psoriatic onycholysis, and palmoplantar pustulosis.

	Psoriasis present	Psoriasis absent	P	Psoriatic onycholysis present	Psoriatic onycholysis absent	P	PPP present	PPP absent	P
Age (years)	52.5 ± 12.5	46.5 ± 13.0	0.001	50.1 ± 14.2	47.7 ± 12.7	0.1	52.4 ± 11.9	47.5 ± 13.2	0.03
Disease duration (years)	4.0 (2.0, 8.0)	6.0 (2.0, 11.0)	0.004	4.0 (2.0, 7.0)	6.5 (2.0, 12.0)	0.009	2.0 (1.0, 5.0)	6.0 (3.0, 11.0)	0.00009
HLA-B27	22	71	0.00004	28	70	0.0004	23	68	0.0008
VAS pain (mm)	43.1 ± 21.0	55.1 ± 25.6	0.001	44.0 ± 22.2	54.2 ± 25.4	0.006	48.6 ± 20.8	51.9 ± 25.6	0.3
BASDAI	3.1 ± 2.4	4.7 ± 2.8	0.00007	2.8 ± 2.5	4.7 ± 2.7	0.00001	4.3 ± 2.6	4.2 ± 2.8	0.04
ASDAS-ESR	1.8 (1.6, 2.1)	2.5 (1.9, 3.2)	0.006	1.9 (1.6, 2.4)	2.5 (1.8, 3.2)	0.06	—	—	—
DAS28	4.25 (3.8, 4.5)	4.05 (3.0, 6.9)	0.3	4.24 (3.8, 4.5)	4.28 (3.7, 4.8)	0.3	—	—	—
CRP (mg/L)	4.1 (1.6, 9.4)	6.35 (2.4, 13.3)	0.07	3.8 (1.6, 9.6)	6.1 (2.1, 13.5)	0.02	4.5 (1.1, 12.4)	5.6 (2.4, 12.0)	0.4
ESR (mm/h)	13.0 (6.0, 22.0)	14.0 (6.0, 28.0)	0.2	13.0 (7.0, 22.0)	14.0 (6.0, 27.0)	0.2	21.0 (10.5, 36.0)	13.0 (6.0, 24.0)	0.004
IL-6 (pg/mL)	2.5 (1.4, 6.2)	3.6 (1.7, 7.9)	0.4	3.4 (1.4, 6.5)	3.3 (1.6, 6.7)	0.2	2.4 (1.0, 6.6)	3.4 (1.5, 6.7)	0.2
IL-18 (pg/mL)	290.1 (225.4, 373.9)	259.7 (205.3, 372.3)	0.3	286.0 (225.4, 408.9)	264.9 (202.9, 370.0)	0.4	265.0 (193.3, 377.9)	278.2 (213.6, 373.1)	0.4
IL-23(pg/mL)	0.0 (0.0, 0.9)	0.0 (0.0, 2.5)	0.3	0.0 (0.0, 0.9)	0.0 (0.0, 2.5)	0.04	0.0 (0.0, 0.3)	0.0 (0.0, 2.5)	0.2
VEGF (pg/mL)	343.5 (211.7, 680.0)	348.9 (229.6, 500.0)	0.5	289.6 (205.7, 759.7)	347.8 (260.0, 500.0)	0.5	333.1 (235.3, 390.0)	348.9.3 (225.9, 612.1)	0.1
EGF (pg/mL)	130.0 (74.0, 196)	95.0 (68.0, 160.0)	0.1	120.0 (74.0, 196.0)	103.0 (66.0, 164.0)	0.23	104.0 (58.0, 168)	107.0 (68.0, 182.0)	0.4
ET-1 (pg/mL)	1.3 ± 0.6	1.2 ± 0.5	0.5	1.21 ± 0.4	1.15 ± 0.6	0.5	1.5 ± 0.7	1.2 ± 0.5	0.001

Data are presented as mean ± standard deviation, median (Q1 and Q3). AAU: acute anterior uveitis, ASDAS-ESR: Ankylosing Spondylitis Disease Activity Score, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, CRP: C-reactive protein, DAS28: Disease Activity Score 28, EGF: epidermal growth factor, ESR: erythrocyte sedimentation rate, ET-1: endothelin-1, IBD: inflammatory bowel disease, IL-6: interleukin-6, IL-18: interleukin-18, IL-23: interleukin-23, PPP: palmoplantar pustulosis, VAS pain: visual analogue scale of patient's pain, and VEGF: vascular endothelial growth factor.

($P = 0.007$). When SpA patients were compared to controls, increased serum levels IL-18 ($P = 0.03$) were associated with an increased risk of IBD (Table 5).

4.3. Skin Psoriasis. Skin psoriasis was present in 32.4% of SpA patients, in 82.7% PsA patients, and in 1.5% AS patients (Table 1). We compared SpA patients with skin psoriasis to those without skin psoriasis. SpA patients with skin psoriasis had shorter disease duration ($P = 0.004$), lower prevalence of HLA-B27 antigen ($P = 0.00004$), and lower disease activity as assessed using the VAS ($P = 0.001$), and BASDAI ($P = 0.00007$) (Table 3). In SpA patients, increased VAS ($P = 0.003$) and BASDAI ($P = 0.002$) were associated with decreased risk of skin psoriasis (Table 4).

There were no differences in serum levels of IL-6, IL-23, IL-18, VEGF, and EGF between SpA patients with and without skin psoriasis (Table 3). There were no correlations between serum IL-6, IL-23, IL-18, and PASI score (all $P > 0.05$). In SpA patients as compared to healthy controls, increased serum levels of IL-18 ($P = 0.0002$) and decreased serum levels of ET-1 ($P = 0.006$) were associated with increased risk of skin psoriasis (Table 5).

4.4. Psoriatic Onychopathy. Psoriatic onychopathy was present in 25.4% SpA patients and 66.4% PsA patients (Table 1).

We compared SpA patients with psoriatic onychopathy to those without psoriatic onychopathy. SpA patients with psoriatic onychopathy had shorter disease duration ($P = 0.009$), lower prevalence of HLA-B27 antigen ($P = 0.0004$), and lower disease activity as assessed using the VAS ($P = 0.006$), BASDAI ($P = 0.00001$), CRP ($P = 0.02$), and IL-23 ($P = 0.04$) (Table 3).

SpA patients with psoriatic onychopathy compared to healthy controls had higher IL-23 level ($P = 0.02$).

SpA patients with increased BASDAI ($P = 0.002$) had lower risk of psoriatic onychopathy (Table 4).

In SpA patients, compared to healthy controls, increased serum levels of IL-18 ($P = 0.0002$) and decreased serum levels of ET-1 ($P = 0.008$) were associated with increased risk of psoriatic onychopathy (Table 5).

Table 4: A logistic regression model of the OR of selected markers of disease activity in spondyloarthritis groups of patients with different extra-articular symptoms compared to controls.

Covariates	AAU		IBD		Psoriasis		Psoriatic onycholysis		PPP	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Disease duration ≥ 5 years	4.05 (1.62–10.12)	0.002	3.7 (0.72–18.84)	0.11	0.58 (0.26–1.25)	0.16	0.29 (0.15–0.59)	0.0004	0.22 (0.09–0.53)	0.0007
VAS ≥ 40 mm	2.28 (0.91–5.7)	0.07	2.22 (0.43–11.30)	0.33	0.31 (0.14–0.68)	0.003	0.67 (0.33–1.36)	0.2	1.22 (0.49–3.08)	0.65
BASDAI ≥ 4	3.56 (1.46–8.66)	0.005	3.10 (0.57–16.86)	0.18	0.24 (0.10–0.61)	0.002	0.33 (0.16–0.67)	0.002	0.61 (0.25–1.47)	0.27
CRP ≥ 10 mg/L	2.90 (1.26–6.67)	0.01	4.03 (0.93–17.51)	0.06	0.52 (0.21–1.29)	0.16	0.59 (0.28–1.24)	0.16	1.05 (0.44–2.49)	0.9

AAU: acute anterior uveitis, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, CRP: C-reactive protein, IBD: inflammatory bowel disease, OR: odds ratio, PPP: palmoplantar pustulosis, and VAS pain: visual analogue scale of patient's pain.

Table 5: A logistic regression model of the OR of serum levels of selected cytokines in spondyloarthritis groups of patients with different extra-articular symptoms compared to controls.

Covariates	AAU		IBD		Psoriasis		Psoriatic onycholysis		PPP	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
IL-6 ≥ 6.64 pg/mL	6.75 (1.23–36.91)	0.02	5.4 (0.61–47.76)	0.12	4.29 (0.81–22.80)	0.08	4.35 (0.87–21.64)	0.07	5.06 (0.91–28.15)	0.06
IL-18 ≥ 227.45 pg/mL	8.17 (2.46–27.03)	0.0006	7.0 (1.17–41.53)	0.03	9.0 (2.87–28.16)	0.0002	6.82 (2.50–18.59)	0.0002	3.69 (1.27–10.71)	0.01
IL-23 ≥ 2.5 pg/mL	23.9 (1.28–444.01)	0.03	13.6 (0.49–373.39)	0.12	15.6 (0.83–291.96)	0.06	11.49 (0.63–209.44)	0.09	7.55 (0.34–166.21)	0.19
ET-1 ≤ 1,081 pg/mL	9.45 (2.56–34.79)	0.0007	0.23 (0.04–1.11)	0.06	5.77 (1.65–20.15)	0.006	4.93 (1.50–16.20)	0.008	1.89 (0.49–7.29)	0.35
VEGF ≥ 420.9 pg/mL	2.5 (0.60–10.26)	0.20	0.83 (0.07–9.68)	0.8	1.18 (0.34–4.07)	0.7	1.71 (0.51–5.82)	0.38	0.5 (0.08–2.99)	0.4
EGF ≥ 172.0 pg/mL	1.28 (0.27–5.93)	0.7	0.33 (0.01–7.23)	0.4	1.34 (0.36–4.94)	0.6	1.34 (0.36–4.94)	0.6	0.96 (0.18–4.92)	0.9

AAU: acute anterior uveitis, EGF: epidermal growth factor, ET-1: endothelin-1, IBD: inflammatory bowel disease, IL-6: interleukin-6, IL-18: interleukin-18, IL-23: interleukin-23, OR: odds ratio, PPP: palmoplantar pustulosis, and VEGF: vascular endothelial growth factor.

4.5. Palmoplantar Pustulosis. PPP was present in 14.6% SpA patients and in 91.3% SAPHO patients (Table 1). We compared SpA patients with PPP with those without PPP. SpA patients with PPP had shorter disease duration ($P = 0.0009$), lower prevalence of HLA-B27 antigen ($P = 0.0008$), and lower disease activity as assessed using the BASDAI ($P = 0.04$). They had higher ESR ($P = 0.004$) and higher serum ET-1 ($P = 0.001$) (Table 3). There were no differences in serum ET-1 between SpA patients with PPP and healthy controls ($P = 0.4$). In SpA patients compared to healthy controls increased serum levels of IL-18 ($P = 0.01$) were associated with increased risk of PPP (Table 5).

