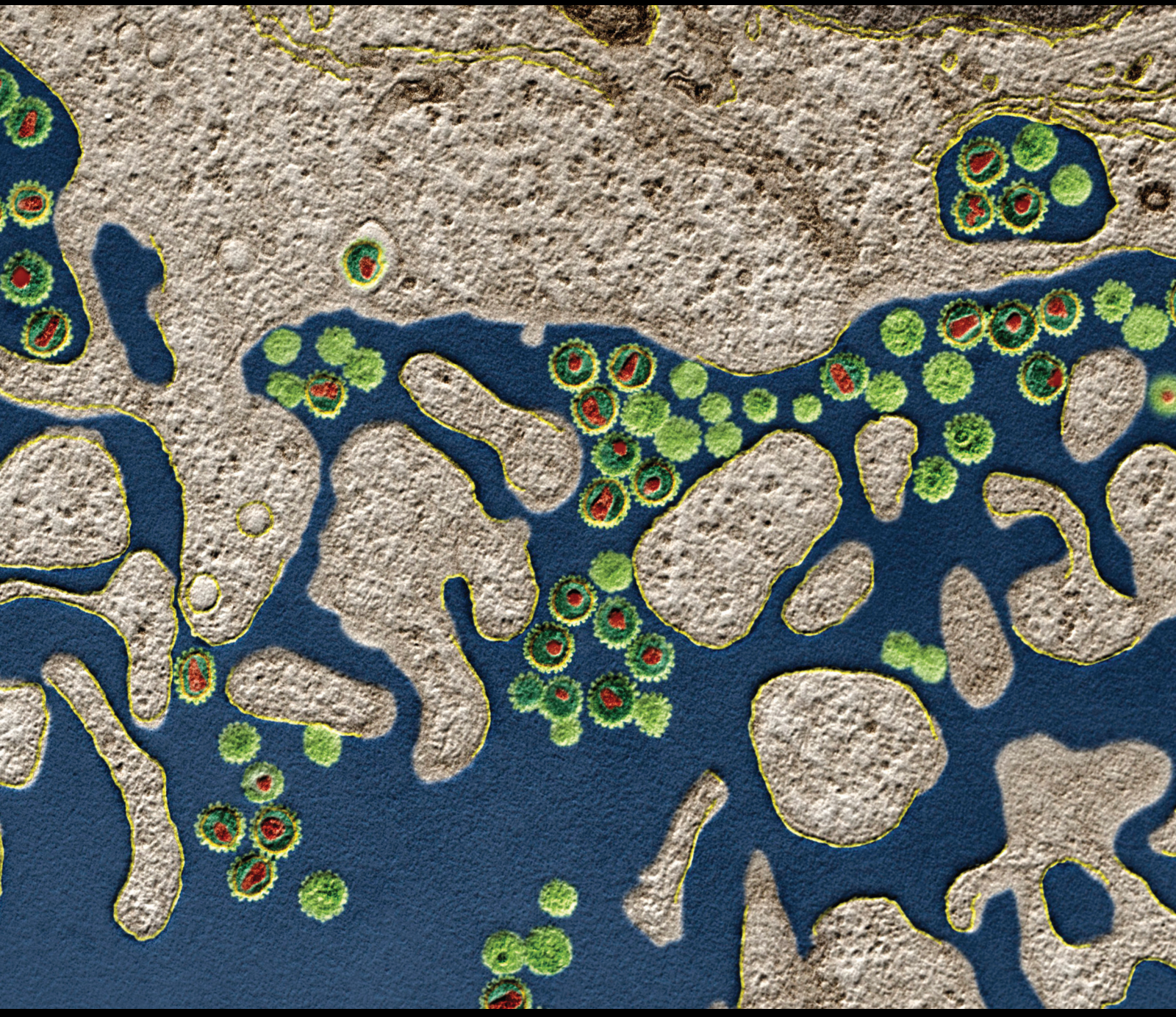
Journal of Immunology Research

Regulation of Inflammation in Autoimmune Diseases 2020

Lead Guest Editor: Lihua Duan
Guest Editors: Keshav Raj Sigdel, Xiaoquan Rao, and Yan Yang



Regulation of Inflammation in Autoimmune Diseases 2020

Journal of Immunology Research

Regulation of Inflammation in Autoimmune Diseases 2020

Lead Guest Editor: Lihua Duan
Guest Editors: Keshav Raj Sigdel, Xiaoquan Rao, and Yan Yang

Contents

Hindawi
Journal of Immunology Research
Volume 2021, Article ID 9473508, 8 pages
https://doi.org/10.1155/2021/9473508

Review Article

The Potential Importance of MicroRNAs as Novel Indicators How to Manage Patients with Juvenile Idiopathic Arthritis More Effectively

Krzysztof Orczyk and Elzbieta Smolewska

Department of Pediatric Cardiology and Rheumatology, Medical University of Lodz, Sporna 36/50, 91-738 Lodz, Poland

Correspondence should be addressed to Elzbieta Smolewska; e.smolewska@wp.pl

Received 15 July 2020; Revised 20 December 2020; Accepted 11 January 2021; Published 29 January 2021

Academic Editor: Lihua Duan

Small, noncoding sequences of ribonucleic acid called microRNAs (miRNAs, miR) are functioning as posttranscriptional regulators of gene expression. As they draw increasing attention of rheumatologists, there is a growing body of evidence concerning specific molecules that may affect the long-term care of patients with inflammatory arthritides. Findings involving children with juvenile idiopathic arthritis (JIA) are still limited though. The aim of the study was to browse the available data on microRNAs which may be utilized as potential biomarkers helpful in diagnosing and monitoring JIA patients. The review contains a brief summary on the most studied sequences: miR-16, miR-125a-5p, miR-146a, miR-155, and miR-223. It is complemented with other miRNAs possibly relevant for JIA (miR-145, miR-23b, miR-27a, and miR-204) and discussion on challenges for using miRNAs in pediatric rheumatology (particularly, issues regarding specificity of biomarkers and measurements involving synovial fluid).

1. Introduction

Small, noncoding sequences of microRNA (miRNA, miR) have already established their position as an innovative part of the diagnostic process in the bulk of disorders, with particular emphasis placed on neoplastic diseases. miRNAs should be considered as negative posttranscriptional regulators affecting the expression of genes involved in both innate and adaptive immune responses [1]. Karami et al. stated that nearly 60% of human genes contain at least one binding site for miRNA [2]. By direct base-pairing with the 3′ untranslated region (3′ UTR) of target mRNAs [3], miRNAs can suppress or impair gene translation leading to the inhibition of target protein synthesis [4]. A brief summary of miRNA biogenesis and its functioning is depicted in Figure 1.

According to Ormseth et al. [5], miRNAs are not limited to intracellular activity, but they can also be transported to recipient cells through plasma, where they are protected from degradation by several mechanisms, involving exosomes, microvesicles, lipoproteins, and RNA-binding proteins. High robustness of cell-free miRNAs in body fluids (e.g., plasma and serum) supports their potential role as reliable biomarkers [6, 7]. However, concentrations of particular miRNAs may differ from each other depending on the source of the examined material. Interestingly, miR-132, which has been observed to be significantly decreased in serum of RA patients, turned out to be overexpressed in synovial PBMCs [8].

Growing popularity and availability of measuring these molecules reflects from PubMed statistics: only in 2019, the number of the registered papers regarding miRNA exceeded 15,000 articles. Of these, just 121 referred to adult patients with rheumatoid arthritis (RA), whereas juvenile idiopathic arthritis (JIA) was involved in merely eight studies. The first study which postulated the role of miRNA in the pathogenesis of RA dates back to 2007, when Bhanji et al. reported that sera of RA patients include antibodies against Argonaute 2 (a protein required for miRNA-mediated gene silencing) [9]. In the following years, Romo-Garcia et al. speculated that the transcriptional arrest present at the early stages of RA may be related to overexpression of miRNAs and subsequent

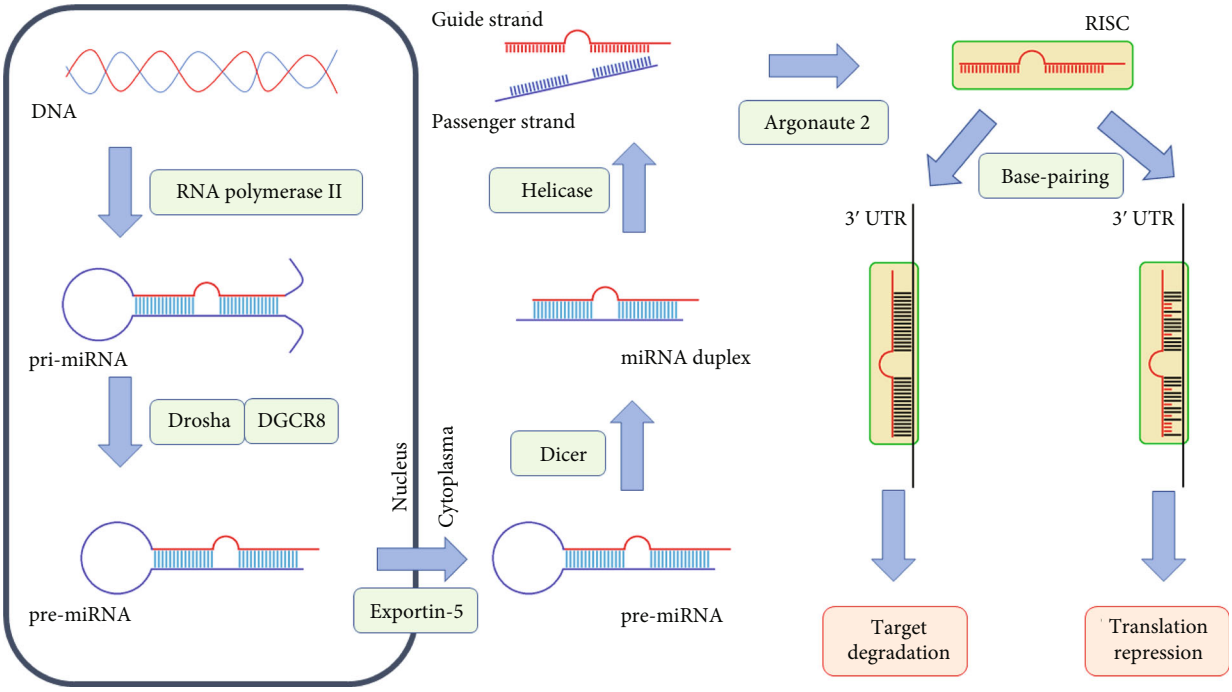


Figure 1: Biogenesis and functioning of miRNA. The figure illustrates the following steps: (a) endonuclear synthesis of the primary double-stranded miRNA transcript (pri-miRNA) of hundreds to thousands of nucleotides in length, mostly by RNA polymerase II; (b) cleavage of pri-miRNA by Drosha endonuclease accompanied by DiGeorge syndrome critical region protein 8 (DGCR8), resulting in formation of 60 to 70 nucleotide long pre-miRNA; (c) export of pre-miRNA from the nucleus into the cytoplasm by Exportin-5 coacting with GTP-binding nuclear protein Ran (Ran-GTP); (d) cleavage of pre-miRNA by Dicer into an asymmetric miRNA duplex containing guide and passenger strand of about 22 nucleotides in length; (e) unwinding the duplex by helicase and release of the passenger miRNA with its subsequent degradation; (f) loading the guide strand by Argonaute 2 into the RNA-induced silencing complex (RISC); (g) binding RISC (containing mature miRNA) to 3′ untranslated region (3′ UTR) of target mRNA; (h) translational repression or target degradation depending on the partial or full complementarity of base-pairing. Based on [60–62].

intensified inhibition of translation [7]. The initial evidence regarding miRNA in JIA, specifically miR-155, was reported in 2014 [10]. A thorough literature review involving the PubMed database provided at least 55 miRNA sequences (listed and briefly described in Supplementary Table 1) which may be applicable to rheumatic diseases. They may affect the pathogenesis of JIA through several mechanisms which include, among others, repressing the expression of genes that are crucial for the TNF/IL-1 pathway [11] or dysregulating the macrophage polarization [12]. Within this paper, we will summarize the available data on the most studied miRNAs in RA and JIA (miR-16, miR-125a-5p, miR-146a, miR-155, and miR-223). Then, we will shed a light on several less known sequences which bear a potential for the future diagnostic process and monitoring disease activity in JIA patients.