5. Discussion

We presented extra-articular symptoms in constellation with disease activity and selected serum cytokines which are involved in disease activity, angiogenesis, and endothelial function in SpA patients.

The percentage of AAU in SpA patients and AS patients in our study was similar to those presented by other authors [4–6, 9].

The most important genetic factor associated with AAU is HLA-B27 [8]. We confirmed these associations in our study too.

In our study AAU was the most frequent in AS patients. Additionally, SpA patients with AAU had higher levels of ASDAS-ESR and lower DAS28. These confirm observations of other authors that AAU is more frequent in axial SpA [6, 8, 9]. These suggest that there is association between severity of axial, but not peripheral arthritis with AAU occurrence in SpA.

We also confirmed as other authors that patients with AAU had longer disease duration and higher disease activity [4, 8].

IL-6 plays role in arthritis but its role in SpA pathogenesis is controversial. Kramer et al. [23] presented increased serum levels of IL-6 in patients with active uveitis. In our study we found that SpA patients with AAU had higher serum levels of IL-6. Additionally, increased serum IL-6 was associated with increased risk of AAU in SpA. In our previous study by Przepiera-Będzak et al. [14] we showed increased serum IL-6 in SpA patients and positive correlation with markers of

disease activity such as ESR and CRP. These could suggest the role of IL-6 in pathogenesis of AAU in SpA.

IL-18 is considered as one of proinflammatory cytokine which also activate and deregulate endothelial function. In our previous study we confirmed higher levels of IL-18 in SpA patients and AS patients [13]. In current study we additionally confirmed that serum IL-18 was higher in SpA patients with AAU and compared to healthy controls. Additionally, increased serum IL-18 increased risk of AAU. These suggest the role of IL-18 in the pathogenesis of AAU in SpA by influence on inflammatory process and endothelial function.

The percentage of IBD in SpA patients in our study was similar to those presented by other authors [4, 5]. Patients with IBD had longer disease duration and higher disease activity, which correspond with other authors observations [6]. Additionally we observed that SpA patients with IBD had lower DAS 28. This confirmed data presented by others that IBD is less frequent in SpA patients with peripheral arthritis [6]. These suggest that there is association between severity of axial, but not peripheral arthritis and IBD occurrence in SpA.

The percentage of SpA patients with skin psoriasis in our study was similar to the results presented by others [6, 9]. Peluso et al. [7] presented that 89.2% of PsA patients had psoriasis, and that PsA patients with skin psoriasis had longer disease duration and more frequent axial disease. In our study the percentage of PsA patients with psoriasis was the same, but data concerning disease activity and disease duration were reversible. We found that patients with skin psoriasis had shorted disease duration and lower ASDAS-ESR which suggest that they had lower activity of axial SpA. On the other hand Essers et al. [4] in study of 216 AS patients found that history of psoriasis was associated with grater age and lower CRP. In our study we found the same associations in SpA patients with skin psoriasis. Additionally, we confirmed that increased disease activity measured by VAS and BASDAI decreased risk of skin psoriasis. These suggest that there is no association between severity of axial and peripheral arthritis and skin psoriasis occurrence in SpA.

We also found that SpA patients with skin psoriasis, compared to SpA group without that symptom, had lower levels of proinflammatory cytokines such as IL-6, IL-23, IL-18, VEGF, and EGF. Nevertheless, we confirmed that increased serum IL-18 levels were associated with increased risk of skin psoriasis in SpA. These foundlings suggest that some markers of disease activity did not influence on prevalence of psoriatic skin changes. On the other hand it can confirm the role of IL-18 in pathogenesis of skin psoriasis by influence on disease activity and endothelial function.

IL-23 is produced by keratinocytes and is considered to play a role in SpA pathogenesis. In our previous study by Przepiera-Będzak et al. [14] we showed increased serum IL-23 levels in SpA patients. It was interesting that we did not find correlation between serum IL-23 and PASI [14]. In current study there were no differences in serum level of IL-23 between patients with and without psoriasis, which suggested that serum IL-23 could influence pathogenesis of skin psoriasis but did not reflect a presence of skin disease in SpA patients.

Psoriatic onychopathy was observed only in PsA group. Peluso et al. have presented psoriatic onychopathy in 89.2% PsA patients [7]. In SpA patients 66.4% had psoriatic onychopathy. In our study we fund that longer disease duration and higher disease activity were associated with decreased risk of psoriatic onychopathy. These suggest that there is no association between severity of axial and peripheral joints arthritis and psoriatic onychopathy occurrence in SpA.

The axis IL-17/ILK-23 is considered to play the crucial role in SpA pathogenesis. In our study we presented association between psoriatic onychopathy and serum IL-23. SpA patients with psoriatic onychopathy had higher serum IL-23 than healthy controls. In our previous study by Przepiera-Będzak et al. [14] we confirmed that SpA patients had higher serum IL-23 than healthy controls. Results of our study confirm the role IL-23 in pathogenesis of psoriatic onychopathy.

There were data which confirmed increased serum IL-18 in SAPHO. These suggested role of IL-18 in pathogenesis of SAHO [12, 13]. In our study increased serum IL-18 was associated with increased risk of PPP. This could confirm the role of IL-18 in pathogenesis of PPP by influence on disease activity and endothelial function.

ET-1 plays a role in inflammation and vasculopathy. Kuryliszyn-Moskal et al. [24] presented increased serum ET-1 in rheumatoid arthritis patients with extra-articular symptoms. In a previous study by Przepiera-Będzak et al. [13], serum ET-1 levels were lower in SpA patients than in controls, but levels were significantly higher in SAPHO than in AS or PsA. In our current study SpA patients with PPP compared with those without PPP had higher ET-1 level. So we can speculate that maybe ET-1 by its influence on vasculopathy and inflammation can play a role in PPP pathogenesis in SpA patients.

6. Conclusion

The analysis of serum levels of selected cytokines in SpA patients with and without extra-articular symptoms and healthy controls confirmed the following:

(i) patients with AAU had higher serum IL-6 and IL-18;

(ii) patients with IBD had higher levels of IL-6;

(iii) patients with nail psoriasis had higher levels of IL-23;

(iv) patients with PPP had higher levels of ET-1.

In SpA patients, increased serum IL-18 and decreased serum ET-1 were associated with an increased risk of extra-articular symptoms. Additionally, increased serum IL-6 and IL-23 increased the risk of AAU. Among SpA patients, increased disease activity was associated with an increased risk of AAU and IBD and a decreased risk of skin psoriasis, psoriatic onycholysis, and PPP.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Hanna Przepiera-Będzak participated in the design and coordination of the study, performed the statistical analysis, and drafted the manuscript. Katarzyna Fischer carried out the immunoassays. Marek Brzosko participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Research Article

Increased IL17A, IFNG, and FOXP3 Transcripts in Moderate-Severe Psoriasis: A Major Influence Exerted by IL17A in Disease Severity

Priscilla Stela Santana de Oliveira,[1] Michelly Cristiny Pereira,[1] Simão Kalebe Silva de Paula,[1] Emerson Vasconcelos Andrade Lima,[2] Mariana Modesto de Andrade Lima,[2] Rodrigo Gomes de Arruda,[3] Wagner Luís Mendes de Oliveira,[1] Ângela Luzia Branco Pinto Duarte,[2] Ivan da Rocha Pitta,[1] Moacyr Jesus Melo Barreto Rêgo,[1] and Maira Galdino da Rocha Pitta[1]

[1]*Laboratório de Imunomodulação e Novas Abordagens Terapêuticas (LINAT), Núcleo de Pesquisa em Inovação Terapêutica Suely Galdino (NUPIT-SG), Universidade Federal de Pernambuco (UFPE), Recife, PE, Brazil*
[2]*Hospital das Clínicas, Universidade Federal de Pernambuco (UFPE), Recife, PE, Brazil*
[3]*Faculdade Nova Roma, Recife, PE, Brazil*

Correspondence should be addressed to Maira Galdino da Rocha Pitta; mgrpitta@gmail.com

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Psoriasis is a chronic and recurrent dermatitis, mediated by keratinocytes and T cells. Several proinflammatory cytokines contribute to formation and maintenance of psoriatic plaque. The Th1/Th17 pathways and some of IL-1 family members were involved in psoriasis pathogenesis and could contribute to disease activity. Therefore, we sought to analyse skin transcript levels of IL17A, IL22, RORC, IL8, IFNG, IL33, IL36A, FOXP3, and IL10 and correlate with clinic of patients with plaque-type psoriasis. In order to conduct that, we collected punch biopsies from lesional skin and obtained tissue RNA. After reverse transcription, qRT-PCR quantified the relative mRNA expression. The main results revealed increased transcripts levels of IL17A, IFNG, and FOXP3 in moderate-severe patients. Despite this, only IL17A can increase the chance to worsen disease severity. We also observed many significant positive correlations between each transcript. In conclusion, IL17A is elevated in lesional skin from psoriasis patients and plays crucial role in disease severity.

1. Introduction

Plaque-type psoriasis is the most prevalent form of psoriasis, corresponding to approximately 90% of cases. Erythematous scaly plaques, well demarcated, raised edges, and varied distribution throughout the body [1] characterize classical lesions. In some cases, systemic diseases such as inflammatory bowel disease and cardiovascular complications present worsening symptoms [2, 3].

The Th1/Th17 pathways are the principal immune components of the disease. The precise mechanism of how the plaque is formed remains uncertain, but when some kind of "triggers" are activated in skin, a cascade of molecules acts in the interaction between keratinocytes and immune cells. At the beginning of the process, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) induce Langerhans cells to produce IL-12 and IL-23 [4]. IL-12 contributes to the strengthening of Th1 profile while IL-23 leads to Th17 differentiation that produces, mainly, IL-17 and IL-22 [5, 6]. In the epidermis, IL-8 operates as a potent chemotactic factor for neutrophils and contributes to the development of the erythema observed in skin lesions [7].

Recent studies have pointed out the active participation of others cytokines from IL-1 family. Between them there is

IL-33, also named IL-1F11, that mediates biological functions through IL-1 orphan receptor ST2 [8]. In psoriasis, TNFα regulates IL-33, which promotes inflammation through mast and keratinocyte activation [9, 10]. In addition, IL-36α is one of the three homology proteins IL-36α (IL-1F6), β (IL-1F8), and γ (IL-1F9) that also belong to the IL-1 family. They are expressed in both dendritic and keratinocytes cells [11]. Evidence of the involvement of IL-36 cytokines in the pathophysiology of psoriasis includes the fact that a nonfunctional receptor antagonist (IL-36Ra) was associated with generalized pustular psoriasis [12]. Furthermore, IL-36 ligands deficient mice were protected from psoriasis form dermatitis model while the absence of IL-36Ra exacerbated the pathology [13].