2. miR-16

miR-16 seems to be one of the crucial factors for the pathogenesis of RA and JIA, as through targeting the silencing mediator for retinoid and thyroid hormone receptor (SMRT), it regulates the expression of proinflammatory cytokines, particularly interleukin- (IL-) 1α, IL-6, and IL-8 [13]. Zhou et al. [14] hypothesized the role of miR-16 as a key regulator of Toll-like receptor-signaled inflammatory response. According to Murata et al. [15], miR-16 levels detected in RA patients were markedly higher than in osteoarthritis patients. Furthermore, Demir et al. [16] reported elevated miR-16 concentrations in JIA patients when compared to healthy controls. Nevertheless, the plasma level of this molecule fluctuates in a disease activity-dependent manner which may lead to contradictory findings varying on the measurement protocol. Hence, miR-16 was significantly upregulated in children at the onset of both poly- and oligoarticular JIA [17], but it inversely correlated with Disease Activity Score 28-Joint Count (DAS28) in patients with the average RA duration of 10.4 years [15]. Interestingly, Filková et al. [18] observed that miR-16 is higher in patients with established RA than in the early stage of the disease. Moreover, they hypothesized that this molecule may be utilized as a biomarker of RA outcome because its pretherapy level correlated with disease activity drop after 9 months of treatment. Correlation between high disease activity and miR-16 concentrations was also observed in inflammatory bowel diseases [19] and in ankylosing spondylitis [20]. Nonetheless, children with juvenile spondyloarthropathy had lower miR-16 concentrations than JIA patients [17]. Regarding JIA, miR-16 tends to have higher values in poly- rather than in oligoarthritis [17]. However, the discrepancy observed

between subtypes was insufficient to differentiate patients basing only on that parameter.

3. miR-125a-5p

Research in rheumatology on miR-125a-5p involved mainly patients with systemic onset of JIA (SoJIA), which is characterized by autoinflammatory rather than autoimmune response. Monocytes isolated from children in the active phase of SoJIA abundantly overexpressed miR-125a-5p when compared to patients in clinical remission or with active polyarthritis [21]. Moreover, Do et al. [22] noted that miR-125a-5p markedly restricted activation of macrophages' anti-inflammatory phenotype involving expression of CD163. Furthermore, Leong et al. [23] observed a positive correlation between miR-125a-5p and indicators of systemic inflammation (particularly ferritin) with no parallel relation to joint involvement. However, due to the distinct pathogenesis of SoJIA, findings from this subtype cannot be directly extrapolated to all JIA patients. Evangelatos et al. [24] reported increased miR-125a-5p levels in early RA patients, regardless of the results of "classic" serological markers (rheumatoid factor and anticitrullinated protein antibodies). It is also worth interjecting here that miR-125b, which shares the seed sequence and target specificity with miR-125a-5p [25], was the first microRNA recognized as a predictor of treatment outcome in RA patients (specifically, response to rituximab) [26]. Therefore, the final position of measuring miR-125a-5p in JIA patients with active joint inflammation merits further investigation.

4. miR-146a

The pathogenetic role of miR-146a in developing inflammatory arthritis is expressed through several pathways of insufficient negative feedback [27]. By targeting inhibin beta A (INHBA), it regulates differentiation of macrophages [28] towards M2 type [29]. It also targets IL-1 receptor-associated kinase 1 and 2 (IRAK1, IRAK2) as well as tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [30], which enables suppression of NFkB signaling [3] and modulation of TNF production [31]. Furthermore, miR-146a promotes the expression of proinflammatory cytokines (including, among others, IL-2 and IL-12) by its upregulation in Th1 [32] and Th17 lymphocytes [24] with concurrent downregulation in Th2 cells [33]. Despite being decreased in early RA when compared to the patients with established diagnosis [34], miR-146a was found to be elevated in oligo-, polyarticular, and SoJIA [17, 28]. Besides, the single-nucleotide polymorphism (SNP) of miR-146a sequence was postulated to be associated with susceptibility to develop enthesitis-related arthritis (JIA-ERA) [35] and ankylosing spondylitis [36]. Concerning disease activity, miR-146a positively correlated with DAS28 [15] and Juvenile Arthritis Disease Activity Score 27-Joint Count (JADAS27) [17]. Moreover, Li et al. [28] found miR-146a levels to be associated with the systemic features. Additionally, the molecule has also been assessed as a good predictor of response to methotrexate therapy (AUC 0.760 in the study conducted by Singh et al. [37]).

5. miR-155

Through targeting the suppressor of cytokine signaling 1 (SOCS1), miR-155 acts as a key promoter of M1 macrophages [38]. At the same time, it directly affects IL-13 receptor alpha 1 (IL-13Rα1) which results in the inhibition of M2 activation [39]. Besides, Kurowska-Stolarska et al. found the significant expression of miR-155 in CD68+ macrophages within the lining layer of the synovial membrane in RA patients [40]. Furthermore, miR-155 enhances the production of IL-2 by peripheral blood mononuclear cells (PBMCs) [10]. Additionally, Vigorito et al. [41] reported the impact of miR-155 on B cell maturation into class-switched plasma cells releasing immunoglobulin G1. Interestingly, transcriptional alterations detected in neutrophils isolated from plasma of children with JIA seem to perpetuate regardless of the disease activity [42]. Demir et al. [16] found that upregulation of miR-155 was even more significant in JIA patients with the improvement in disease activity after 6 months of therapy. However, in adults with ankylosing spondylitis, the elevated miR-155 levels correlated with an increase in disease activity index [43]. miR-155 concentrations tended to be higher in children with polyarthritis, but the difference did not reach statistical significance [16]. Moreover, Ma et al. [17] obtained similar miR-155 values in patients diagnosed with JIA or juvenile spondyloarthropathy which further diminished its specificity. As summarized by Su et al. [44], overexpression of miR-155 in RA patients correlated with the inflammatory markers, DAS28 as well as with TNF and IL-1β levels. Similar to miR-146a, miR-155 appeared to be a promising prognostic marker of methotrexate effectiveness (AUC 0.728 in the same study [37]).

6. miR-223

The upregulation of miR-223 in peripheral naïve CD4+ T lymphocytes [45] plays a pivotal role in the development of local joint inflammation. Given that miR-223 is involved in the activation of M2 macrophages through targeting PBX/Knotted 1 Homeobox 1 (Pknox 1) [46] and in the suppression of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome activity [47], its detected overexpression may be hypothesized as an indicator of malfunctioning inflammatory response. Intriguingly, miR-223 applies not only to the articular manifestations, as the elevated serum levels of the molecule were found also in children with SoJIA [6]. Moreover, miR-223 concentrations were associated with CRP levels [18] and titer of rheumatoid factor (RF) [48] in RA patients. It also appeared to be a reliable predictor of change in disease activity within a 3- and 12-month observation [18]. Furthermore, elevated miR-223 values before treatment initiation were directly correlated with better response to methotrexate and inversely correlated with anti-TNF efficacy [24]. miR-223 seems to be the first epigenetic factor that was speculated as a novel RA therapeutic target by

Li et al. [49], who observed that miR-223 inhibition reduced the disease severity of murine collagen-induced arthritis.

7. Other miRNAs Possibly Relevant for JIA

None of the aforementioned, widely studied miRNA sequences were characterized as being specific to oligoarticular JIA, which is considered as the most common subtype of the disease. Nevertheless, Sun et al. [50] noted that miR-145 is upregulated in oligoarthritis comparing to polyarthritis and SoJIA. Besides, miR-23b appeared to be overexpressed in RA patients with the relevant titer of antinuclear antibodies (ANA) [51]. Given that the variability of gene expression may be correlated with a child's age at disease onset [52], such findings may contribute to the task of defining early onset ANA-positive arthritis which is planned to be discerned in the upcoming reclassification of JIA [53].

Current approach to JIA therapy includes prompt administration of biological agents in patients who did not respond to the first-line treatment, namely, methotrexate [54]. Interestingly, miR-27a may serve as an efficient predictor of whether a patient is likely to improve on anti-TNF therapy. Increased initial miR-27a levels in adults with early RA were associated with better response to a combination therapy of methotrexate with adalimumab, particularly when the concentrations of the molecule decreased within the first 3 months of such treatment [55]. On the other hand, suspending methotrexate in JIA children that achieved clinical remission on medication is another frequent concern of pediatric rheumatologists. Nonetheless, miR-204 turned out to be markedly decreased in patients with inactive disease who continue methotrexate in monotherapy [16]; therefore, it may become a biomarker helpful in therapeutic decisions.

8. Challenges for miRNA in Pediatric Rheumatology

JIA is the most common arthropathy in childhood, though it is not characterized by homogenous natural history of the disease. Patients differ from each other with regard to the disease subtype, number and location of the inflamed joints, time of developing new symptoms and complications, and treatment response. One of the major objectives of research in pediatric rheumatology is to seek for and determine effective markers (mainly serological and genetic) which may facilitate prognosing the disease course and therefore optimize JIA treatment. However, insufficient specificity is a frequent drawback of biomarkers utilized to measure the activity of inflammation. The most studied serological biomarkers in JIA include S100 proteins, particularly S100A8/A9 [56] and S100A12 [57], which are very sensitive in detecting high disease activity. Nevertheless, chronic neutrophil activation may be observed as well in other entities with a distinct origin. For instance, Hu et al. obtained similar S100A8/A9 values in both JIA and cystic fibrosis patients [42]. miRNAs as a class of molecules are not free of such limitations. Despite the potential usefulness of miR-23b in diagnosing rheumatic diseases, this molecule is also related to fibrogenesis within chronic liver injury and diabetic nephropathy [51]. Nevertheless, there are several miRNAs which have already been tested whether they are specific for systemic autoimmune diseases. Jin et al. reported three sequences (namely, miR-124, miR-448, and miR-551b) which had altered values in patients with RA, systemic lupus erythematosus, Sjögren's syndrome, and ulcerative colitis; the authors did not find significant differences of the miRNAs' levels between healthy controls and patients with pneumonia, sepsis, and HBV hepatitis [58].

There are several studies underlining the importance of a distinct route of miRNA expression in fibroblast-like synoviocytes (FLS) [15, 59]. Nziza et al. reported a set of miRNAs extracted from the synovial fluid (miR-6764-5p, miR-155, and miR-146a) that perfectly distinguish (AUC 1.0) children with JIA from patients with *Kingella kingae* septic arthritis [60]. However, such proceedings are more invasive than the examination of blood samples and exclude the possibility of comparison with healthy controls due to ethical issues. Therefore, further investigation of miRNAs in JIA should focus on the sequences that may ease the differentiation of patients basing on plasma and/or serum levels.

9. Conclusions

miRNAs seem to open a new chapter of diagnosing and monitoring activity of childhood arthropathies on the epigenetic level. They might provide the probable answer which patients are more likely to develop a more aggressive course of disease or respond properly to the administered therapy. The authors recommend to continue validation of the already well-known sequences (miR-16, miR-125a-5p, miR-146a, miR-155, and miR-223) and to further investigate the alternative promising molecules (miR-145, miR-23b, miR-27a, and miR-204). Studies including patients that are supposed to be recognized as early onset ANA-positive JIA are of utmost importance.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

KO collected literature and drafted the manuscript. ES supervised the overall study design and contributed to the manuscript preparation. All authors revised and approved the final version of the manuscript.

Acknowledgments

The study is a part of the National Science Centre research task MINIATURA 4 (decision No. 2020/04/X/NZ5/00117) and was supported by the Medical University of Lodz, Poland (grant No. 503/8-000-01/503-81-001-19-00).

Supplementary Materials

Supplementary Table 1: list of microRNAs studied in rheumatology. ACPA: anti-citrullinated protein antibodies; ADA: adalimumab; ANA: antinuclear antibodies; AOSD: adult-onset Still's disease; AS: ankylosing spondylitis; BTRC: beta-transducin repeat containing gene; CD: cluster of differentiation; CF: cystic fibrosis; CIA: collagen-induced arthritis; CRP: C-reactive protein; DAS28: Disease Activity Score 28-Joint Count; DMARD: disease-modifying antirheumatic drug; ERA: enthesitis-related arthritis; ESR: erythrocytes sedimentation rate; FLS: fibroblast-like synoviocytes; foxp3: forkhead box P3; FMF: familial Mediterranean fever; GCS: glucocorticosteroids; IL: interleukin; IFNβ: interferon beta; INHBA: inhibin beta A; IRAK: interleukin-1 receptor-associated kinase; JADAS27: Juvenile Arthritis Disease Activity Score 27-Joint Count; JAK/STAT: Janus kinase/signal transducers and activators of transcription; JDM: juvenile dermatomyositis; JIA: juvenile idiopathic arthritis; JSA: juvenile spondyloarthropathy; miR: microRNA; MMP: matrix metalloproteinase; MRI: magnetic resonance imaging; MTX: methotrexate; NLRP3: NLR family pyrin domain containing protein 3; NFkB: nuclear factor kappa of activated B cells; OA: osteoarthritis; PBMC: peripheral blood mononuclear cell; PLT: platelet count; PsA: psoriatic arthritis; PTGS2: prostaglandin-endoperoxide synthase 2; RA: rheumatoid arthritis; RF: rheumatoid factor; RORyt: retinoic-acid-receptor-related orphan nuclear receptor gamma; RTX: rituximab; SLE: systemic lupus erythematosus; SNP: single-nucleotide polymorphism; SOCS3: suppressor of cytokine signaling 3; SoJIA: systemic onset of juvenile idiopathic arthritis; SS: Sjögren syndrome; SSZ: sulfasalazine; TAK1: transforming growth factor-beta-activated kinase 1; Th: helper T cells; TLR: Toll-like receptor; TNF: tumor necrosis factor; TRAF6: tumor necrosis factor receptor-associated factor 6; Treg: regulatory T cells; UC: ulcerative colitis; WBC: white blood count. *(Supplementary Materials)*

References

[1] F. Cirillo, P. Lazzeroni, C. Sartori, and M. Street, "Inflammatory diseases and growth: effects on the GH-IGF axis and on growth plate," *International Journal of Molecular Sciences*, vol. 18, no. 9, p. 1878, 2017.