On the other hand, regulatory T cells seem to fail in their peripheral anti-inflammatory control. Several researchers found decreased $CD4^+CD25^+FOXP3^+$ T circulating cells both numerically and functionally in psoriasis patients [14, 15]. In order to balance the deficient anti-inflammatory response, clinical trials have been done using IL-10 recombinant human (rh) therapy protocols [16].

The aim of this study was to evaluate the pro- and anti-inflammatory psoriasis panel of molecules. Here, we sought to quantify Th17-related (IL17A, IL22, and RORC), Th1-related (IFNG and IL8), Treg-related (FOXP3 and IL10), and IL-1 family (IL33 and IL36A) skin transcripts and correlate with disease activity, systemic comorbidities, and methotrexate use in samples of Brazilian patients affected by psoriasis.

2. Materials and Methods

2.1. Ethics Committee. The human ethics committee of the Health Sciences Center of the Federal University of Pernambuco, located in Recife, Brazil, approved the study protocol (process number: 723.390).

2.2. Population Study. The study included twenty-one patients (11 male and 10 female) with plaque-type psoriasis attended and randomly selected in the Dermatology and Rheumatology Outpatient Clinic at Universidade Federal de Pernambuco. Only patients diagnosed with plaque-type psoriasis in strict accordance with the diagnostic criteria of Nestle et al. [17] with no prior immunobiologic therapy and no coexistent autoimmune disorders were considered. Psoriasis Area and Severity Index (PASI) was measured and classified as mild (0–10) and moderate-severe (>10) according to Menter et al. [18]. Other clinical parameters as comorbidities, disease duration, and previous systemic treatment were also questioned.

2.3. Skin Samples and RNA Extraction. Four milimeters (4 mm) punch biopsies were taken from lesional skin of psoriatic patients. They were stored up to 24 hours at 4°C RNA later stabilization solution (Invitrogen Life Technologies, CA, EUA) until extraction. RNA was isolated using QIAGEN RNeasy Kit (Qiagen, Valencia, CA) and the amount was measured by nanodrop 2000 (Thermo Fisher Scientific, Carlsbad, CA, EUA). The maximum of 500 ng of total tissue RNA was reverse transcribed using High-capacity cDNA archive Kit 2X (Applied Biosystems Warrington, UK) according to manufacturer's instruction.

2.4. Quantitative Real-Time Polymerase Chain Reaction Analysis. qRt-pcr was carried out using predesigned Taqman probes gene expression assay (Applied Biosystems, Warrington, UK), using ABI Prism 7900 HT sequence detection PCR machine (Applied Biosystems, Warrington, UK). We evaluated IL8 (Hs00174103_m1), IFNG (Hs00989291), IL-33 (Hs00369211_m1), IL36A (Hs00205367), IL17A (Hs00174383), IL22 (Hs01574154), IL10 (Hs00961622), RORC (Hs01076122_m1), FOXP3 (Hs01085834_m1), and 18S (Hs03928990) as a housekeeping gene. The cycling condition consisted of 2 minutes at 50°C followed by 10 minutes at 95°C. After these steps, there are 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute.

2.5. Statistical Analysis. We used GraphPad PRISM 6.01 software (GraphPad Software Inc., San Diego, CA) and STATA 12 (StataCorp LP., Texas, USA) for data plotting and analysis. To ascertain the sample's normality, we performed D'Agostino & Pearson omnibus normality test. The Mann–Whitney test and Spearman rank correlation were used when the variables did not follow Gaussian distribution. For variables that passed normality test, we applied unpaired t-test. We considered correlation (R^2) strength as follows: $0 < R^2 \leq 0.35$ = weak correlation; $0.35 < R^2 \leq 0.67$ = moderate correlation; $0.67 < R^2 \leq 1$ = strong correlation. To evaluate the association between transcripts levels and PASI, we performed multiple logistic regression for the clinical variables with dichotomous scores. The statistical significance was accepted when $p < 0.05$.

3. Results

3.1. Patients Cohort. A group of 21 patients, 11 men and 10 women, was included in this study. The mean age was 52 years with men showing a lower mean age than women (46.9 and 57.7, resp.). We stratified the PASI in accordance with the classification postulated by Menter and colleagues (2008), in the Journal of American Academy of Dermatology. PASI showed 8 as the lower punctuation and 28 as the highest score. General mean of PASI was 18±7.2. According to that, men showed more severe disease (mean 21.3 ± 5.8) than women (14.4 ± 7.2) and it was statistically significant, $p = 0.026$. Thus, we wanted to know if the other clinical parameters interfered in disease activity. The findings showed that neither prior use of methotrexate nor presence or absence of comorbidities interfered in disease activity, $p = 4823$ and $p = 1182$, respectively (Data not shown). Table 1 details the clinical features of patients.

3.2. IL17A, IFNG, and FOXP3 mRNA Levels Were Increased in Skin Biopsy from Moderate-Severe Psoriatic Patients. We observed that in our sample only five individuals showed disease activity considered mild and the others had more

Table 1: Clinical features of psoriatic patients from Brazil northeast[a].

Characteristics	All individuals ($n = 21$)
Age (yrs.)	
Mean ± SD/range	52.05 ± 13.48/23–74
Gender *N* (%)	
Male	11 (52.3)
Female	10 (47.7)
Disease duration (years)	
Mean ± SD (range), all	8.1 ± 6.5 (0.5–22)
0–5 years *N* (%)—mean ± SD	9 (42.8)—2.1 ± 1.2
6–10 years *N* (%)—mean ± SD	6 (28.6)—9.1 ± 1.3
>10 years *N* (%)—mean ± SD	6 (28.6)—16.3 ± 4.2
PASI clinical subgroups *N* (%)	
Mild (PASI < 10)—mean ± SD	5 (23.8)—8.8 ± 1
Moderate-severe (PASI ≥ 10)—mean ± SD	16 (76.2)—20.8 ± 5.7
Clinical comorbidities *N* (%)	
Diabetes	3 (14.3)
Dyslipidemia	3 (14.3)
Hypertension	3 (14.3)
Treatment *N* (%)	
Methotrexate	9 (42.9)

[a]Considering a Gaussian distribution, clinical values were represented by mean ± SD.

severe clinical presentation. As we can see in Figures 1(a), 1(b), and 1(c), only three of the nine transcripts analysed showed statistical significance in moderate-severe disease compared with mild activity. IL17A, IFNG, and FOXP3 showed $p = 0.0192$, $p = 0.0237$, and $p = 0.0239$, respectively. Figures 1(d), 1(e), 1(f), 1(g), 1(h), and 1(i) provide the other analysed transcripts graphs.

3.3. Correlation between Cytokines in Skin Biopsies from Psoriatic Patients. After analysing the PASI score and their influence in expression of a panel of both pro- and anti-inflammatory transcripts, we evaluated the importance that each cytokine could play over the expression of the others. We summarized the significances and correlation's coefficients in Table 2.

In our study, we identified several statistically significant positive correlations between the molecules analysed in different magnitudes. With $p < 0.05$, IL17A correlated with its own transcription factor, RAR-related orphan receptor C (RORC), and other cytokines, such as IL22 and IL8. We also correlated IFNG versus IL10 and FOXP3; IL22 versus IL8 and IL10; RORC versus IL10; and lastly IL8 versus IL10.

We observed more significant correlation ($p < 0.01$) between IL36A versus IL17A, IFNG, IL8, and IL10. Similarly, FOXP3 showed correlation with IL10 and IL17A with IFNG. Strongly significant correlation ($p < 0.001$) was observed between the two transcription factors RORC versus FOXP3. This last one also correlated with IL36A and IL17A. Finally, IL10 showed significant correlation with IL17A.

3.4. Men and Women Have Different Profile of Cytokines Expressed in the Skin. As previously mentioned, men and women exhibited different gravity of disease. Therefore, we investigated whether this fact influences the cytokine expression in the skin, separating the groups by gender. We observed that the male group showed significant high levels of IL17A transcripts compared to woman as well as more severe disease (Figures 2(a) and 2(b)). However, there was no significant statistical when we correlated each other (Figure 3(a)). In contrast, it occurred in female group. IL17A and FOXP3 transcripts showed significant statistical positive correlation with PASI (Figures 3(b) and 3(c)).

Among the fifteen statistically significant correlations described above, eight correlations only remained in the male group. They were IL17A versus, IL8, IL22, IL36A, and IL10, IL10 versus IL8 and IL22, IL36A with IL8, and RORC versus FOXP3. In the opposite, three correlations occurred only in female group: IL17A versus IFNG and FOXP3 and this last one with IL36A (Table 3).

3.5. IL17A Exhibits Greater Influence on Disease Severity. Finally, in order to understand the relationship between transcripts levels and PASI score, we conducted a multiple logistic regression. We found that IL17A high levels increased the chance to have moderate-severe disease as shown in Table 4. Due to the multicollinearity existence, IFNG and IL10 could not be included in the regression model.

4. Discussion

Recently, we demonstrated that IL-17A, IL-22, and IL-6 cytokines were more elevated in serum from psoriasis patients than in heathy controls. However, we have not found any correlation between systemic cytokine expression and disease severity [19]. So, we decided to investigate the lesion microenvironment and investigate if there was a correlation with disease severity and if it occurred in a local level in our group.

Over the past years, researches focused in Th1/Th17 pathways and described high levels of IFNγ, IL-17, and its isoforms in lesional psoriatic skin in both gene and protein levels [20–23]. Despite the availability of studies of psoriasis large-scale genomic and transcriptomes platforms [24–26], there is still a gap between the current knowledge and the clinical progression.

Our study showed increased expression of IL17A, IFNG, and FOXP3 in patients who exhibited severe clinical disease profile. Just as we did, Kim and Colleagues (2016) found significant difference in IL17A transcript expression between their clinically stratified groups [27]. In contrast to our study, they showed that patients classified with mild disease activity had the highest levels of IL17A. Suárez-Fariñas et al. (2012) also detected greater expression of IL23 p19 and p40 subunits IL17, IL22, and IFNG in lesions of moderate-severe patients using real-time reverse transcriptase method [28].