[2] J. Karami, S. Aslani, M. N. Tahmasebi et al., "Epigenetics in rheumatoid arthritis; fibroblast-like synoviocytes as an emerging paradigm in the pathogenesis of the disease," *Immunology and Cell Biology*, vol. 98, no. 3, pp. 171–186, 2020.

[3] L. Yan, M. Liang, X. Hou et al., "The role of microRNA-16 in the pathogenesis of autoimmune diseases: a comprehensive review," *Biomedicine & Pharmacotherapy*, vol. 112, article 108583, 2019.

[4] N. Iwamoto and A. Kawakami, "Recent findings regarding the effects of microRNAs on fibroblast-like synovial cells in rheumatoid arthritis," *Immunological Medicine*, vol. 42, no. 4, pp. 156–161, 2019.

[5] M. J. Ormseth, J. F. Solus, Q. Sheng et al., "Development and validation of a microRNA panel to differentiate between patients with rheumatoid arthritis or systemic lupus erythematosus and controls," *The Journal of Rheumatology*, vol. 47, no. 2, pp. 188–196, 2020.

[6] Y. Kamiya, J. Kawada, Y. Kawano et al., "Serum microRNAs as potential biomarkers of juvenile idiopathic arthritis," *Clinical Rheumatology*, vol. 34, no. 10, pp. 1705–1712, 2015.

[7] M. F. Romo-García, Y. Bastian, M. Zapata-Zuñiga et al., "Identification of putative miRNA biomarkers in early rheumatoid arthritis by genome-wide microarray profiling: a pilot study," *Gene*, vol. 720, article 144081, 2019.

[8] S. Mi, J. Zhang, W. Zhang, and R. S. Huang, "Circulating microRNAs as biomarkers for inflammatory diseases," *MicroRNA*, vol. 2, no. 1, pp. 64–72, 2013.

[9] R. A. Bhanji, T. Eystathioy, E. K. Chan, D. B. Bloch, and M. J. Fritzler, "Clinical and serological features of patients with autoantibodies to GW/P bodies," *Clinical Immunology*, vol. 125, no. 3, pp. 247–256, 2007.

[10] Y. A. Lashine, S. Salah, H. R. Aboelenein, and A. I. Abdelaziz, "Correcting the expression of miRNA-155 represses PP2Ac and enhances the release of IL-2 in PBMCs of juvenile SLE patients," *Lupus*, vol. 24, no. 3, pp. 240–247, 2014.

[11] D. Hammaker and G. S. Firestein, "Epigenetics of inflammatory arthritis," *Current Opinion in Rheumatology*, vol. 30, no. 2, pp. 188–196, 2018.

[12] J. Świdrowska-Jaros, K. Orczyk, and E. Smolewska, "Macrophages - silent enemies in juvenile idiopathic arthritis," *Postępy Higieny i Medycyny Doświadczalnej*, vol. 70, pp. 743–750, 2016.

[13] R. Zhou, X. Li, G. Hu, A. Y. Gong, K. M. Drescher, and X. M. Chen, "miR-16 targets transcriptional corepressor SMRT and modulates NF-kappaB-regulated transactivation of interleukin-8 gene," *PLoS One*, vol. 7, no. 1, article e30772, 2012.

[14] R. Zhou, G. Hu, A. Y. Gong, and X. M. Chen, "Binding of NF-kappaB p65 subunit to the promoter elements is involved in LPS-induced transactivation of miRNA genes in human biliary epithelial cells," *Nucleic Acids Research*, vol. 38, no. 10, pp. 3222–3232, 2010.

[15] K. Murata, H. Yoshitomi, S. Tanida et al., "Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis," *Arthritis Research & Therapy*, vol. 12, no. 3, 2010.

[16] F. Demir, A. H. Çebi, and M. Kalyoncu, "Evaluation of plasma microRNA expressions in patients with juvenile idiopathic arthritis," *Clinical Rheumatology*, vol. 37, no. 12, pp. 3255–3262, 2018.

[17] X. Ma, F. Wu, L. Xin et al., "Differential plasma microRNAs expression in juvenile idiopathic arthritis," *Modern Rheumatology*, vol. 26, no. 2, pp. 224–232, 2016.

[18] M. Filková, B. Aradi, L. Šenolt et al., "Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 73, no. 10, pp. 1898–1904, 2014.

[19] K. Schönauen, N. Le, U. von Arnim, C. Schulz, P. Malfertheiner, and A. Link, "Circulating and fecal microRNAs as biomarkers for inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 24, no. 7, pp. 1547–1557, 2018.

[20] M. Wang, L. Wang, X. Zhang et al., "Overexpression of miR-31 in peripheral blood mononuclear cells (PBMC) from patients with ankylosing spondylitis," *Medical Science Monitor*, vol. 23, pp. 5488–5494, 2017.

[21] G. S. Schulert, N. Fall, J. B. Harley et al., "Monocyte microRNA expression in active systemic juvenile idiopathic arthritis implicates microRNA-125a-5p in polarized monocyte

phenotypes," *Arthritis & Rhematology*, vol. 68, no. 9, pp. 2300–2313, 2016.

[22] T. Do, R. Tan, M. Bennett et al., "MicroRNA networks associated with active systemic juvenile idiopathic arthritis regulate CD163 expression and anti-inflammatory functions in macrophages through two distinct mechanisms," *Journal of Leukocyte Biology*, vol. 103, no. 1, pp. 71–85, 2018.

[23] J. Y. Leong, Y. J. Guan, S. Albani, and T. Arkachaisri, "Recent advances in our understanding of the pathogenesis of juvenile idiopathic arthritis and their potential clinical implications," *Expert Review of Clinical Immunology*, vol. 14, no. 11, pp. 933–944, 2018.

[24] G. Evangelatos, G. E. Fragoulis, V. Koulouri, and G. I. Lambrou, "MicroRNAs in rheumatoid arthritis: from pathogenesis to clinical impact," *Autoimmunity Reviews*, vol. 18, no. 11, article 102391, 2019.

[25] Y. M. Sun, K. Y. Lin, and Y. Q. Chen, "Diverse functions of miR-125 family in different cell contexts," *Journal of Hematology & Oncology*, vol. 6, no. 1, p. 6, 2013.

[26] I. Duroux-Richard, Y. M. Pers, S. Fabre et al., "Circulating miRNA-125b is a potential biomarker predicting response to rituximab in rheumatoid arthritis," *Mediators of Inflammation*, vol. 2014, Article ID 342524, 9 pages, 2014.

[27] N. S. Lai, M. Koo, C. L. Yu, and M. C. Lu, "Immunopathogenesis of systemic lupus erythematosus and rheumatoid arthritis: the role of aberrant expression of non-coding RNAs in T cells," *Clinical & Experimental Immunology*, vol. 187, no. 3, pp. 327–336, 2017.

[28] D. Li, M. Duan, Y. Feng, L. Geng, X. Li, and W. Zhang, "MiR-146a modulates macrophage polarization in systemic juvenile idiopathic arthritis by targeting INHBA," *Molecular Immunology*, vol. 77, pp. 205–212, 2016.

[29] C. Huang, X. J. Liu, QunZhou et al., "MiR-146a modulates macrophage polarization by inhibiting Notch1 pathway in RAW264.7 macrophages," *International Immunopharmacology*, vol. 32, pp. 46–54, 2016.

[30] J. Hou, P. Wang, L. Lin et al., "MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2," *Journal of Immunology*, vol. 183, no. 3, pp. 2150–2158, 2009.

[31] K. M. Pauley, M. Satoh, A. L. Chan, M. R. Bubb, W. H. Reeves, and E. K. Chan, "Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients," *Arthritis Research & Therapy*, vol. 10, no. 4, 2008.

[32] Y. Liu, Y. Han, H. Qu, J. Fang, M. Ye, and W. Yin, "Correlation of microRNA expression profile with clinical response to tumor necrosis factor inhibitor in treating rheumatoid arthritis patients: a prospective cohort study," *Journal of Clinical Laboratory Analysis*, vol. 33, no. 7, article e22953, 2019.

[33] S. Monticelli, K. M. Ansel, C. Xiao et al., "MicroRNA profiling of the murine hematopoietic system," *Genome Biology*, vol. 6, no. 8, 2005.

[34] N. Mookherjee and H. S. El-Gabalawy, "High degree of correlation between whole blood and PBMC expression levels of miR-155 and miR-146a in healthy controls and rheumatoid arthritis patients," *Journal of Immunological Methods*, vol. 400-401, pp. 106–110, 2013.

[35] S. Singh, G. Rai, and A. Aggarwal, "Association of microRNA-146a and its target gene IRAK1 polymorphism with enthesitis related arthritis category of juvenile idiopathic arthritis," *Rheumatology International*, vol. 34, no. 10, pp. 1395–1400, 2014.

[36] Y. Xiao, H. Liu, L. Chen, Y. Wang, X. Yao, and X. Jiang, "Association of microRNAs genes polymorphisms with arthritis: a systematic review and meta-analysis," *Bioscience Reports*, vol. 39, no. 7, 2019.

[37] A. Singh, P. S. Patro, and A. Aggarwal, "MicroRNA-132, miR-146a, and miR-155 as potential biomarkers of methotrexate response in patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 38, no. 3, pp. 877–884, 2019.

[38] X. Niu and G. S. Schulert, "Functional regulation of macrophage phenotypes by microRNAs in inflammatory arthritis," *Frontiers in Immunology*, vol. 10, 2019.

[39] R. T. Martinez-Nunez, F. Louafi, and T. Sanchez-Elsner, "The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Rα1)," *The Journal of Biological Chemistry*, vol. 286, no. 3, pp. 1786–1794, 2011.

[40] M. Kurowska-Stolarska, S. Alivernini, L. E. Ballantine et al., "MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis," *Proceedings of the National Academy of Sciences*, vol. 108, no. 27, pp. 11193–11198, 2011.

[41] E. Vigorito, K. L. Perks, C. Abreu-Goodger et al., "microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells," *Immunity*, vol. 27, no. 6, pp. 847–859, 2007.

[42] Z. Hu, K. Jiang, M. B. Frank, Y. Chen, and J. N. Jarvis, "Complexity and specificity of the neutrophil transcriptomes in juvenile idiopathic arthritis," *Scientific Reports*, vol. 6, no. 1, 2016.

[43] B. P. Qian, M. L. Ji, Y. Qiu et al., "Identification of serum miR-146a and miR-155 as novel noninvasive complementary biomarkers for ankylosing spondylitis," *Spine*, vol. 41, no. 9, pp. 735–742, 2016.

[44] L. C. Su, A. F. Huang, H. Jia, Y. Liu, and W. D. Xu, "Role of microRNA-155 in rheumatoid arthritis," *International Journal of Rheumatic Diseases*, vol. 20, no. 11, pp. 1631–1637, 2017.

[45] V. Fulci, G. Scappucci, G. D. Sebastiani et al., "miR-223 is overexpressed in T-lymphocytes of patients affected by rheumatoid arthritis," *Human Immunology*, vol. 71, no. 2, pp. 206–211, 2010.

[46] Q. Chen, H. Wang, Y. Liu et al., "Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3," *PLoS One*, vol. 7, no. 8, article e42971, 2012.

[47] F. Bauernfeind, A. Rieger, F. A. Schildberg, P. A. Knolle, J. L. Schmid-Burgk, and V. Hornung, "NLRP3 inflammasome activity is negatively controlled by miR-223," *Journal of Immunology*, vol. 189, no. 8, pp. 4175–4181, 2012.

[48] M. C. Lu, C. L. Yu, H. C. Chen, H. C. Yu, H. B. Huang, and N. S. Lai, "Increased miR-223 expression in T cells from patients with rheumatoid arthritis leads to decreased insulin-like growth factor-1-mediated interleukin-10 production," *Clinical and Experimental Immunology*, vol. 177, no. 3, pp. 641–651, 2014.

[49] Y. T. Li, S. Y. Chen, C. R. Wang et al., "Brief report: amelioration of collagen-induced arthritis in mice by lentivirus-mediated silencing of microRNA-223," *Arthritis & Rheumatism*, vol. 64, no. 10, pp. 3240–3245, 2012.