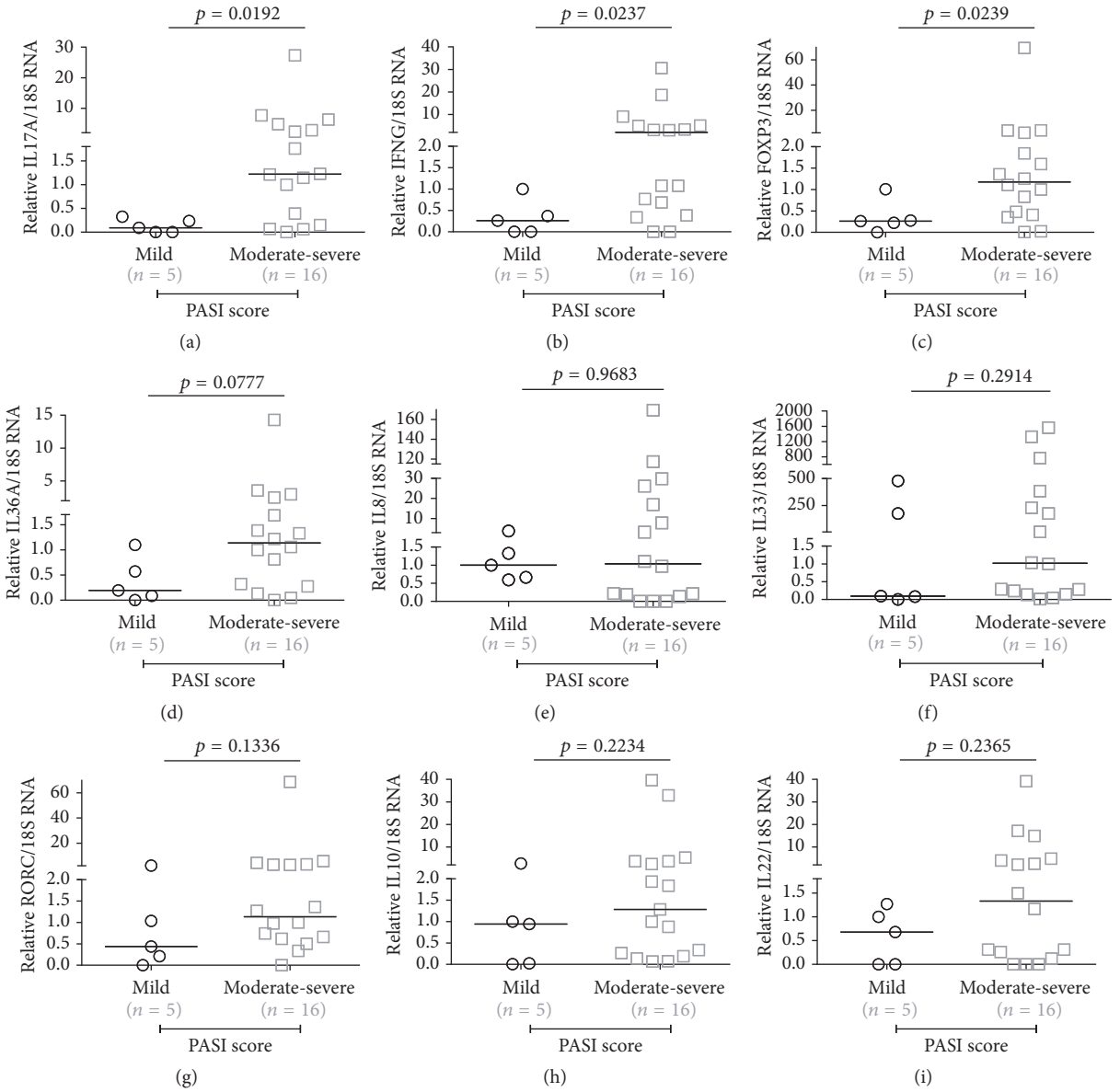

Figure 1: Transcripts levels of (a) IL17A, (b) IFNG, (c) FOXP3, (d) IL36A, (e) IL8, (f) IL33, (g) RORC, (h) IL10, and (i) IL22 according to PASI's severity disease.

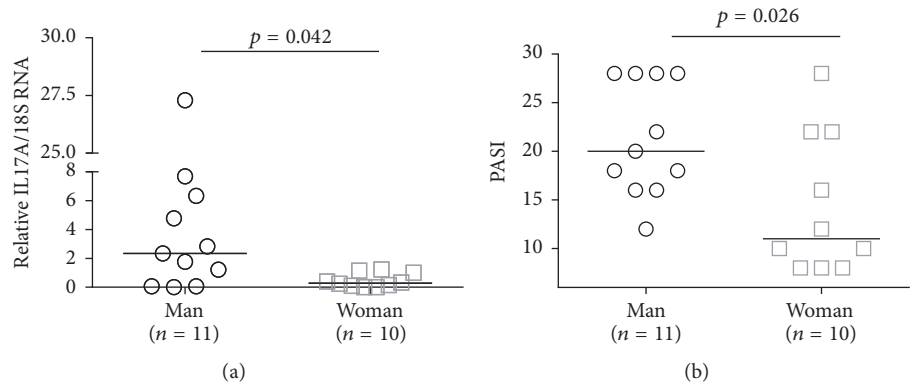

Figure 2: Different IL17A biopsy profile expression (a) and disease severity (b) between man and woman.

Table 2: Interplay among cytokines in human psoriasis lesions[a].

	IL8	IL-33	IL36A	IL17A	FOXP3	RORC	IL22	IFNG	IL10
IL8		$p = 0.443$ $r = -0.176$	$p = 0.006$ $r = 0.574$	**p = 0.037* r = 0.457**	$p = 0.064$ $r = 0.410$	$p = 0.798$ $r = 0.059$	**p = 0.044* r = 0.442**	$p = 0.087$ $r = 0.382$	**p = 0.039* r = 0.452**
IL33	$p = 0.443$ $r = -0.176$		$p = 0.106$ $r = 0.362$	$p = 0.126$ $r = 0.344$	$p = 0.097$ $r = 0.371$	$p = 0.278$ $r = 0.248$	$p = 0.857$ $r = -0.041$	$p = 0.279$ $r = 0.247$	$p = 0.211$ $r = 0.284$
IL36A	**p = 0.006** r = 0.574**	$p = 0.106$ $r = 0.362$		**p = 0.001** r = 0.651**	**p < 0.001*** r = 0.774**	$p = 0.096$ $r = 0.372$	$p = 0.117$ $r = 0.351$	**p = 0.007** r = 0.563**	**p = 0.002** r = 0.622**
IL17A	$p = 0.037$ $r = 0.457$	$p = 0.126$ $r = 0.344$	$p = 0.001$ $r = 0.651$		**p < 0.001*** r = 0.697**	**p = 0.017* r = 0.511**	**p = 0.049* r = 0.434**	**p = 0.004** r = 0.598**	**p < 0.001*** r = 0.795**
FOXP3	$p = 0.064$ $r = 0.410$	$p = 0.097$ $r = 0.371$	**p < 0.001*** r = 0.774**	**p < 0.001*** r = 0.697**		**p < 0.001*** r = 0.686**	$p = 0.109$ $r = 0.359$	**p = 0.020* r = 0.500**	**p = 0.006** r = 0.574**
RORC	$p = 0.798$ $r = 0.059$	$p = 0.278$ $r = 0.248$	$p = 0.096$ $r = 0.372$	**p = 0.017* r = 0.511**	**p < 0.001*** r = 0.686**		$p = 0.821$ $r = 0.052$	$p = 0.415$ $r = 0.187$	**p = 0.049* r = 0.433**
IL22	**p = 0.044 r = 0.442**	$p = 0.857$ $r = -0.041$	$p = 0.117$ $r = 0.351$	**p = 0.049* r = 0.434**	$p = 0.109$ $r = 0.359$	$p = 0.821$ $r = 0.052$		$p = 0.179$ $r = 0.304$	**p = 0.024* r = 0.487**
IFNG	$p = 0.087$ $r = 0.382$	$p = 0.279$ $r = 0.247$	**p = 0.007** r = 0.563**	**p = 0.004** r = 0.598**	**p = 0.020* r = 0.500**	$p = 0.415$ $r = 0.187$	$p = 0.179$ $r = 0.304$		**p = 0.028* r = 0.476**
IL10	**p = 0.039* r = 0.452**	$p = 0.211$ $r = 0.284$	**p = 0.002** r = 0.622**	**p < 0.001*** r = 0.795**	**p = 0.006** r = 0.574**	**p = 0.049* r = 0.433**	**p = 0.024* r = 0.487**	**p = 0.028* r = 0.476**	

[a]Determination of statistical correlations was made according to Spearman's rank correlation test and represented by p value. $*$ equals $p < 0.05$, $**$ $p < 0.01$, and $***$ $p < 0.001$. The correlation coefficients are represented by "r."

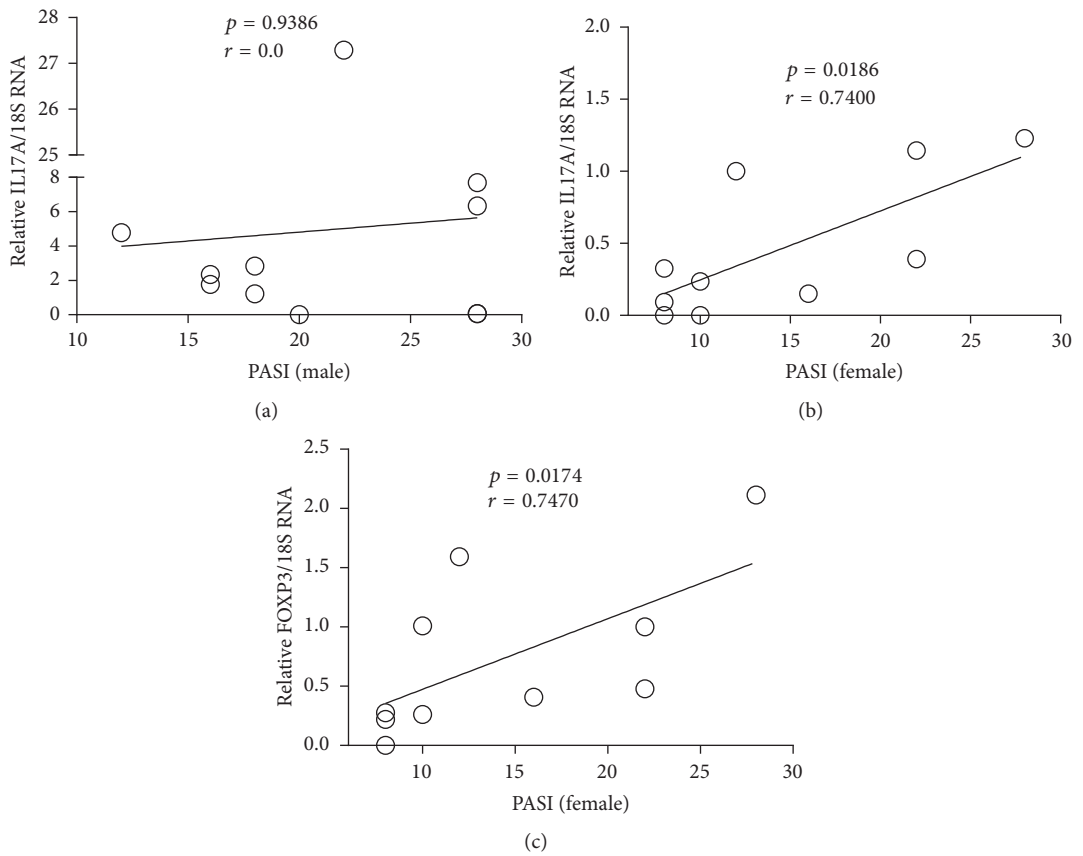

Figure 3: Correlation of IL17A (a, b) and FOXP3 (c) with disease severity between man and woman.

Table 3: Correlation coefficients and p values by gender[a].

Gene	Man	Gene	Woman
IL17A vs IL8	$p = 0.017$ $r = 0.716$	IL17A vs IFNG	$p < 0.001$ $r = 0.976$
IL17A vs IL22	$p = 0.041$ $r = 0.633$	IL17A vs FOXP3	$p = 0.049$ $r = 0.644$
IL17A vs IL36A	$p = 0.031$ $r = 0.664$	FOXP3 vs IL36A	$p < 0.001$ $r = 0.806$
IL17A vs IL10	$p = 0.001$ $r = 0.882$	—	—
IL10 vs IL8	$p = 0.042$ $r = 0.633$	—	—
IL10 vs IL22	$p = 0.034$ $r = 0.651$	—	—
IL36A vs IL8	$p = 0.024$ $r = 0.688$	—	—
RORC vs FOXP3	$p = 0.005$ $r = 0.800$	—	—

[a]Determination of statistical correlations was made according to Spearman's rank correlation test and represented by p value. $p < 0.05$, $p < 0.01$, and $p < 0.001$. The correlation coefficient is represented by "r." "vs" means versus.

Table 4: Association of transcripts expression and disease activity[a].

PASI score	Odds ratio	95% CI$^+$	p value
IL22	0.189	0.005–6.247	0.351
RORC	0.388	0.053–2.825	0.350
FOXP3	0.014	6.18e − 07–344.728	0.411
IL17A	8.16e − 06	5.49e − 10–0.121	*0.017*
IL36A	21.499	0.191–2414.25	0.203
IL33	1.000	0.989–1.012	0.881
IL8	1.686	0.984–2.888	0.057

[a]CI$^+$: confidence interval.

Further, we verified significant levels of FOXP3 in psoriasis lesions from severe patients. Considering that it is reported as a T regulatory cell transcription factor, we did not expect this increase. Soler and McCormick (2011) demonstrated that psoriasis patients really had regulatory T cells presenting FOXP3, although most of them were nonfunctional [29].

We did not observe significant differences in expression of IL8, IL22, IL10, RORC, IL33, and IL36A. However, we detected their signature in psoriatic skin at a transcript level and this corroborated with previous studies[9, 30, 31].

Continuing our analysis, we conducted all possible correlations between cytokines. Then, we found statistically significant correlation between cytokines from all three pathways with each other. It is important to notice that IL17A showed correlation with almost all analysed transcripts. Carrier and colleagues (2011) previously identified the interregulation among Th17 cytokines and IL-36 homologous forms. They also verified significance in correlation between IL36A versus IL17A and IFNG [32], as we did.

Curiously, we noted that IL17A had strong significant correlation with anti-inflammatory FOXP3 and IL10. We did not expect increased FOXP3 transcripts in moderate-severe patients, since this correlation seems antagonistic. Nevertheless, recent studies demonstrated another side of Treg cells. Bovenschen et al. (2011) found positive triple CD4$^+$ IL-17$^+$ FOXP3$^+$ in lesions from psoriasis patients indicating copathogenic profile. Soler and colleagues (2013) also pointed the connection between IL17 and FOXP3. They defended that Treg cells in psoriasis readily turn into IL-17-expressing cells [33, 34].

We also verified that when we considered gender, some correlations between cytokines only occurred in one of the groups. The male group preserved most of correlations especially those involving Th17 pathway. We could associate it to the fact that male group showed PASI mean 21.3 ± 5.8 and it is statistically higher than in women PASI 14.4 ± 7.2. It is worth pointing out that the severity of psoriasis in female patients may fluctuate with hormonal changes with worsening in puberty and peak at menopause [35]. Taking into consideration that population in this study consisted of eldered women (57.7 ± 9.8), they presented milder disease.

It is important to recognize our limitations in this study. We identified some failures such as small sample size, elevated cases of patients with moderate-severe disease, absence of healthy controls, and lack of proteomic analysis to confirm our findings. However, we know that, in psoriasis research, there are few studies with emphasis on experimental immunology in Brazil. Therefore, we hope that these data may open doors for future investigations.

5. Conclusion

Our study revealed increased transcripts levels of IL17A, IFNG, and FOXP3 in moderate-severe psoriasis patients. IL17A seems to play major influence in disease severity. In addition, we also showed complex network correlations between Th1, Th17, Treg, and IL-1 family related transcripts.

Competing Interests

All authors declare no conflict of interests.

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Research Article

Effect of Interleukin-15 on CD11b, CD54, and CD62L Expression on Natural Killer Cell and Natural Killer T-Like Cells in Systemic Lupus Erythematosus

Syh-Jae Lin,[1] Ji-Yih Chen,[2] Ming-Ling Kuo,[3,4,5] Hsiu-Shan Hsiao,[1] Pei-Tzu Lee,[1] and Jing-Long Huang[1]

[1]*Division of Asthma, Allergy, and Rheumatology, Department of Pediatrics, Chang Gung Children's Hospital, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[2]*Department of Medicine, Division of Allergy, Immunology and Rheumatology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taiwan*
[3]*Department of Microbiology and Immunology, Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[4]*Division of Allergy, Asthma, and Rheumatology, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan, Taiwan*
[5]*Chang Gung Immunology Consortium, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan*

Correspondence should be addressed to Jing-Long Huang; long@adm.cgmh.org.tw

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Adhesion molecules may play an important role in systemic lupus erythematosus (SLE) pathogenesis. We investigated the effect of interleukin- (IL-) 15 on CD11b, CD54, and CD62L expression on natural killer (NK) cells, T cells, and $CD56^+CD3^+$ NKT-like cells from SLE subjects and healthy controls. SLE patients had decreased circulating NK cells and NKT-like cells compared to controls. NK cells from SLE patients showed higher CD11b and CD62L expression compared to controls. IL-15 enhanced CD11b and CD54 but downregulated CD62L expression on NK cells from SLE patients. Similar observations were found for T cells and NKT-like cells. NK cells from SLE patients expressed higher CD56 than controls; both could be further enhanced by IL-15. IL-15 also enhanced CD56 expression of NKT-like cells from SLE patients. A greater degree of IL-15 induced downregulation of CD62L on NKT-like cells noted in SLE patients compared to controls. The percentage of CD11b expressing NK cells and the % inhibition of CD62L expression on NKT-like cells by IL-15 correlated with serum anti-dsDNA levels in SLE patients, respectively. Taken together, we demonstrated the dysfunctional NK and NKT-like cells in SLE patients with regard to CD11b and CD62L expression and their response to IL-15.

1. Introduction

Systemic lupus erythematosus (SLE) is characterized by many immunologic abnormalities involving various immune cells like T and B cells [1, 2]. Natural killer (NK) cells, defined by expression of CD56 and lack of CD3, are important effector cells in the innate immune response against infections and tumors [3]. Two subsets of human peripheral blood NK cells have been identified: $CD56^{dim}$ $CD16^+$ NK subset is more cytotoxic, while $CD56^{bright}$ subset has the capacity to produce abundant cytokines and plays an important immunoregulatory role [4]. Previous studies have found a decrease in NK cell numbers, impaired NK cytotoxicity, and defects of NK differentiation in SLE patients [5–8].

$CD3^+CD56^+$ NKT-like cells refer to a subset of $\alpha\beta$ T cells expressing NK activation receptors exhibiting an effector memory phenotype [9, 10]. Like NK cells, NKT-like cells expand in response to viral infection by producing inflammatory cytokines, such as IFN-γ [11]. Similar to NK cells, they possess antitumor activity and lyse target cells by secreting perforin and granzyme [12]. NKT-like cells were reported to be decreased in SLE patients. The number of

NKT-like cells correlated inversely with SLE disease activity [13].

Interleukin- (IL-) 15 is a pleiotropic common gamma chain signaling cytokine that is important for the activation of $CD8^+$ T cells and NK cells [14, 15]. IL-15 plays a *crucial* role in NK differentiation and survival, as in IL-15-deficient mice; the development of NK cells is severely compromised [16]. Patients with SLE have increased serum levels of IL-15, which did not correlate with disease severity [17, 18]. It remains uncertain whether IL-15 may contribute to the pathogenesis of SLE.

Cell adhesion molecules that mediate the leukocyte recruitment to the inflamed tissue and regulate lymphocyte homing may play a pathogenic role in SLE [19, 20]. CD11b is an important integrin that marks NK cell maturation and cytotoxicity [21]. CD54 (ICAM-1) belongs to the immunoglobulin gene superfamily and plays an important role in various inflammatory conditions [22]. CD62L belongs to a member of selectins that is important for NK cells homing to the lymph nodes and also an important marker for NK maturation and response to viral infections [23]. Previous studies have shown that circulating soluble CD54 and CD62L correlated with SLE disease activity [24, 25].

In the present study we examined the expression of CD11b, CD54, and CD62L on NK, T, and NKT-like cells from the peripheral blood of both SLE patients and healthy controls. We sought to determine whether IL-15 would influence the expression of these molecules and their relationship to SLE disease activity.

2. Materials and Methods

2.1. Study Subjects. Study subjects include 33 SLE patients ($n = 33$) and 17 healthy controls recruited from Chang Gung Memorial Hospital (CGMH), Linkou, Taiwan. The diagnosis of SLE fulfills the 1997 American College of Rheumatology classification criteria [26]. We evaluated the severity of our SLE patients using the systemic lupus erythematosus disease activity index (SLEDAI) scoring method [27]. Laboratory parameters such as C3, C4, and anti-dsDNA were recorded. We obtained heparinized whole blood from each study individual under the preapproval by the institutional research committee at CGMH. Informed consent was provided for all blood donors.