[50] J. Sun, M. Feng, F. Wu et al., "Plasma miR-26a as a diagnostic biomarker regulates cytokine expression in systemic juvenile

idiopathic arthritis," *The Journal of Rheumatology*, vol. 43, no. 8, pp. 1607–1614, 2016.

[51] X. Liu, S. Ni, C. Li et al., "Circulating microRNA-23b as a new biomarker for rheumatoid arthritis," *Gene*, vol. 712, p. 143911, 2019.

[52] G. Giancane, A. Alongi, and A. Ravelli, "Update on the pathogenesis and treatment of juvenile idiopathic arthritis," *Current Opinion in Rheumatology*, vol. 29, no. 5, pp. 523–529, 2017.

[53] A. Martini, A. Ravelli, T. Avcin et al., "Toward new classification criteria for juvenile idiopathic arthritis: first steps, pediatric rheumatology international trials organization international consensus," *The Journal of Rheumatology*, vol. 46, no. 2, pp. 190–197, 2019.

[54] S. Ringold, S. T. Angeles-Han, T. Beukelman et al., "2019 American College of Rheumatology/Arthritis Foundation guideline for the treatment of juvenile idiopathic arthritis: therapeutic approaches for non-systemic polyarthritis, sacroiliitis, and enthesitis," *Arthritis & Rheumatology*, vol. 71, no. 6, pp. 846–863, 2019.

[55] J. Sode, S. B. Krintel, A. L. Carlsen et al., "Plasma microRNA profiles in patients with early rheumatoid arthritis responding to adalimumab plus methotrexate vs methotrexate alone: a placebo-controlled clinical trial," *The Journal of Rheumatology*, vol. 45, no. 1, pp. 53–61, 2018.

[56] J. Bojko, "Measurement of blood calprotectin (MRP-8/MRP-14) levels in patients with juvenile idiopathic arthritis," *Reumatologia*, vol. 1, no. 1, pp. 18–22, 2017.

[57] K. Orczyk and E. Smolewska, "A granulocyte-specific protein S100A12 as a potential prognostic factor affecting aggressiveness of therapy in patients with juvenile idiopathic arthritis," *Journal of Immunology Research*, vol. 2018, Article ID 5349837, 7 pages, 2018.

[58] F. Jin, H. Hu, M. Xu et al., "Serum microRNA profiles serve as novel biomarkers for autoimmune diseases," *Frontiers in Immunology*, vol. 9, article 2381, 2018.

[59] Z. Zakeri, A. Salmaninejad, N. Hosseini et al., "MicroRNA and exosome: key players in rheumatoid arthritis," *Journal of Cellular Biochemistry*, vol. 120, pp. 10930–10944, 2019.

[60] N. Nziza, E. Jeziorski, M. Delpont et al., "Synovial-fluid miRNA signature for diagnosis of juvenile idiopathic arthritis," *Cells*, vol. 8, no. 12, article 1521, 2019.

[61] I. Duroux-Richard, C. Jorgensen, and F. Apparailly, "miRNAs and rheumatoid arthritis - promising novel biomarkers," *Swiss Medical Weekly*, vol. 141, article w13175, 2011.

[62] F. Cirillo, P. Lazzeroni, C. Catellani, C. Sartori, S. Amarri, and M. E. Street, "MicroRNAs link chronic inflammation in childhood to growth impairment and insulin-resistance," *Cytokine & Growth Factor Reviews*, vol. 39, pp. 1–18, 2018.

[63] N. Nziza, I. Duroux-Richard, and F. Apparailly, "MicroRNAs in juvenile idiopathic arthritis: can we learn more about pathophysiological mechanisms?," *Autoimmunity Reviews*, vol. 18, no. 8, pp. 796–804, 2019.

[64] N. Mu, J. Gu, T. Huang et al., "A novel NF-κB/YY1/microRNA-10a regulatory circuit in fibroblast-like synoviocytes regulates inflammation in rheumatoid arthritis," *Scientific Reports*, vol. 6, article 20059, 2016.

[65] Z. T. Feng, J. Li, J. Ren, and Z. Lv, "Expression of miR-146a and miR-16 in peripheral blood mononuclear cells of patients with rheumatoid arthritis and their correlation to the disease activity," *Nan Fang YiKe Da XueXueBao*, vol. 31, no. 2, pp. 320–323, 2011.

[66] M. Talari, B. Kapadia, V. Kain et al., "MicroRNA-16 modulates macrophage polarization leading to improved insulin sensitivity in myoblasts," *Biochimie*, vol. 119, pp. 16–26, 2015.

[67] A. Paradowska-Gorycka and B. Stypińska, "The role of cell-free circulating microRNA in diagnostics in patients with rheumatoid arthritis," *Reumatologia*, vol. 3, no. 3, pp. 95-96, 2016.

[68] Y. H. Wu, W. Liu, B. Xue et al., "Upregulated expression of microRNA-16 correlates with Th17/Treg cell imbalance in patients with rheumatoid arthritis," *DNA and Cell Biology*, vol. 35, no. 12, pp. 853–860, 2016.

[69] N. Akhtar, A. K. Singh, and S. Ahmed, "MicroRNA-17 suppresses TNF-α signaling by interfering with TRAF2 and cIAP2 association in rheumatoid arthritis synovial fibroblasts," *Journal of Immunology*, vol. 197, no. 6, pp. 2219–2228, 2016.

[70] H. W. Li, Y. Xie, F. Li, G. C. Sun, Z. Chen, and H. S. Zeng, "Effect of miR-19a and miR-21 on the JAK/STAT signaling pathway in the peripheral blood mononuclear cells of patients with systemic juvenile idiopathic arthritis," *Experimental and Therapeutic Medicine*, vol. 11, no. 6, pp. 2531–2536, 2016.

[71] F. J. Sheedy, E. Palsson-McDermott, E. J. Hennessy et al., "Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21," *Nature Immunology*, vol. 11, no. 2, pp. 141–147, 2010.

[72] Y. Y. Wang, G. Sun, H. Luo et al., "MiR-21 modulates hTERT through a STAT3-dependent manner on glioblastoma cell growth," *CNS Neuroscience & Therapeutics*, vol. 18, no. 9, pp. 722–728, 2012.

[73] L. Ouboussad, L. Hunt, E. M. A. Hensor et al., "Profiling microRNAs in individuals at risk of progression to rheumatoid arthritis," *Arthritis Research & Therapy*, vol. 19, no. 1, p. 288, 2017.

[74] J. Zheng, H. Y. Jiang, J. Li et al., "MicroRNA-23b promotes tolerogenic properties of dendritic cells in vitro through inhibiting Notch1/NF-κB signalling pathways," *Allergy*, vol. 67, no. 3, pp. 362–370, 2012.

[75] K. Murata, M. Furu, H. Yoshitomi et al., "Comprehensive microRNA analysis identifies miR-24 and miR-125a-5p as plasma biomarkers for rheumatoid arthritis," *PLoS One*, vol. 8, no. 7, article e69118, 2013.

[76] F. Niederer, M. Trenkmann, C. Ospelt et al., "Down-regulation of microRNA-34a$*$ in rheumatoid arthritis synovial fibroblasts promotes apoptosis resistance," *Arthritis and Rheumatism*, vol. 64, no. 6, pp. 1771–1779, 2012.

[77] Q. Hu, W. Gong, J. Gu et al., "Plasma microRNA profiles as a potential biomarker in differentiating adult-onset Still's disease from sepsis," *Frontiers in Immunology*, vol. 9, article 3099, 2019.

[78] Q. Wei, F. Lv, H. Zhang et al., "MicroRNA-101-3p inhibits fibroblast-like synoviocyte proliferation and inflammation in rheumatoid arthritis by targeting PTGS2," *Bioscience Reports*, vol. 40, no. 1, article BSR20191136, 2020.

[79] V. Anaparti, I. Smolik, X. Meng, V. Spicer, N. Mookherjee, and H. El-Gabalawy, "Whole blood microRNA expression pattern differentiates patients with rheumatoid arthritis, their seropositive first-degree relatives, and healthy unrelated control subjects," *Arthritis Research & Therapy*, vol. 19, no. 1, p. 249, 2017.

[80] Z. D. Fan, Q. Cao, N. Huang et al., "MicroRNA-125b regulates Th17/Treg cell differentiation and is associated with juvenile idiopathic arthritis," *World Journal of Pediatrics*, vol. 16, no. 1, pp. 99–110, 2020.

[81] E. Kim, J. Cook-Mills, G. Morgan, S. T. Sredni, and L. M. Pachman, "Increased expression of vascular cell adhesion molecule 1 in muscle biopsy samples from juvenile dermatomyositis patients with short duration of untreated disease is regulated by miR-126," *Arthritis & Rheumatism*, vol. 64, no. 11, pp. 3809–3817, 2012.

[82] D. Lagos, G. Pollara, S. Henderson et al., "miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator," *Nature Cell Biology*, vol. 12, no. 5, pp. 513–519, 2010.

[83] S. Bandyopadhyay, T. Lane, R. Venugopal et al., "MicroRNA-133a-1 regulates inflammasome activation through uncoupling protein-2," *Biochemical and Biophysical Research Communications*, vol. 439, no. 3, pp. 407–412, 2013.

[84] C. Liu, A. Pan, X. Chen, J. Tu, X. Xia, and L. Sun, "MiR-5571-3p and miR-135b-5p, derived from analyses of microRNA profile sequencing, correlate with increased disease risk and activity of rheumatoid arthritis," *Clinical Rheumatology*, vol. 38, no. 6, pp. 1753–1765, 2019.

[85] N. S. Lai, H. C. Yu, C. H. Tung, K. Y. Huang, H. B. Huang, and M. C. Lu, "The role of aberrant expression of T cell miRNAs affected by TNF-α in the immunopathogenesis of rheumatoid arthritis," *Arthritis Research & Therapy*, vol. 19, no. 1, p. 261, 2017.

[86] B. J. Andonian, C.-H. Chou, O. R. Ilkayeva et al., "Plasma microRNAs in established rheumatoid arthritis relate to adiposity and altered plasma and skeletal muscle cytokine and metabolic profiles," *Frontiers in Immunology*, vol. 10, article 1475, 2019.

[87] Y. Chen, X. Wang, M. Yang et al., "miR-145-5p increases osteoclast numbers in vitro and aggravates bone erosion in collagen-induced arthritis by targeting osteoprotegerin," *Medical Science Monitor*, vol. 24, pp. 5292–5300, 2018.

[88] K. D. Taganov, M. P. Boldin, K. J. Chang, and D. Baltimore, "NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 33, pp. 12481–12486, 2006.

[89] X. Chen, Y. Ba, L. Ma et al., "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Research*, vol. 18, no. 10, pp. 997–1006, 2008.

[90] T. Niimoto, T. Nakasa, M. Ishikawa et al., "MicroRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients," *BMC Musculoskeletal Disorders*, vol. 11, p. 209, 2010.

[91] Q. Zhou, S. Haupt, J. T. Kreuzer et al., "Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 74, no. 6, pp. 1265–1274, 2015.

[92] S. H. Lin, J. C. Ho, S. C. Li, J. F. Chen, C. C. Hsiao, and C. H. Lee, "MiR-146a-5p expression in peripheral $CD14^+$ monocytes from patients with psoriatic arthritis induces osteoclast activation, bone resorption, and correlates with clinical response," *Journal of Clinical Medicine*, vol. 8, no. 1, p. 110, 2019.

[93] J. Stanczyk, D. M. Leslie Pedrioli, F. Brentano et al., "Altered expression of microRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 58, no. 4, pp. 1001–1009, 2008.

[94] X. M. Chen, Y. Zhao, X. D. Wu et al., "Novel findings from determination of common expressed plasma exosomal microRNAs in patients with psoriatic arthritis, psoriasis vulgaris, rheumatoid arthritis, and gouty arthritis," *Discovery Medicine*, vol. 28, no. 151, pp. 47–68, 2019.

[95] S. Alivernini, B. Tolusso, L. Petricca et al., "Synovial predictors of differentiation to definite arthritis in patients with seronegative undifferentiated peripheral inflammatory arthritis: microRNA signature, histological, and ultrasound features," *Frontiers in Medicine*, vol. 5, p. 186, 2018.

[96] J. C. Fernández-Ruiz, C. Ramos-Remus, J. Sánchez-Corona et al., "Analysis of miRNA expression in patients with rheumatoid arthritis during remission and relapse after a 5-year trial of tofacitinib treatment," *International Immunopharmacology*, vol. 63, pp. 35–42, 2018.

[97] M. J. Ormseth, J. F. Solus, K. C. Vickers, A. M. Oeser, P. Raggi, and C. M. Stein, "Utility of select plasma microRNA for disease and cardiovascular risk assessment in patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 42, no. 10, pp. 1746–1751, 2015.