2.2. PBMC Incubation. Peripheral blood mononuclear cells (PBMCs) were collected by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Uppsala, Sweden) within 6 hours of blood drawing. PBMCs were then incubated in RPMI-1640, 10% fetal calf serum in the presence or absence of IL-15 at the concentration of 10 ng/mL (Peprotech, Rocky Hill, USA) for eighteen hours. NK cell viability remains >98% after incubation with IL-15 at 10 ng/mL for 18 hours, while IL-15 at 50 or 100 ng/mL may induce the apoptosis of NK cells (data not shown).

2.3. Flow Cytometric Analysis. Following incubation with or without IL-15, PBMCs were harvested, washed, and resuspended for staining. For each experiment, cells were stained with APC-conjugated anti-CD3 antibody (BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-CD56 antibody (BD Biosciences, San Jose, CA, USA), and PE-conjugated anti-CD54, anti-CD11b, and anti-CD62L antibody (Beckman Coulter, Fullerton, CA, USA) for 30 min at 4°C. Cells were then washed twice and analyzed by a Becton Dickinson FACScan analyzer. First, the lymphocyte population was gated to identify CD3-positive and CD3-negative lymphocyte populations. Secondly, the CD3-positive and CD3-negative lymphocyte populations were gated for further analysis of the expression patterns of CD56 and the adhesion molecules. In some experiments when NK cell numbers are adequate, $CD56^+CD3^-$ NK cells were further divided by CD16 and CD56 staining in to 2 groups: $CD16^+CD56^{dim}$ NK cells (more than 80%) and $CD16^{dim}$ $CD56^{bright}$ NK cells according to the mean fluorescence intensity (MFIs) of CD56 (Figure 3). The % inhibition of CD62L expression by IL-15 was calculated as [MFI of CD62L in medium − MFI of CD62L in the presence of IL-15 (10 ng) for 18 hours/MFI of CD62L in medium] × 100.

2.4. Statistics. The Wilcoxon signed rank test was applied for analysis of the responses before and after a treatment, using SPSS 9.0 software. The Mann-Whitney U test was used to compare SLE and healthy donor responses. Spearman's rank correlation was applied to detect the association between different parameters. The data are presented as means ± standard error of mean. Data were considered significantly different if p was less than 0.05.

3. Results

3.1. Patient Characteristics and Percentages of NK, T, and NKT-Like Cells. The characteristics of the controls and SLE patients were shown in Table 1. Patients were predominantly female, ages between 12 and 32 years, and had an average disease duration of 8.9 ± 0.7 years. Approximately 57.6% of patients were taking regular corticosteroids and some were receiving methotrexate, azathioprine, or mycophenolate. The percentages of $CD56^+CD3^-$ NK cells from the peripheral blood of SLE patients were lower than those from healthy controls ($p = 0.008$). The percentages of $CD56^+CD3^+$ NKT-like cells from SLE patients were also lower than controls ($p = 0.038$). There was no difference of the percentages of $CD56^-CD3^+$ T cells between SLE patients and controls ($p = 0.38$).

3.2. CD11b, CD54, and CD62L Expression on $CD56^+CD3^-$ NK Cells. Figure 1(a) shows the CD11b, CD54, and CD62L expression on NK cells from SLE patients and controls. NK cells from SLE expressed higher CD11b compared to controls (42.9 ± 3.1% versus 31.0 ± 5.1%, $p = 0.032$). The expression of CD54 on NK cells from SLE patients was not different from controls (18.2 ± 2.2% versus 14.3 ± 2.5%, $p = 0.302$). As the majority of NK, T, and NKT-like cells expressed CD62L (data not shown), CD62L expression was presented as MFI. The MFI of CD62L on NK cells from SLE patients was higher than controls (9226 ± 1395 versus 5617 ± 658, $p = 0.033$).

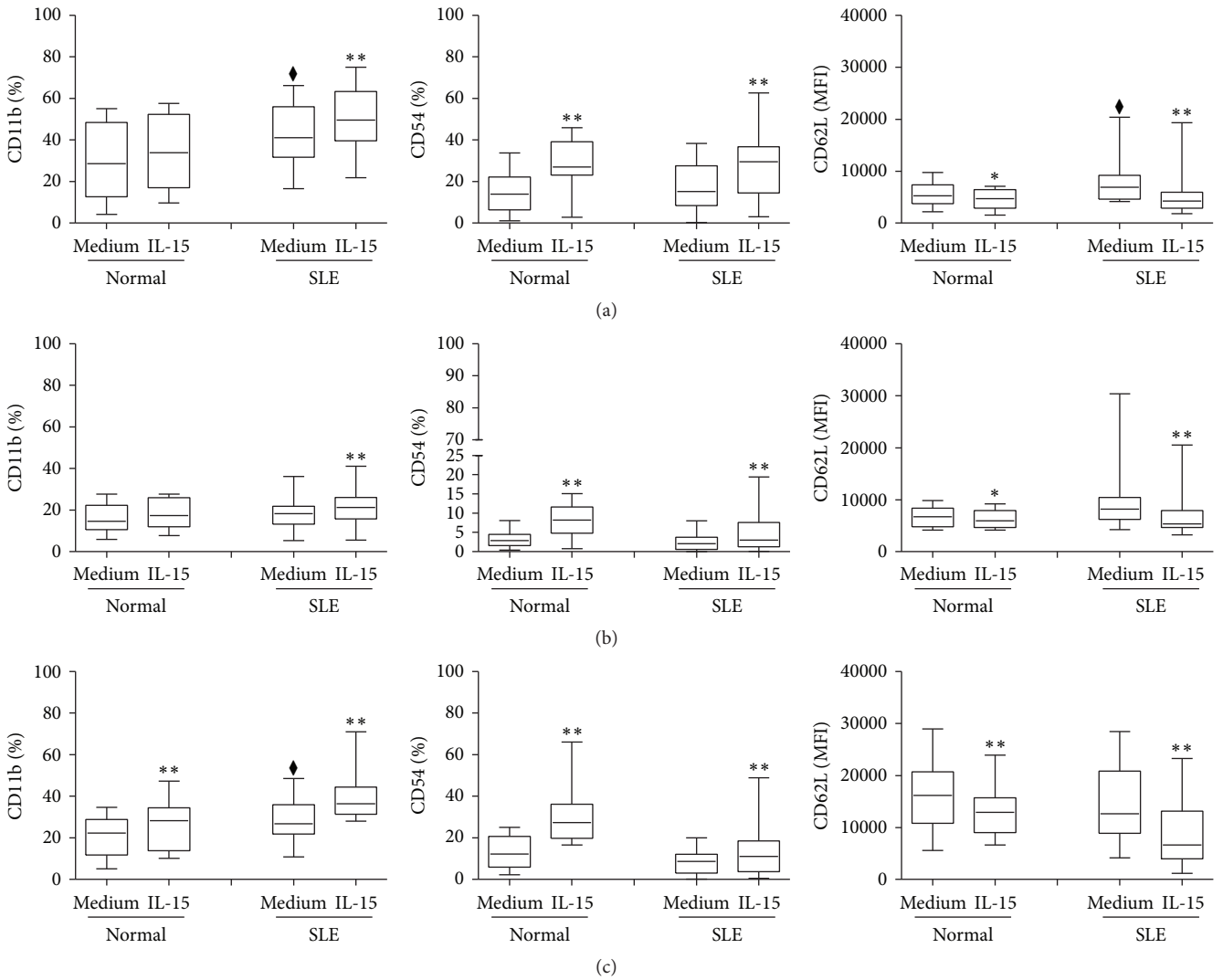

Figure 1: The CD11b, CD54, and CD62L expression on (a) $CD56^+CD3^-$ NK cells, (b) $CD56^-CD3^+$ T cells, and (c) $CD56^+CD3^+$ NKT-like cells from SLE patients and healthy controls. PBMCs were stimulated with IL-15 (10 ng/mL) for 18 hrs. Cells were stained by anti-CD3 and anti-CD56 antibodies and segregated into $CD56^+CD3^-$ NK cells, $CD56^-CD3^+$ T cells, and $CD56^+CD3^+$ NKT-like cells. Surface marker (CD11b, CD54, or CD62L) expression on each cell population was analyzed by flow cytometry. Data was expressed as percent expression (%) ± SEM. $^\blacklozenge p < 0.05$ compared to normal; $^* p < 0.05/^{**} p < 0.01$ compared to medium (normal, $n = 17$; SLE, $n = 33$).

IL-15 enhanced the CD11b expression of NK cells from SLE patients (50.1 ± 2.9% versus 42.9 ± 3.1%, $p = 0.012$) but had no effect on CD11b expression of NK cells from controls (34.8 ± 4.9% versus 31.0 ± 5.0%, $p = 0.055$). IL-15 enhances the expression of CD54 on NK cells from both SLE patients (27.4 ± 2.8% versus 18.2 ± 2.2%, $p < 0.001$) and controls (28.2 ± 2.9% versus 14.3 ± 2.5%, $p = 0.002$), respectively. In contrast, IL-15 resulted in a decrease in CD62L expression on NK cells from both SLE patients (5619 ± 1357 versus 9226 ± 1395, $p = 0.028$) and controls (4482 ± 552 versus 5617 ± 658, $p = 0.001$).

3.3. CD11b, CD54, and CD62L Expression on $CD56^-CD3^+$ T Cells. CD11b, CD54, and CD62L expression on T cells from SLE patients and controls are shown in Figure 1(b). T cells expressed much lower CD11b and CD54 than did NK cells, both SLE patients and controls alike. T cells from SLE patients expressed comparable CD11b (17.9 ± 1.5% versus 16.1 ± 1.9%, $p = 0.528$), CD54 (2.4 ± 0.4% versus 3.3 ± 0.6%, $p = 0.151$), and CD62L (65.7 ± 3.1% versus 59.0 ± 3.1%, $p = 0.193$) compared to controls. IL-15 enhanced CD54 expression on T cells from SLE patients (4.8 ± 0.9% versus 2.4 ± 0.4%, $p < 0.001$) and controls (8.3 ± 1.0% versus 3.3 ± 0.6%, $p = 0.001$) alike. IL-15 resulted in an increase of CD11b expression (20.7 ± 1.7% versus 17.9 ± 1.5%, $p = 0.003$) and a decrease of CD62L MFI (7205.7 ± 904.7 versus 10767.9 ± 1424.9, $p < 0.001$) on T cells from SLE patients.