[98] A. Legesse-Miller, O. Elemento, S. J. Pfau, J. J. Forman, S. Tavazoie, and H. A. Coller, "let-7 overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts," *The Journal of Biological Chemistry*, vol. 284, no. 11, pp. 6605–6609, 2009.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 7206451, 8 pages
https://doi.org/10.1155/2020/7206451

Review Article

Nuclear Alarmin Cytokines in Inflammation

Lili Jiang,[1] Yijia Shao,[1] Yao Tian,[1] Changsheng Ouyang,[2] and Xiaohua Wang[3]

[1]*Department of Rheumatology and Clinical Immunology, Jiangxi Provincial People's Hospital Affiliated to Nanchang University, Nanchang, China*
[2]*Department of Cardiology, Jiangxi Provincial People's Hospital Affiliated to Nanchang University, Nanchang, China*
[3]*Geriatric Medical Center, Jiangxi Provincial People's Hospital Affiliated to Nanchang University, China*

Correspondence should be addressed to Changsheng Ouyang; oycs20011023@163.com and Xiaohua Wang; jxlnyxzx@163.com

Received 24 July 2020; Revised 25 October 2020; Accepted 29 October 2020; Published 4 December 2020

Academic Editor: Xiaoquan Rao

Pathogen-associated molecular patterns (PAMPs) are some nonspecific and highly conserved molecular structures of exogenous specific microbial pathogens, whose products can be recognized by pattern recognition receptor (PRR) on innate immune cells and induce an inflammatory response. Under physiological stress, activated or damaged cells might release some endogenous proteins that can also bind to PRR and cause a harmful aseptic inflammatory response. These endogenous proteins were named damage-associated molecular patterns (DAMPs) or alarmins. Indeed, alarmins can also play a beneficial role in the tissue repair in certain environments. Besides, some alarmin cytokines have been reported to have both nuclear and extracellular effects. This group of proteins includes high-mobility group box-1 protein (HMGB1), interleukin (IL)-33, IL-1α, IL-1F7b, and IL-16. In this article, we review the involvement of nuclear alarmins such as HMGB1, IL-33, and IL-1α under physiological state or stress state and suggest a novel activity of these molecules as central initiators in the development of sterile inflammation.

1. Introduction

The mechanism of the immune system sensing exogenous pathogens and internal tissue damage has attracted increasing attention. There are two main modes to activate the body's immune defense system when confronted with damage caused by various factors: one is ectogenic Pathogen-associated molecular patterns (PAMPs), and the other one is endogenic damage-associated molecular patterns (DAMPs) or alarmins. Pathogen-associated molecular patterns (PAMPs) are some nonspecific and highly conserved molecular structures that are necessary for the survival and pathogenicity of a class or a group of specific microbial pathogens [1]. Pattern recognition receptors (PRRs) are germline-encoded receptors that can recognize PAMP, thus triggers innate and adaptive immunity through activating a series of signaling pathways. One of the most important responses is to induce the synthesis of proinflammatory cytokines and the activation of inflammasomes downstream [2]. DAMPs or alarmins are endogenous proteins or peptides released by leukocytes and epithelial cells when stimulated by danger signals. They strengthen the innate and adaptive immunity by recruiting and activating the antigen-presenting cells (APCs) [3]. These DAMPs include high-mobility group box-1 (HMGB1), defensins, antimicrobial peptides, eosinophilic neurotoxins, heat shock proteins, and some cytokines like IL-1α and IL-33. It was thought that the biological effects of cytokines were only to transmit signals through specific receptors on the cell membrane, but increasing studies suggest that certain cytokines also play a role in the nucleus, such as IL-33, HMGB1, and IL-1α [4–6]. Here, we review the involvement of three representative nuclear alarmins, HMGB1, IL-33, and IL-1α, in the development of inflammation.

2. Members of Nuclear Alarmins Involved in Inflammation

2.1. HMGB1. HMGB1 was named due to its low molecular weight and fast swimming in electrophoresis and was first recognized as an intranuclear protein [7]. It is present in almost all eukaryotic cells and is highly conserved between

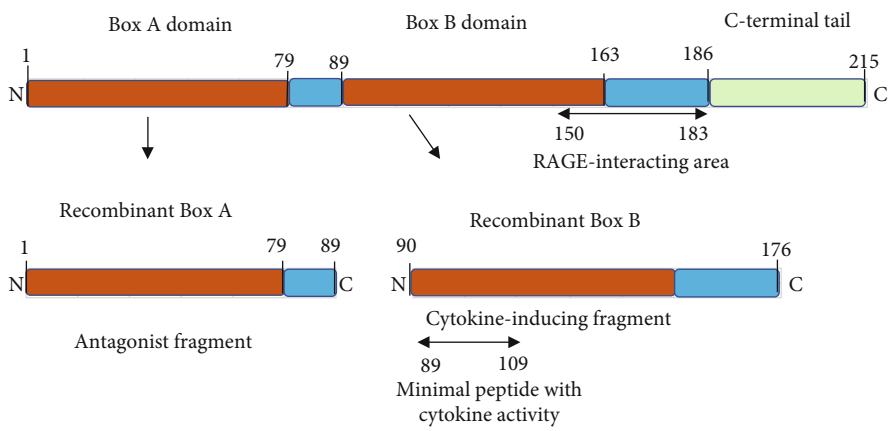


Figure 1: Molecular structure of HMGB1.

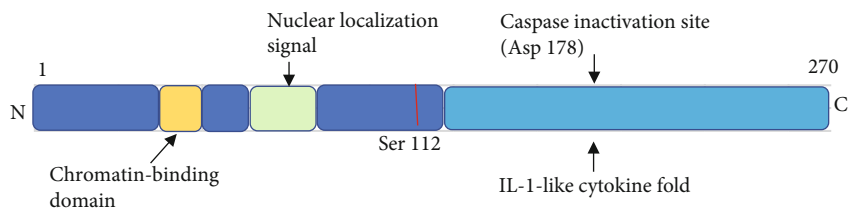


Figure 2: Molecular structure of IL-33.

species [8]). Structurally, it is divided into three regions: Box A, Box B, and C-terminal domain. Both Box A and Box B are capable of binding to DNA; C-terminal is a residual terminal with a negative charge (Figure 1) [9]. Structure and function analysis showed that Box B had the biological activity of HMGB1, while Box A is an antagonist of HMGB1 and Box B, which can block the inflammatory effect of HMGB1 [10]. HMGB1 is a widely expressed nuclear protein and affects transcription regulation. It binds to the DNA grooves and loosens the DNA wrapped in the nucleosome, thus promoting chromatin remodeling [11]. HMGB1 can also bend the DNA significantly and promote the combination of DNA and relevant transcription factors, such as p53, NF-κB, and steroid receptor [12, 13]. HMGB1-deficient mice die soon after birth suggesting the key role of HMGB1 in the nucleus in maintaining life [14]. HMGB1 stays very short at specific DNA binding sites and moves quickly in the nucleus. The stimulation of inflammation can lead to the acetylation of lysine residues in HMGB1 and prevent it from moving into the nucleus [15].

2.2. IL-33. Interleukin-33 (IL-33), also known as NF-HEV (nuclear factor from high endothelial venules), IL-1F11, is a new member of the IL-1 family originally reported by Schmitz et al. in 2005 [16]. It is widely expressed in the whole body, especially in the central nervous system and gastrointestinal [16]. It is composed of 270 amino acids, with an IL-1-like cytokine folding region at the C-terminal and a nuclear localization signal peptide and chromatin binding region at the N-terminal (Figure 2) [17]. IL-33 is synthesized at 30 KD in cellular and then cut into 18 KD by hydrolase as a mature form while secreted to extracellular [18]. Recent studies indicate that human IL-33 is processed at Asp178, not Asp112 as previously claimed [19, 20], and IL-33 is processed into bioactive forms and secreted to extracellular by neutrophil elastase and cathepsin G [21]. Recently, it has been reported that IL-33 is expressed in the nucleus, such as the human endothelial cells [22, 23]. The function of IL-33 in the nucleus is associated with the attachment to heterochromatin [24, 25].

IL-33 is derived from a wide range of tissues, but there are relatively few researches on which cell secreted IL-33 and its role in the disease. It has been reported that vascular endothelial cells (VECs) are the main source of IL-33. IL-33 are released from the nucleus when VECs are stimulated by inflammatory cytokines [26]. IL-33 is also expressed in the epithelial cells of the mucosa and the keratinocytes of skin [27–29], as well as some immune cells such as macrophages [30]. The secretory pathway of IL-33 is still unclear. It has been reported that it may be affected by the proteolytic enzyme, similar to that of IL-1β [31]. Researches also showed that cardiac fibroblasts stimulated by PMA and monocytes stimulated by LPS can secrete mature IL-33 [32, 33]. Recent studies suggested that the precursor IL-33 has biological activity, and its biological activity is reduced after proteolytic enzyme cleavage [19, 20, 30, 34].

2.3. IL-1α. IL-1α is also an important member of the IL-1 family. IL-1α lacks secretory protein as a signal peptide, so it can only be transformed from its precursor molecule. When the cell is stimulated, proteases (calpain, Granzyme B, etc.) cut pro-IL-1α into the 17 kDa mature form of IL-1α, both of which have biological activities [35, 36]. Pro-IL-1α is a 31 KD protein, which can be expressed in most

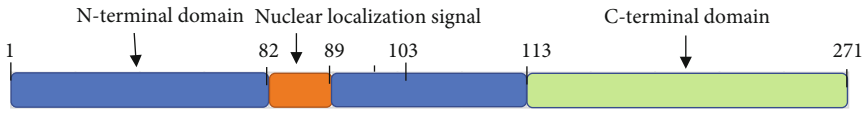


Figure 3: Molecular structure of IL-1α.

Table 1: Alarmins and relative receptors.

Alarmin	Relative receptors
HMGB1	RAGE, TLR2, TLR4
IL-33	ST2L
IL-1α	IL-1R

dormant nonhematopoietic cells of humans, such as the epithelial cells of the gastrointestinal tract, liver, kidney, and skin [37, 38]. It consists of the N-terminal domain (NTD), nuclear localization signal (NLS), and C-terminal domain (CTD) (Figure 3) [39]. NLS induced pro-IL-1α to migrate to the nucleus as an intranuclear transcription factor and participates in gene regulation [40, 41]. Mature IL-1α plays a biological role by binding to IL-1R [42].

3. Alarmin Receptors

Studies have shown that alarmins play a role in chemotaxis and activation of immune cells through G protein-coupled receptors (GPCRs) and non-G protein-coupled receptors (non-G PCR s) (Table 1).

3.1. Receptors for HMGB1. The Receptor for Advanced Glycation End Products (RAGE) is considered to be the receptor of HMGB1 [43]. RAGE is expressed on antigen-presenting cells (APC) [44–46], as well as endothelial cells and smooth muscle cells (SMCs) [47–51]. RAGE deficiency can significantly prolong the survival time of endotoxin mice. However, the deletion of RAGE does not completely prevent HMGB1 from stimulating macrophages to secrete inflammatory factors [52]. Other studies suggested that TLR2 and TLR4 are HMGB1's receptors as well [53]. However, there is no difference in the response of macrophages to HMGB1, whether the macrophages comes from TLR2-deficient mice or wild-type mice [54]. This suggests that TLR2, TLR4, and RAGE can bind to HMGB1, but RAGE may play a more important role for HMGB1.

Recent studies suggested that HMGB1 combined with other immune-stimulators, such as LPS, IL-1β, and DNA, can enhance its biological effect. This suggests that HMGB1 can simultaneously promote the activation of two receptors and produce biological effects. For example, HMGB1/DNA complex is easier to bind to RAGE than HMGB1, because the anchoring of DNA and TLR9 strengthens the combination of HMGB1 and RAGE [55, 56].

3.2. Receptors for IL-33. As the only specific receptor of ILl-33, ST2L is mainly expressed in Th2 lymphocytes [57], mast cells, and NKT, but not in Th1 lymphocytes [58]. IL-1 receptor accessory protein (IL-1RAcP) is essential for IL-33/ST2L to activate downstream signal pathways; IL-1RAcP-deficient mast cells cannot be stimulated to secrete IL-6 by IL-33 [59, 60]. IL-33 activates downstream signal pathways through ERK1/2, p38MAPK, and JNKs [16]; the TRAF6 pathway plays a key role in activating NF-κB and inducing Th2 cytokines by IL-33 [61]. However, the relationship between ST2L and NF-κB activation is controversial. It has been reported that the activation of ST2L has an anti-NF-κB effect, as in cardiomyocytes; IL-33-activated NF-κB inhibits angiotension II-induced NF-κB activation, thus alleviating the cardiac hypertrophy [62–64]. Soluble ST2 (sST2) is the extracellular segment of ST2L, which acts as a decoy receptor and binds to IL-33 competitively, thus blocking the effect of IL-33 [65]. In animal experiments, injection of sST2 or ST2 blocking antibody can alleviate asthma mediated by IL-33 and block the proinflammatory effect of IL-33 on rheumatoid arthritis [66–68].

4. Alarmins in Inflammation

4.1. HMGB1 in Inflammation. HMGB1 shows a strong proinflammatory effect when released into the extracellular environment, mainly through the following two mechanisms. First, necrotic cells release HMGB1 and activate the immune system [69, 70]. Recent studies indicated that apoptotic cells can also release HMGB1, but the reactive oxygen species produced by the activation of intracellular hydrolase can inactivate HGMB1 and block its proinflammatory activity [71]. Second, monocytes or macrophages can secret HMGB1 when activated by LPS, proinflammatory factors, or NO [72]. In endotoxemia, HMGB1 is considered a lethal factor in the late stage of endotoxic shock [73–75]. Increasing inflammatory factors, such as LPS, TNF-α, and IL-1, induce macrophages or DCs secrete HMGB1, which further stimulates macrophages or DCs to secrete inflammatory factors, thus forming a vicious circle [76].

4.2. IL-33 in Inflammation. Recent studies suggested that IL-33 is involved in the occurrence and progress of various diseases, and its mechanism is complex. It can promote the pathophysiological progress of asthma [77, 78], rheumatoid arthritis [79], and systemic lupus erythematosus [80], while in atherosclerosis, allogeneic transplantation, endotoxic shock, and parasitic infection, it inhibits the occurrence and development of diseases [81].

The dual function of IL-33 is mainly due to the different types of immune responses on different cells. IL-33 induce Th2 cells [82], mast cells [83], and basophils to secrete large amounts of IL-4, IL-5, IL-13, IgE, and IgA [83], which induce the pathological changes related to Th2 immune response. In vivo administration of recombinant IL-33 can cause histological changes in the lung and gastrointestinal tract, such as increased mucus secretion, epithelial hyperplasia, and

overgrowth, which were considered to be related to Th2 immune response induced by IL-33 [16]. Previous studies also reported that IL-33 can induce the tolerance of allografts, which may be related to the differentiation of Th2 cells, MDSCs, and Treg cells induced by IL-33 [84–86]. The specific role of IL-33 in the cell nucleus is still not very clear, but studies have suggested that it can regulate gene expression. First, IL-33 would be lost when stimulated by inflammation in the resting vascular endothelial cell (VEC) nucleus [22]; second, when binding to NF-κB, IL-33 can block the related gene transcription induced by it [87]; and third, a short sequence of IL-33 precursor is involved in the formation of histone dimer, which is the components of higher-order chromatin structure [24].

4.3. IL-1α in Inflammation. IL-1α is an important alarmin that mediates aseptic inflammation. Studies have shown that the IL-1α expression can be upregulated in cells in the hypoxic environment, which activates aseptic inflammation. This is mainly due to the fact that hypoxia-inducible factor (HIF) induced by hypoxia can regulate the IL-1α transcription, thus affects the IL-1α-related inflammation by regulating the expression of IL-1α [88]. The expression and nuclear localization of IL-1α depend on the redox reaction. Overexpression of manganese superoxide dismutase leads to a corresponding increase of H2O2; meanwhile, a significant elevation of IL-1α is observed, in both mRNA and protein levels, as well as an increased localization of IL-1α in the nucleus [89].

5. Alarmins and Inflammatory Diseases

5.1. HMGB1 and Inflammatory Diseases. As a natural alarmin, HMGB1 is involved in the inflammatory response of acute local organ injury, as well as Th17-mediated autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), and its animal model-experimental autoimmune encephalomyelitis (EAE). HMGB1 is highly expressed in lesions of MS patients and EAE, and its three receptors RAGE, TLR2, and TLR4 are upregulated in macrophages or microglia. Besides, there is a positive feedback effect between HMGB1 and microglia, which promotes disease progression [90–92]. In the allograft rejection model, the expression of HMGB1 gradually increased over time. Notably, there was an ischemia-reperfusion injury in the process of obtaining the graft from the donor and during the surgery [93–96], which leads to the HMGB1 release from necrotic cells. These HMGB1 may be immediately involved in early and late graft rejection. The overexpression of HMGB1 is observed in colon cancer, breast cancer, and prostate cancer. With RAGE or HMGB1 blocked, tumor growth and metastasis are inhibited in animal models [97–99].

5.2. IL-33 and Inflammatory Diseases. In TNBS-induced enteritis, IL-33 upregulates CD103+IDO+ DCs through intestinal epithelial cells (IECs) and produces inhibitory Tregs to alleviate pathological changes mediated by Th1/Th17 [100]. IL-33 inhibits cardiac hypertrophy caused by AngII through activating NF-κB, and as a decoy receptor of IL-33, the serum expression of sST2 increases in patients with myocardial hypertrophy and heart failure caused by it [62], and the expression was correlated with the grade of heart failure.

5.3. IL-1α and Inflammatory Diseases. IL-1α is an important dual inflammatory factor, mainly involved in a variety of autoimmune diseases, as well as in anti-infection, anti-tumor, and other processes [101]. By inducing the release of TNF-α, G-CSF, and other inflammatory factors and recruiting concentrated granulocytes [102, 103], IL-1α can promote the progress of acute lung injury [104, 105], DSS-induced intestinal inflammation, and psoriasis. In addition, IL-1α can also be used as a prognostic indicator for distant metastasis of head and neck squamous cell carcinoma and promote the growth of melanoma, pancreatic ductal adenocarcinoma, and other tumors [105, 106].

6. Conclusions

DAMP or alarmin is actively released by cells or directly released by necrotic tissues when the tissue is stimulated or damaged, then produce certain biological effects by binding to relative receptors. Alarmins may play different roles in different locations of cells or in the microenvironment of different diseases. Researchers hope to achieve the goal of curing diseases by regulating alarmins and their relative signal pathways. However, before achieving this goal, the mechanism of these cytokines still needs further research. Does DAMP affect each other? How is DAMP released from intracellular to extracellular? Is there any difference in the function between DAMP that is actively released or passively released? Are there any differences between DAMP that is released by apoptotic cells or necrotic cells? All in all, there is still a long way to go to clarify the biological effects and related mechanisms of DAMP.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

LJ, YS, and YT reviewed the literature and wrote the first draft. LJ and XW reviewed the literature and finalized the manuscript. CO, XW, and LJ revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

This work was supported by the Interdisciplinary Innovation Team, Frontier Science Key Research Project of Jiangxi Provincial People's Hospital (19-008), and Jiangxi Provincial Natural Science Foundation (20192ACB21006).

References

[1] T. Kawai and S. Akira, "Pathogen recognition with Toll-like receptors," *Current Opinion in Immunology*, vol. 17, no. 4, pp. 338–344, 2005.

[2] X. Cao, "Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease," *Nature Reviews. Immunology*, vol. 16, no. 1, pp. 35–50, 2016.

[3] Y. Nie, D. Yang, and J. J. Oppenheim, "Alarmins and antitumor immunity," *Clinical Therapeutics*, vol. 38, no. 5, pp. 1042–1053, 2016.

[4] M. Ferhat, A. Robin, S. Giraud et al., "Endogenous IL-33 contributes to kidney ischemia-reperfusion injury as an alarmin," *J Am Soc Nephrol*, vol. 29, no. 4, pp. 1272–1288, 2018.

[5] H. Yang, H. Wang, and U. Andersson, "Targeting inflammation driven by HMGB1," *Frontiers in Immunology*, vol. 11, p. 484, 2020.

[6] A. Malik and T. D. Kanneganti, "Function and regulation of IL-1α in inflammatory diseases and cancer," *Immunological Reviews*, vol. 281, no. 1, pp. 124–137, 2018.

[7] L. Einck and M. Bustin, "The intracellular distribution and function of the high mobility group chromosomal proteins," *Experimental Cell Research*, vol. 156, no. 2, pp. 295–310, 1985.

[8] J. Zlatanova and K. Holde, "Binding to four-way junction DNA: a common property of architectural proteins?," *The FASEB Journal*, vol. 12, no. 6, pp. 421–431, 1998.

[9] M. E. Bianchi, L. Falciola, S. Ferrari, and D. M. Lilley, "The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins," *The EMBO Journal*, vol. 11, no. 3, pp. 1055–1063, 1992.

[10] A. D. Baxevanis and D. Landsman, "The HMG-1 box protein family: classification and functional relationships," *Nucleic Acids Research*, vol. 23, no. 9, pp. 1604–1613, 1995.

[11] C. Ritt, R. Grimm, S. Fernandez, J. C. Alonso, and K. D. Grasser, "Four differently chromatin-associated maize HMG domain proteins modulate DNA structure and act as architectural elements in nucleoprotein complexes," *The Plant Journal*, vol. 14, no. 5, pp. 623–631, 1998.

[12] K. M. Livesey, R. Kang, P. Vernon et al., "p53/HMGB1 complexes regulate autophagy and apoptosis," *Cancer Research*, vol. 72, no. 8, pp. 1996–2005, 2012.

[13] V. S. Melvin, S. C. Roemer, M. E. A. Churchill, and D. P. Edwards, "The C-terminal extension (CTE) of the nuclear hormone receptor DNA binding domain determines interactions and functional response to the HMGB-1/-2 co-regulatory proteins," *The Journal of Biological Chemistry*, vol. 277, no. 28, pp. 25115–25124, 2002.

[14] S. Calogero, F. Grassi, A. Aguzzi et al., "The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice," *Nature Genetics*, vol. 22, no. 3, pp. 276–280, 1999.

[15] R. Assenberg, M. Webb, E. Connolly et al., "A critical role in structure-specific DNA binding for the acetylatable lysine residues in HMGB1," *The Biochemical Journal*, vol. 411, no. 3, pp. 553–561, 2008.

[16] J. Schmitz, A. Owyang, E. Oldham et al., "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines," *Immunity*, vol. 23, no. 5, pp. 479–490, 2005.

[17] D. E. Smith, "IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma," *Clinical and Experimental Allergy*, vol. 40, no. 2, pp. 200–208, 2010.

[18] C. A. Dinarello, "An IL-1 family member requires caspase-1 processing and signals through the ST2 receptor," *Immunity*, vol. 23, no. 5, pp. 461-462, 2005.

[19] A. U. Lüthi, S. P. Cullen, E. A. McNeela et al., "Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases," *Immunity*, vol. 31, no. 1, pp. 84–98, 2009.

[20] D. Talabot-Ayer, C. Lamacchia, C. Gabay, and G. Palmer, "Interleukin-33 is biologically active independently of caspase-1 cleavage," *The Journal of Biological Chemistry*, vol. 284, no. 29, pp. 19420–19426, 2009.

[21] E. Lefrancais, S. Roga, V. Gautier et al., "IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G," in *Proceedings of the National Academy of Sciences*, vol. 109no. 5, pp. 1673–1678, 2012.

[22] A. M. Küchler, J. Pollheimer, J. Balogh et al., "Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation," *The American Journal of Pathology*, vol. 173, no. 4, pp. 1229–1242, 2008.

[23] C. Moussion, N. Ortega, and J. P. Girard, "The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'?," *PLoS One*, vol. 3, no. 10, article e3331, 2008.

[24] L. Roussel, M. Erard, C. Cayrol, and J. P. Girard, "Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket," *EMBO Reports*, vol. 9, no. 10, pp. 1006–1012, 2008.

[25] V. Carriere, L. Roussel, N. Ortega et al., "IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 1, pp. 282–287, 2007.

[26] X. Gao, X. Wang, Q. Yang et al., "Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells," *Journal of Immunology*, vol. 194, no. 1, pp. 438–445, 2014.

[27] W. A. Verri, A. T. G. Guerrero, S. Y. Fukada et al., "IL-33 mediates antigen-induced cutaneous and articular hyperno-ciception in mice," in *Proceedings of the National Academy of Sciences*, vol. 105no. 7, pp. 2723–2728, 2008.

[28] L. Zhang, R. Lu, G. Zhao, S. C. Pflugfelder, and D. Q. Li, "TLR-mediated induction of pro-allergic cytokine IL-33 in ocular mucosal epithelium," *The International Journal of Biochemistry & Cell Biology*, vol. 43, no. 9, pp. 1383–1391, 2011.

[29] F. Cevikbas and M. Steinhoff, "IL-33: a novel danger signal system in atopic dermatitis," *The Journal of Investigative Dermatology*, vol. 132, no. 5, pp. 1326–1329, 2012.

[30] T. Ohno, K. Oboki, N. Kajiwara et al., "Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages," *Journal of Immunology*, vol. 183, no. 12, pp. 7890–7897, 2009.

[31] M. Keller, A. Rüegg, S. Werner, and H. D. Beer, "Active caspase-1 is a regulator of unconventional protein secretion," *Cell*, vol. 132, no. 5, pp. 818–831, 2008.