3.4. CD11b, CD54, and CD62L Expression on $CD56^+CD3^+$ NKT-Like Cells. CD11b, CD54, and CD62L expressions on NKT-like cells from SLE and controls are shown in Figure 1(c). NKT-like cells from SLE patients expressed higher

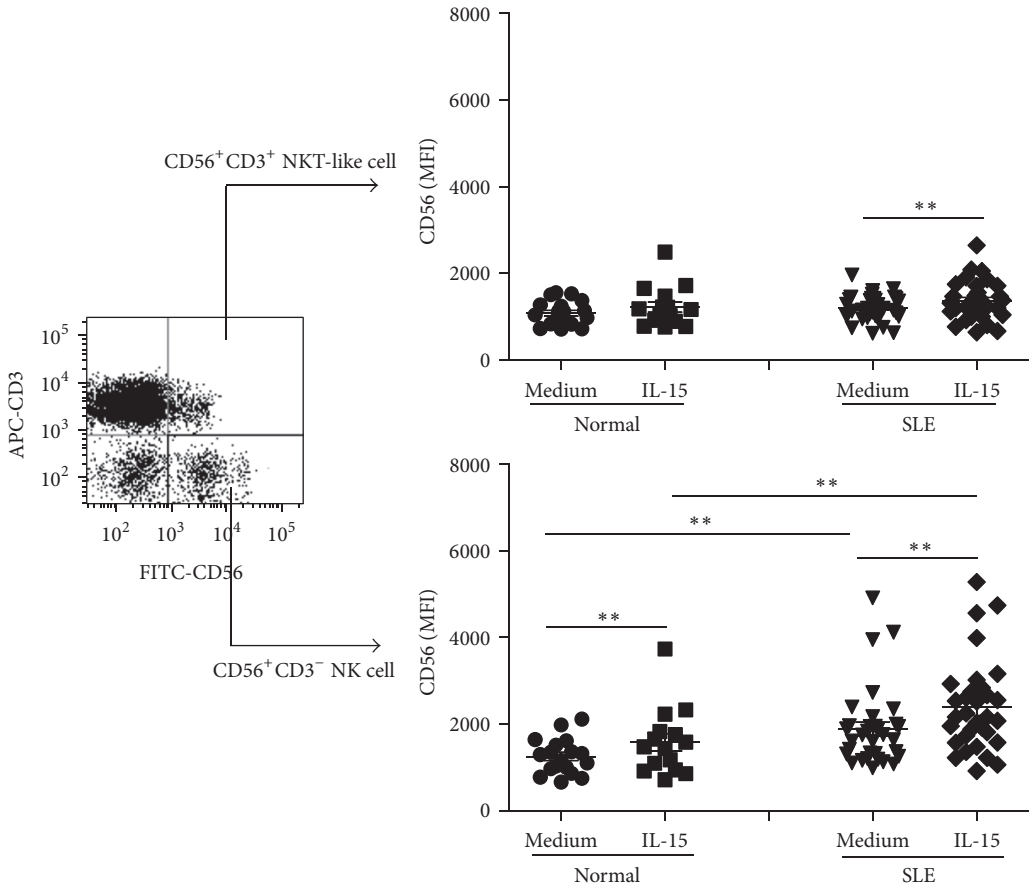

Figure 2: Effect of interleukin- (IL-) 15 on CD56 expression of $CD56^+CD3^-$ NK cells and $CD56^+CD3^+$ NKT-like cells from SLE patients and healthy controls. PBMCs were stimulated with IL-15 (10 ng/mL) for 18 hrs and stained with anti-CD3 and anti-CD56. The $CD56^+CD3^-$ NK and $CD56^+CD3^+$ NKT-like cell population were separated by flow cytometry as illustrated. Data was expressed as mean fluorescence intensity (MFI) ± SEM. $^{**}p < 0.01$ (normal, $n = 17$; SLE, $n = 33$).

CD11b (28.7 ± 1.9% versus 21.0 ± 2.7%, $p = 0.043$) but comparable CD54 (8.5 ± 1.2% versus 13.2 ± 2.5%, $p = 0.075$) and CD62L (14565 ± 1392 versus 16676 ± 1630 [MFI], $p = 0.317$) compared to controls. Similar to that observed in NK cells, IL-15 enhanced CD11b and CD54 expression on NKT-like cells from both SLE patients (CD11b: 38.4 ± 1.9% versus 28.7 ± 1.9%, $p < 0.001$; CD54: 14.5 ± 2.9% versus 8.5 ± 1.2%, $p = 0.005$) and controls (CD11b: 26.0 ± 3.1% versus 21.0 ± 2.7%, $p = 0.007$; CD54: 30.6 ± 4.2% versus 13.2 ± 2.5%, $p = 0.007$), respectively. IL-15 resulted in a decrease in CD62L MFI on NKT-like cells from both SLE patients (9213 ± 1150 versus 14565 ± 1392, $p < 0.001$) and controls (13151 ± 1214 MFI versus 16676 ± 1630, $p = 0.002$).

3.5. IL-15 Enhances CD56 Expression on NK and NKT-Like Cells from SLE. We next examine the expression of CD56, another NK marker responsible for adhesive function [28], on NK cells from SLE patients compared to controls. As shown in Figure 2, although NK cells are deficient in numbers compared to controls (Table 1), NK cells from SLE patients exhibited higher CD56 MFI on $CD3^-CD56^+$ NK cells (1886.4 ± 156.7 versus 1250.9 ± 96.8, $p = 0.002$). IL-15 enhanced CD56 MFI on NK cells from both SLE patients and controls. The CD56 MFI on NKT-like cells from SLE patients was comparable to that from controls. IL-15 enhanced CD56 MFI of NKT-like cells from SLE patients (1363 ± 79 versus 1199 ± 52, $p = 0.001$) and had no effect on controls (1218 ± 120 versus 1079 ± 67, $p = 0.211$).

3.6. $CD56^{bright}$ NK Cells Were More Responsive to IL-15 Induced Downregulation of CD62L. When NK cells are further divided into $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ subsets (Figure 3), we found that there is no difference of CD11b, CD54, and CD62L expression between $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in SLE patients and controls alike. For SLE patients, IL-15 enhanced CD11b expression of $CD56^{bright}$ NK subsets (58.8 ± 4.2% versus 50.4 ± 4.9%, $p = 0.002$) but did not affect that of the $CD56^{dim}$ subsets (52.1 ± 4.2% versus 47.8 ± 4.6%, $p = 0.096$). No significant difference of CD54 expression and its response to IL-15 was noted between $CD56^{bright}$ and $CD56^{dim}$ NK subsets. $CD56^{bright}$ NK cells expressed greater CD62L than did $CD56^{dim}$ subsets. IL-15 downregulates CD62L expression on $CD56^{bright}$ NK

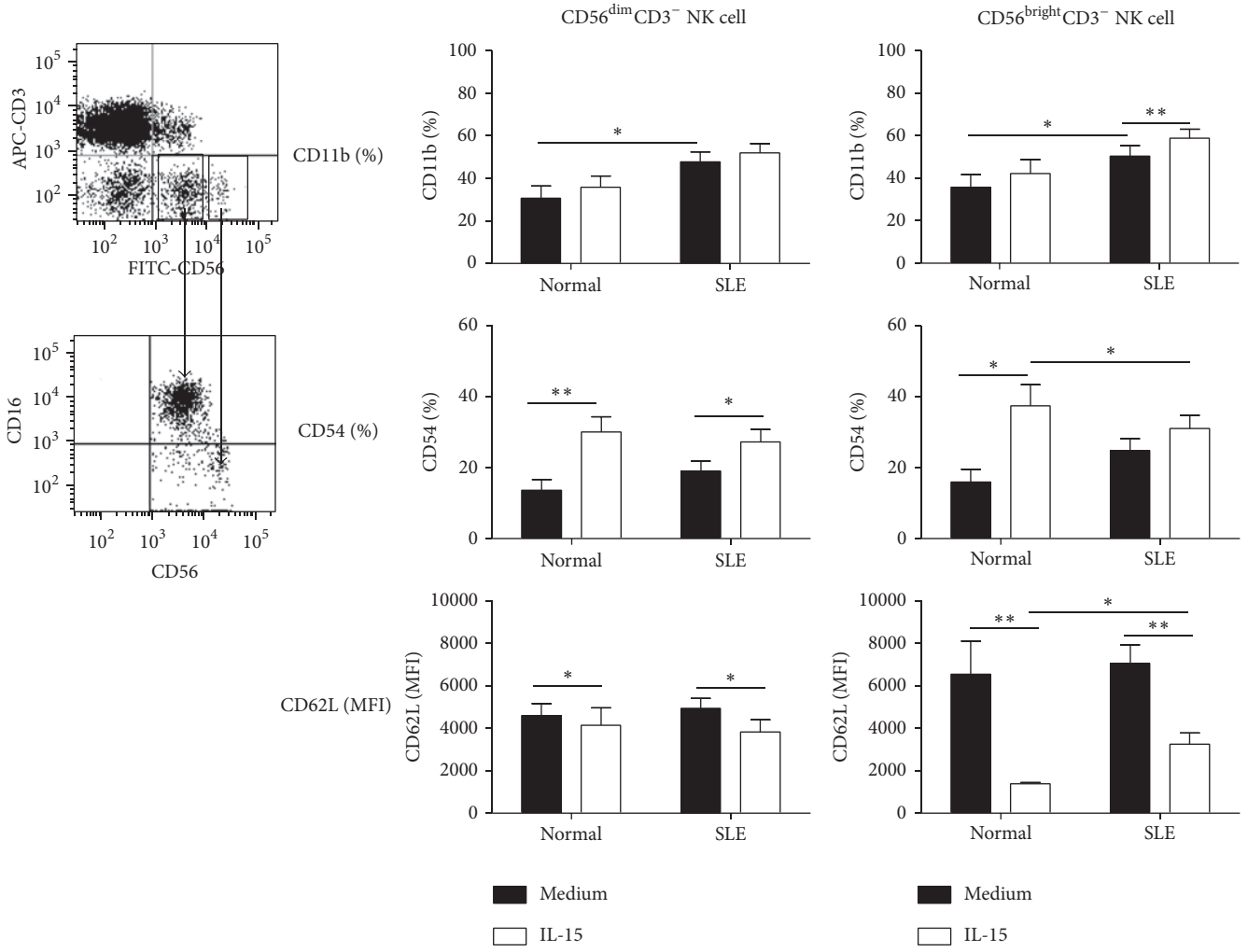

Figure 3: The CD11b, CD54, and CD62L expression on $CD56^{dim}CD3^{-}$ and $CD56^{bright}CD3^{-}$ NK cells from SLE and healthy donors. PBMCs were stimulated with IL-15 (10 ng/mL) for 18 hrs and stained with anti-CD3 and anti-CD56. A representative profile of how the $CD56^{dim}$ and $CD56^{bright}$ NK populations were gated under flow cytometry is shown. Data was expressed as percent expression (%) ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ (normal, $n = 10$; SLE, $n = 17$).

subsets to a greater extent than on that of on $CD56^{dim}$ subsets from SLE patients (28.3 ± 4.7% versus 20.6 ± 3.4%, $p = 0.023$).