[32] C. J. Nile, E. Barksby, P. Jitprasertwong, P. M. Preshaw, and J. J. Taylor, "Expression and regulation of interleukin-33 in human monocytes," *Immunology*, vol. 130, no. 2, pp. 172–180, 2010.

[33] J. Zhu and W. Carver, "Effects of interleukin-33 on cardiac fibroblast gene expression and activity," *Cytokine*, vol. 58, no. 3, pp. 368–379, 2012.

[34] C. Cayrol and J. P. Girard, "The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 22, pp. 9021–9026, 2009.

[35] O. Groß, A. S. Yazdi, C. J. Thomas et al., "Inflammasome activators induce interleukin-1α secretion via distinct pathways with differential requirement for the protease function of caspase-1," *Immunity*, vol. 36, no. 3, pp. 388–400, 2012.

[36] C. Garlanda, C. A. Dinarello, and A. Mantovani, "The interleukin-1 family: back to the future," *Immunity*, vol. 39, no. 6, pp. 1003–1018, 2013.

[37] E. T. Osei, C.-A. Brandsma, W. Timens, I. H. Heijink, and T.-L. Hackett, "Current perspectives on the role of interleukin-1 signalling in the pathogenesis of asthma and COPD," *European Respiratory Journal*, vol. 55, no. 2, p. 1900563, 2020.

[38] Y. E. Hernandez-Santana, E. Giannoudaki, G. Leon, M. B. Lucitt, and P. T. Walsh, "Current perspectives on the interleukin-1 family as targets for inflammatory disease," *European Journal of Immunology*, vol. 49, no. 9, pp. 1306–1320, 2019.

[39] J. H. Wessendorf, S. Garfinkel, X. Zhan, S. Brown, and T. Maciag, "Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor," *The Journal of Biological Chemistry*, vol. 268, no. 29, pp. 22100–22104, 1993.

[40] M. Buryskova, M. Pospisek, A. Grothey, T. Simmet, and L. Burysek, "Intracellular interleukin-1alpha functionally interacts with histone acetyltransferase complexes," *The Journal of Biological Chemistry*, vol. 279, no. 6, pp. 4017–4026, 2004.

[41] B. Zamostna, J. Novak, V. Vopalensky, T. Masek, L. Burysek, and M. Pospisek, "N-terminal domain of nuclear IL-1α shows structural similarity to the C-terminal domain of Snf1 and binds to the HAT/core module of the SAGA complex," *PLoS One*, vol. 7, no. 8, article e41801, 2012.

[42] W. P. Arend, G. Palmer, and C. Gabay, "IL-1, IL-18, and IL-33 families of cytokines," *Immunological Reviews*, vol. 223, no. 1, pp. 20–38, 2008.

[43] J. R. van Beijnum, W. A. Buurman, and A. W. Griffioen, "Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1)," *Angiogenesis*, vol. 11, no. 1, pp. 91–99, 2008.

[44] R. Kokkola, A. Andersson, G. Mullins et al., "RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages," *Scandinavian Journal of Immunology*, vol. 61, no. 1, pp. 1–9, 2005.

[45] F. Zhang, X. Su, G. Huang et al., "sRAGE alleviates neutrophilic asthma by blocking HMGB1/RAGE signalling in airway dendritic cells," *Scientific Reports*, vol. 7, no. 1, p. 14268, 2017.

[46] P. M. LeBlanc, T. A. Doggett, J. Choi et al., "An immunogenic peptide in the A-box of HMGB1 protein reverses apoptosis-induced tolerance through RAGE receptor," *The Journal of Biological Chemistry*, vol. 289, no. 11, pp. 7777–7786, 2014.

[47] R. Ramasamy, S. F. Yan, and A. M. Schmidt, "The RAGE axis and endothelial dysfunction: maladaptive roles in the diabetic vasculature and beyond," *Trends in Cardiovascular Medicine*, vol. 15, no. 7, pp. 237–243, 2005.

[48] W. Huang, Y. Liu, L. Li et al., "HMGB1 increases permeability of the endothelial cell monolayer via RAGE and Src family tyrosine kinase pathways," *Inflammation*, vol. 35, no. 1, pp. 350–362, 2012.

[49] M. Yang, X. Yang, S. Wang et al., "HMGB1-induced endothelial cell pyroptosis is involved in systemic inflammatory response syndrome following radiofrequency ablation of hepatic hemangiomas," *American Journal of Translational Research*, vol. 11, no. 12, pp. 7555–7567, 2019.

[50] Z. Wang, J. Zhang, L. Chen, J. Li, H. Zhang, and X. Guo, "Glycine Suppresses AGE/RAGE Signaling Pathway and Subsequent Oxidative Stress by Restoring Glo1 Function in the Aorta of Diabetic Rats and in HUVECs," *Oxidative Medicine and Cellular Longevity*, vol. 2019, Article ID 4628962, 14 pages, 2019.

[51] Q. Lin, C. Fan, J. Gomez-Arroyo et al., "HIMF (hypoxia-induced mitogenic factor) signaling mediates the HMGB1 (high mobility group box 1)-dependent endothelial and smooth muscle cell crosstalk in pulmonary hypertension," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 39, no. 12, pp. 2505–2519, 2019.

[52] B. Liliensiek, M. A. Weigand, A. Bierhaus et al., "Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response," *The Journal of Clinical Investigation*, vol. 113, no. 11, pp. 1641–1650, 2004.

[53] M. Yu, H. Wang, A. Ding et al., "HMGB1 signals through toll-like receptor (TLR) 4 and TLR2," *Shock*, vol. 26, no. 2, pp. 174–179, 2006.

[54] J. S. Park, D. Svetkauskaite, Q. He et al., "Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein," *The Journal of Biological Chemistry*, vol. 279, no. 9, pp. 7370–7377, 2004.

[55] J. Tian, A. M. Avalos, S. Y. Mao et al., "Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE," *Nature Immunology*, vol. 8, no. 5, pp. 487–496, 2007.

[56] A. M. Avalos, K. Kiefer, J. Tian et al., "RAGE-independent autoreactive B cell activation in response to chromatin and HMGB1/DNA immune complexes," *Autoimmunity*, vol. 43, no. 1, pp. 103–110, 2009.

[57] A. Li, R. H. Herbst, D. Canner et al., "IL-33 Signaling Alters Regulatory T Cell Diversity in Support of Tumor Development," *Cell Reports*, vol. 29, no. 10, pp. 2998–3008.e8, 2019.

[58] I. S. Junttila, C. Watson, L. Kummola et al., "Efficient cytokine-induced IL-13 production by mast cells requires both IL-33 and IL-3," *Journal of Allergy and Clinical Immunology*, vol. 132, no. 3, pp. 704–712.e10, 2013.

[59] A. Lingel, T. M. Weiss, M. Niebuhr et al., "Structure of IL-33 and its interaction with the ST2 and IL-1RAcP receptors–insight into heterotrimeric IL-1 signaling complexes," *Structure*, vol. 17, no. 10, pp. 1398–1410, 2009.

[60] A. A. Chackerian, E. R. Oldham, E. E. Murphy, J. Schmitz, S. Pflanz, and R. A. Kastelein, "IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex," *Journal of Immunology*, vol. 179, no. 4, pp. 2551–2555, 2007.

[61] M. Funakoshi-Tago, K. Tago, M. Hayakawa et al., "TRAF6 is a critical signal transducer in IL-33 signaling pathway," *Cellular Signalling*, vol. 20, no. 9, pp. 1679–1686, 2008.

[62] S. Sanada, D. Hakuno, L. J. Higgins, E. R. Schreiter, A. N. J. McKenzie, and R. T. Lee, "IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system," *The Journal of Clinical Investigation*, vol. 117, no. 6, pp. 1538–1549, 2007.

[63] A. Bhardwaj and J. L. Januzzi Jr., "ST2: a novel biomarker for heart failure," *Expert Review of Molecular Diagnostics*, vol. 10, no. 4, pp. 459–464, 2014.

[64] P. Kunes, Z. Holubcova, M. Kolackova, and J. Krejsek, "Interleukin-33, a novel member of the IL-1/IL-18 cytokine family, in cardiology and cardiac surgery," *The Thoracic and Cardiovascular Surgeon*, vol. 58, no. 8, pp. 443–449, 2010.

[65] G. Bandara, M. A. Beaven, A. Olivera, A. M. Gilfillan, and D. D. Metcalfe, "Activated mast cells synthesize and release soluble ST2-a decoy receptor for IL-33," *European Journal of Immunology*, vol. 45, no. 11, pp. 3034–3044, 2015.

[66] H. Hayakawa, M. Hayakawa, A. Kume, and S. I. Tominaga, "Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation," *The Journal of Biological Chemistry*, vol. 282, no. 36, pp. 26369–26380, 2007.

[67] G. Palmer, D. Talabot-Ayer, C. Lamacchia et al., "Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis," *Arthritis and Rheumatism*, vol. 60, no. 3, pp. 738–749, 2009.

[68] T. Kamradt and S. Drube, "A complicated liaison: IL-33 and IL-33R in arthritis pathogenesis," *Arthritis Research & Therapy*, vol. 15, no. 3, p. 115, 2013.

[69] P. Scaffidi, T. Misteli, and M. E. Bianchi, "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation," *Nature*, vol. 418, no. 6894, pp. 191–195, 2002.

[70] R. Dorostkar, T. Bamdad, M. Parsania, and H. Pouriayevali, "An endogenous immune adjuvant released by necrotic cells for enhancement of DNA vaccine potency," *Iranian Journal of Immunology*, vol. 9, no. 4, pp. 215–225, 2012.

[71] H. Kazama, J. E. Ricci, J. M. Herndon, G. Hoppe, D. R. Green, and T. A. Ferguson, "Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein," *Immunity*, vol. 29, no. 1, pp. 21–32, 2008.

[72] D. S. Pisetsky and W. Jiang, "Role of Toll-like receptors in HMGB1 release from macrophages," *Annals of the New York Academy of Sciences*, vol. 1109, no. 1, pp. 58–65, 2007.

[73] H. Wang, O. Bloom, M. Zhang et al., "HMG-1 as a late mediator of endotoxin lethality in mice," *Science*, vol. 285, no. 5425, pp. 248–251, 1999.

[74] M. Deng, Y. Tang, W. Li et al., "The Endotoxin Delivery Protein HMGB1 Mediates Caspase-11-Dependent Lethality in Sepsis," *Immunity*, vol. 49, no. 4, pp. 740–753.e7, 2018.

[75] H. Zhou, Y. Li, H. Gui et al., "Antagonism of Integrin CD11b Affords Protection against Endotoxin Shock and Polymicrobial Sepsis via Attenuation of HMGB1 Nucleocytoplasmic Translocation and Extracellular Release," *The Journal of Immunology*, vol. 200, no. 5, pp. ji1701285–ji1701780, 2018.

[76] M. T. Lotze and K. J. Tracey, "High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal," *Nature Reviews. Immunology*, vol. 5, no. 4, pp. 331–342, 2005.

[77] M. Fux, T. Pecaric-Petkovic, A. Odermatt et al., "IL-33 is a mediator rather than a trigger of the acute allergic response in humans," *Allergy*, vol. 69, no. 2, pp. 216–222, 2014.

[78] Z. Guo, J. Wu, J. Zhao et al., "IL-33 promotes airway remodeling and is a marker of asthma disease severity," *The Journal of Asthma*, vol. 51, no. 8, pp. 863–869, 2014.

[79] S. Chen, B. Chen, Z. Wen, Z. Huang, and L. Ye, "IL-33/ST2-mediated inflammation in macrophages is directly abrogated by IL-10 during rheumatoid arthritis," *Oncotarget*, vol. 8, no. 20, pp. 32407–32418, 2017.

[80] P. Li, W. Lin, and X. Zheng, "IL-33 neutralization suppresses lupus disease in lupus-prone mice," *Inflammation*, vol. 37, no. 3, pp. 824–832, 2014.

[81] R. Kakkar and R. T. Lee, "The IL-33/ST2 pathway: therapeutic target and novel biomarker," *Nature Reviews. Drug Discovery*, vol. 7, no. 10, pp. 827–840, 2008.

[82] N. Murakami-Satsutani, T. Ito, T. Nakanishi et al., "IL-33 promotes the induction and maintenance of Th2 immune responses by enhancing the function of OX40 ligand," *Allergology International*, vol. 63, no. 3, pp. 443–455, 2014.

[83] R. Joulia, F.-E. L'Faqihi, S. Valitutti, and E. Espinosa, "IL-33 fine tunes mast cell degranulation and chemokine production at the single-cell level," *Journal of Allergy and Clinical Immunology*, vol. 140, no. 2, pp. 497–509.e10, 2017.

[84] H. Yin, X. Y. Li, X. B. Jin et al., "IL-33 prolongs murine cardiac allograft survival through induction of TH2-type immune deviation," *Transplantation*, vol. 89, no. 10, pp. 1189–1197, 2010.