3.7. Greater CD62L Downregulation by IL-15 in NKT-Like Cells from SLE Patients. We next compare the degree of CD62L inhibition by IL-15 of NK cells and NKT-like cells (Figure 4). The % downregulation of CD62L MFI by IL-15 in NK cells was comparable between SLE patients and controls (21.3 ± 3.3% versus 23.8 ± 4.4%, $p = 0.66$). However, NKT-like cells from SLE were more susceptible to IL-15 induced downregulation compared to controls (22.4 ± 5.2% versus 8.4 ± 2.5%, $p = 0.018$).

3.8. Correlation of CD11b and CD62L Expression on NK and NKT Cells and Disease Activity. To evaluate the clinical relevance of adhesion molecule expression on NK and NKT-like cells in SLE patients, we investigate relationships of the percentages of NK and NKT-like cell bearing CD11b and CD62L and SLE-related laboratory parameters by regression analysis. As shown in Figure 5, univariate analysis showed that the percentages of $CD11b^{+}$ NK cells correlated with serum anti-dsDNA levels, in SLE patients ($r = 0.428$, $p = 0.041$), while the % inhibition of CD62L MFI by IL-15 of NKT-like cells also correlates with serum anti-ds DNA levels ($r = 0.374$, $p = 0.043$).

4. Discussion

In the present study, we compared the expression of adhesion molecules CD11b, CD54, and CD62L on NK, T, and NKT-like cells from SLE patients and healthy controls. We also determined the effect of IL-15, an immunoregulatory cytokine, on adhesion molecule expression. IL-15 may directly affect immune cells or indirectly by upregulating other inflammatory mediators. We have shown that IL-15 could enhance

Table 1: Characteristics of controls and patients with SLE.

Characteristics	Controls ($n = 17$)	SLE patients ($n = 33$)
Sex (male/female)	0/17	0/33
Age, mean (range)	29.3 ± 0.9 (27–34)	22.5 ± 0.9 (12–32)
SLEDAI score, median (range)	NA	7 (0~25)
C3, median (range)	NA	66.7 (21–106)
C4, median (range)	NA	9.7 (2.1–32.9)
Anti-dsDNA, median (range)	NA	309.9 (79.6–632.8)
Average disease duration	NA	8.9 ± 0.7
Taking regular corticosteroids	NA	57.6%
Cell population		
$CD56^+CD3^-$ NK cells (%)[#]	7.3 ± 0.9%	4.4 ± 0.6%**
$CD56^-CD3^+$ T cells (%)	71.8 ± 1.4%	67.2 ± 2.8%
$CD56^+CD3^+$ NKT-like cells (%)	3.5 ± 0.6%	2.2 ±0.3%*

*$p < 0.05$ and **$p < 0.01$ compared to controls; [#]the percentages of NK cells, T cells, and NKT-like cells in total lymphocytes.

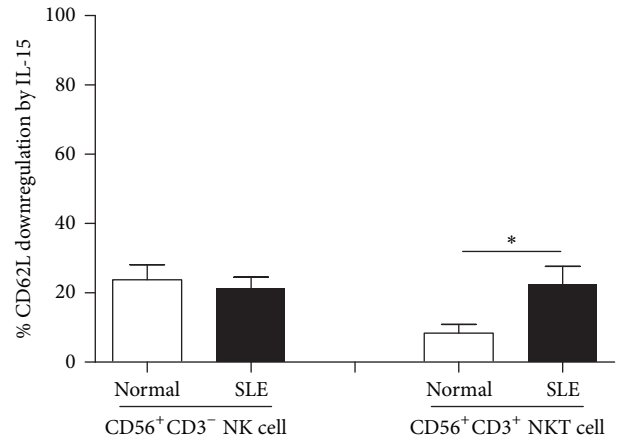

Figure 4: The inhibition of CD62L expression by IL-15 on $CD56^+CD3^-$ NK cells and $CD56^+CD3^+$ NKT-like cells from SLE and healthy donors. PBMCs were stained with anti-CD3, anti-CD56, and anti-CD62L antibodies and were analyzed under flow cytometry. % CD62L downregulation by IL-15 is calculated as [MFI of CD62L in medium − MFI of CD62L in the presence of IL-15 (10 ng/mL) for 18 hours/MFI of CD62L in medium] × 100. *$p < 0.05$ (normal, $n = 10$; SLE, $n = 17$).

tumor necrosis factor-alpha and interferon-gamma production of NK cells [29]. We have also demonstrated that IL-15 induced interferon-gamma but not IL-4 production from human iNKT cells [30].

In agreement with the previous studies [31, 32], we found that the percentages of NK cells and NKT-like cells are decreased in PBMC from SLE patients compared to controls. However, The percentages of T cells were comparable to controls. CD11b, but not CD54 and CD62L expression on NK cells from SLE patients, was increased compared to controls. NK cells progress from an immature $CD27^+CD11b^-$ stage to an intermediate $CD27^+CD11b^+$ stage and finally to a $CD27^-CD11b^+$ stage [33]. $CD11b^+$ NK cells are more readily responsive to cytokine-mediated activation during viral infection [34]. Our findings suggest that $CD11b^+$ bearing NK cells may play a pathogenic role in SLE.

T cells from SLE have been shown to have aberrant signaling, abnormal cytokine secretion [35]; we found however that CD11b, CD54, and CD62L expression of T cells from SLE was comparable to controls. $CD3^+CD56^+$ NKT-like cells have been reported to be increased in certain autoimmune conditions like Behçet's diseases [36, 37]. Similar to that observed with NK cells, we found that $CD3^+CD56^+$ NKT-like cells from SLE patients exhibited higher CD11b expression compared to the corresponding controls.

Consistent with our previous work [38, 39], IL-15 enhanced CD54 expression of NK, T, and NKT-like cells from SLE patients and controls alike, suggesting its ability to promote cell migration and cytotoxicity. We found that IL-15 enhanced CD11b expression of NK cells and T cells from SLE patients, respectively, an effect not observed with healthy controls. Contrary to that observed in CD11b and CD54, we found that IL-15 downregulates CD62L expression on NKT cells and NKT-like cells, from SLE patients and controls.

Although the percentages of NK cells decreased, we found that the CD56 MFI was elevated on SLE NK cells compared to controls, consistent with Schepis et al. [7]. We further demonstrated that CD56 expression on NK cells from SLE patients could be further enhanced with IL-15 stimulation. IL-15 also preferentially enhanced MFI of CD56 of NKT-like cells from SLE patients. Thus, IL-15 may play a disease-promoting role by enhancing CD56 expression on NK and NKT-like cells of SLE patients.

Previous studies have shown the dysregulation of $CD56^{bright}$ NK cells in SLE patients [7, 31]. We found that $CD56^{bright}$ NK cells from SLE patients expressed higher CD11b and CD62L than the $CD56^{dim}$ counterparts. CD11b on $CD56^{bright}$ NK cells from SLE patients but not controls could be enhanced by IL-15. $CD56^{bright}$ NK cells were also more readily susceptible to IL-15 compared to their $CD56^{dim}$ counterparts with regard to CD62L downregulation.

There was no difference in the % inhibition of CD62L by IL-15 on NK cells between SLE patients and controls. However, a greater degree of IL-15 induced downregulation of CD62L on NKT-like cells was noted in SLE patients compared to controls. IL-15 resulted in shedding of CD62L on NK cells and especially NKT-like cells to the circulation which may aggravate the tissue inflammation in SLE. Serum soluble CD62L has been reported to be an SLE disease marker [40, 41].

Correlation analysis revealed that CD11b expression on NK cells correlated with serum anti-dsDNA levels in SLE patients. The % inhibition of CD62L MFI by IL-15 of NKT-like cells also correlated with serum anti-dsDNA levels. IL-15

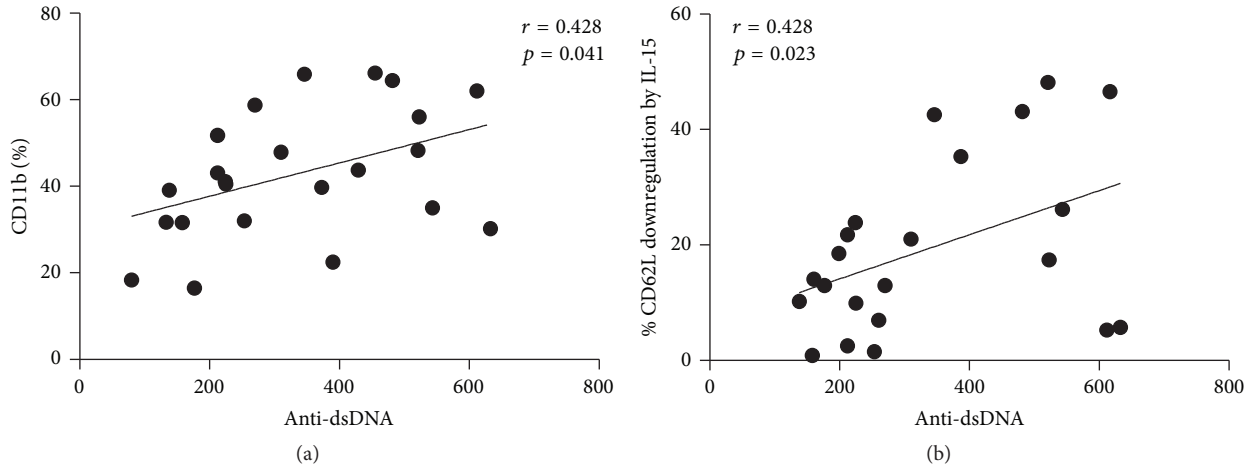

Figure 5: Correlation between (a) serum anti-dsDNA and the percentages of $CD11b^+$ NK cells and (b) serum anti-dsDNA and the % inhibition of CD62L MFI on NKT-like cells by IL-15 calculated as [MFI of CD62L in medium − MFI of CD62L in the presence of IL-15 (10 ng/mL) for 18 hours/MFI of CD62L in medium] × 100.

may aggravate SLE disease severity by promoting CD62L shedding from the surface of NKT-like cells.

Cytokine inhibition may be used as a strategy for treating SLE. Ma et al. demonstrated a beneficial effect of anti-IL-15 in the treatment of murine lupus [42]. Humax-IL15, a human IgG1 anti-IL-15 monoclonal antibody, has been shown to improve symptoms in patients with rheumatoid arthritis [43]. Our finding suggests that IL-15 may be a potential target for immunotherapy against SLE.

Taken together, we demonstrated the dysfunctional NK and NKT-like cells in SLE patients with regard to CD11b and CD62L expressions and their response to IL-15. IL-15 may aggravate inflammation by preferentially upregulating CD11b and CD56 and downregulating CD62L on NK and NKT-like cells in SLE patients. Antagonist to IL-15 may provide a therapeutic option to ameliorate the progression of SLE.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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