[85] H. R. Turnquist, Z. Zhao, B. R. Rosborough et al., "IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival," *Journal of Immunology*, vol. 187, no. 9, pp. 4598–4610, 2011.

[86] S. M. Brunner, G. Schiechl, W. Falk, H. J. Schlitt, E. K. Geissler, and S. Fichtner-Feigl, "Interleukin-33 prolongs allograft survival during chronic cardiac rejection," *Transplant International*, vol. 24, no. 10, pp. 1027–1039, 2011.

[87] S. Ali, A. Mohs, M. Thomas et al., "The dual function cytokine IL-33 interacts with the transcription factor NF-κB to dampen NF-κB-stimulated gene transcription," *Journal of Immunology*, vol. 187, no. 4, pp. 1609–1616, 2011.

[88] P. Rider, I. Kaplanov, M. Romzova et al., "The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells," *Frontiers in Immunology*, vol. 3, 2012.

[89] D. A. McCarthy, A. Ranganathan, S. Subbaram et al., "Redox-control of the alarmin, interleukin-1α," *Redox Biology*, vol. 1, no. 1, pp. 218–225, 2013.

[90] Y. Sun, H. Chen, J. Dai et al., "Glycyrrhizin protects mice against experimental autoimmune encephalomyelitis by inhibiting high-mobility group box 1 (HMGB1) expression and neuronal HMGB1 release," *Frontiers in Immunology*, vol. 9, p. 1518, 2018.

[91] Y. Sun, H. Chen, J. Dai et al., "HMGB1 expression patterns during the progression of experimental autoimmune encephalomyelitis," *Journal of Neuroimmunology*, vol. 280, pp. 29–35, 2015.

[92] A. P. Robinson, M. W. Caldis, C. T. Harp, G. E. Goings, and S. D. Miller, "High-mobility group box 1 protein (HMGB1) neutralization ameliorates experimental autoimmune encephalomyelitis," *Journal of Autoimmunity*, vol. 43, pp. 32–43, 2013.

[93] W. Wang, L. Wu, J. Li et al., "Alleviation of hepatic ischemia reperfusion injury by oleanolic acid pretreating via reducing

HMGB1 release and inhibiting apoptosis and autophagy," *Mediators of Inflammation*, vol. 2019, Article ID 3240713, 10 pages, 2019.

[94] H. Chung, S. J. Hong, S. W. Choi et al., "High mobility group box 1 secretion blockade results in the reduction of early pancreatic islet graft loss," *Biochemical and Biophysical Research Communications*, vol. 514, no. 4, pp. 1081–1086, 2019.

[95] F. Braza, S. Brouard, S. Chadban, and D. R. Goldstein, "Role of TLRs and DAMPs in allograft inflammation and transplant outcomes," *Nature Reviews. Nephrology*, vol. 12, no. 5, pp. 281–290, 2016.

[96] M. E. Cornide-Petronio, E. Negrete-Sánchez, M. Mendes-Braz et al., "The effect of high-mobility group box 1 in rat steatotic and nonsteatotic liver transplantation from donors after brain death," *American Journal of Transplantation*, vol. 16, no. 4, pp. 1148–1159, 2016.

[97] M. Wang, A. Gauthier, L. A. Daley et al., "The role of HMGB1, a nuclear damage-associated molecular pattern molecule, in the pathogenesis of lung diseases," *Antioxidants & Redox Signaling*, vol. 31, no. 13, pp. 954–993, 2019.

[98] K. Wang, S. Shan, S. Wang, X. Gu, X. Zhou, and T. Ren, "HMGB1-containing nucleosome mediates chemotherapy-induced metastasis of human lung cancer," *Biochemical and Biophysical Research Communications*, vol. 500, no. 3, pp. 758–764, 2018.

[99] G. Gdynia, S. W. Sauer, J. Kopitz et al., "The HMGB1 protein induces a metabolic type of tumour cell death by blocking aerobic respiration," *Nature Communications*, vol. 7, no. 1, 2016.

[100] Z. Wang, L. Shi, S. Hua, C. Qi, and M. Fang, "IL-33 ameliorates experimental colitis involving regulation of autophagy of macrophages in mice," *Cell & Bioscience*, vol. 9, no. 1, 2019.

[101] N. C. Di Paolo and D. M. Shayakhmetov, "Interleukin 1α and the inflammatory process," *Nature Immunology*, vol. 17, no. 8, pp. 906–913, 2016.

[102] P. Menghini, D. Corridoni, L. F. Buttó et al., "Neutralization of IL-1α ameliorates Crohn's disease-like ileitis by functional alterations of the gut microbiome," in *Proceedings of the National Academy of Sciences of the United States of America*, vol. 116no. 52, pp. 26717–26726, 2019.

[103] P. Martin, G. Palmer, E. Rodriguez et al., "Intracellular IL-1 receptor antagonist isoform 1 released from keratinocytes upon cell death acts as an inhibitor for the alarmin IL-1α," *Journal of Immunology*, vol. 204, no. 4, pp. 967–979, 2020.

[104] C. Michaudel, I. Maillet, L. Fauconnier et al., "Interleukin-1α mediates ozone-induced myeloid differentiation factor-88-dependent epithelial tissue injury and inflammation," *Frontiers in Immunology*, vol. 9, p. 916, 2018.

[105] V. Rabolli, A. A. Badissi, R. Devosse et al., "The alarmin IL-1α is a master cytokine in acute lung inflammation induced by silica micro- and nanoparticles," *Particle and Fibre Toxicology*, vol. 11, no. 1, p. 69, 2014.

[106] W. Zhang, N. Borcherding, and R. Kolb, "IL-1 signaling in tumor microenvironment," *Advances in Experimental Medicine and Biology*, vol. 1240, pp. 1–23, 2020.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 4297354, 8 pages
https://doi.org/10.1155/2020/4297354

Research Article

Blocking Interleukin-33 Alleviates the Joint Inflammation and Inhibits the Development of Collagen-Induced Arthritis in Mice

Yan Li,[1] Yeqin Fu,[1] Huan Chen,[1] Xiaojin Liu,[2] and Mingcai Li[1]

[1]*Department of Immunology, Ningbo University School of Medicine, Ningbo, China*
[2]*Department of Clinical Laboratory, The Fifth Hospital of Shijiazhuang, Shijiazhuang, China*

Correspondence should be addressed to Mingcai Li; mingcaili@126.com

Received 3 July 2020; Revised 8 October 2020; Accepted 19 October 2020; Published 5 November 2020

Academic Editor: Yan Yang

Rheumatoid arthritis (RA) is considered a systemic chronic inflammatory joint disease characterized by chronic synovitis and cartilage and bone destruction. Interleukin-33 (IL-33) is a proinflammatory cytokine which is highly expressed in the synovium of RA patients and the joints of mice with collagen-induced arthritis (CIA) and exacerbates CIA in mice. However, the role of the IL-33-neutralizing antibody in the murine model of CIA remains unclear. In the present study, CIA mice were given intraperitoneally with polyclonal rabbit anti-murine IL-33 antibody (anti-IL-33) or normal rabbit IgG control after the first signs of arthritis. Administration of anti-IL-33 after the onset of disease significantly reduced the severity of CIA and joint damage compared with controls treated with normal rabbit IgG. Anti-IL-33 treatment also significantly decreased the serum levels of interferon-γ(IFN-γ),IL-6, IL-12, IL-33, and tumor necrosis factor-α (TNF-α). Moreover, anti-IL-33 treatment significantly downregulated the production of IFN-γ, IL-6, IL-12, IL-33, and TNF-α in ex vivo-stimulated spleen cells. Together, our results indicate that the IL-33-neutralizing antibody may provide a therapeutic strategy for RA by inhibiting the release of proinflammatory cytokines.

1. Introduction

Interleukin-33 (IL-33) is a new member of the IL-1 family, which plays a biological role by binding the orphan receptor suppression of tumorigenicity 2 (ST2) (also called ST2L, IL-33Rα, DER4, Fit-1, T1, IL-1RL1, or IL-1R4) [1]. Upon binding to IL-33, the ST2 receptor together with the IL-1 receptor accessory protein (IL-1RAcP) forms a functional signaling heterodimeric complex [1]. There are two forms of ST2, the soluble ST2 (sST2) and the membrane-bound ST2 (ST2L). The sST2 functions as a decoy receptor that can block IL-33/ST2 interaction [2]. ST2 is specifically expressed on mast cells and T helper 2 (Th2) cells and has been proved to be an important effector molecule of Th2 responses in murine collagen-induced arthritis (CIA) [3, 4]. IL-33 can activate nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) through binding with ST2 receptor and induce Th2 cytokine expression, leading to serious pathological changes in mucosal tissues [1]. IL-33 has many effects on inflammatory cells. It can induce mast cells and Th2 cells to produce proinflammatory and Th2 cytokines [1, 5–11], induce Th2 cell chemotaxis [12] and neutrophil migration [13], promote eosinophil and basophil adhesion, and enhance eosinophil survival and basophil migration [14–16]. IL-33 is involved in the pathogenesis of rheumatoid arthritis (RA) [17].

IL-33 levels are increased in sera and synovial fluid of patients with RA [18–20]. Increased IL-33 in sera and synovial fluid is associated with disease activity [21, 22] and bone erosion [23, 24] in RA. Elevated levels of sST2 are associated with RA disease activity and ameliorated inflammation in synovial fibroblasts [25]. In animal studies, IL-33 exacerbates antigen-induced arthritis (AIA) and autoantibody-induced arthritis by activating mast cells [26, 27]. In addition, Palmer et al. [4] demonstrated blockade of IL-33 signaling using an anti-ST2 antibody attenuates the severity of experimental arthritis. However, several studies showed that the development of AIA and CIA, as assessed by clinical or histological evaluation, is not impaired in IL-33-deficient mice [28], and administration of IL-33 or an IL-33 receptor (ST2L) antibody inhibited cartilage destruction, systemic bone loss, and

osteoclast differentiation [29]. These studies indicate that the effect of IL-33 on experimental arthritis seems to be contradictory. The role of IL-33 in the pathogenesis of RA needs further study.

We have previously reported that polyclonal rabbit anti-murine IL-33 antibody (anti-IL-33) reduced cigarette smoke-induced lung inflammation [30] and allergic airway inflammation [31] in mice. In the present study, we provide novel evidence that anti-IL-33 suppresses the expression of interferon-γ (IFN-γ), IL-6, IL-12, IL-33, and tumor necrosis factor-α (TNF-α) and the subsequent joint destruction in collagen-induced arthritic mice. Our data suggest that the treatment of arthritis with the IL-33-neutralizing antibody is a promising new method, which may help to prevent joint damage.

2. Materials and Methods

2.1. Animals. Male DBA/1 mice (8–10 weeks old and weighting 20–24 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The mice were housed in pathogen-free conditions at the Animal Center of Ningbo University. Water and food were provided ad libitum. All experiments were approved by the Animal Ethics Committee of Ningbo University. The anti-IL-33 antibody and normal rabbit IgG were prepared as previously described [31, 32].

2.2. Induction of CIA and Administration of Anti-IL-33. CIA was elicited in DBA/1 mice as previously described with a slight modification [4]. Briefly, bovine type II collagen (CII; Chondrex) was diluted in 0.05 M acetic acid to a concentration of 2 mg/ml. It was emulsified in an equal amount of complete Freund's adjuvant (CFA) (5 mg/ml *Mycobacterium tuberculosis*, strain H37Ra; Difco, Detroit, MI). Mice were immunized intradermally (i.d.) at the base of the tail with 100 μg of bovine CII. On day 21, mice were intraperitoneally (i.p.) injected with 100 μg of CII dissolved in phosphate-buffered saline (PBS), and arthritis usually occurred a few days after the booster injection (Figure 1).

To study the effect of anti-IL-33 in murine CIA, DBA/1 mice ($n = 10$) were injected i.p. with anti-IL-33 from days 25 to 27 daily at a dose of 150 μg/mouse and then once every 3 days until day 42. Control mice ($n = 10$) were injected with the same amount of normal rabbit IgG (Figure 1). The dose of 150 μg anti-IL-33/mouse was based on previous studies by us [31] and Palmer et al. [4]. Mice were sacrificed by cervical dislocation at day 43 after immunization, and serum samples were obtained by aortic puncture. The joints and spleens were collected for histological examination and in vitro studies.

2.3. Assessment of Arthritis. Mice were monitored for signs of arthritis as previously described [3]. From day 20 after the first immunization onward, mice were examined daily for the onset of clinical arthritis. Mice were thought to have arthritis when significant redness and/or swelling were found in the digits or in other parts of the paws. Scores were assigned based on erythema, swelling or loss of function present in each paw on a scale of 0-3, giving a maximum score of 12 per mouse [3]. Paw thickness was measured with a dial caliper.

2.4. Collagen-Specific In Vitro Culture. Spleens were removed on day 43 after primary immunization. Single-cell suspensions were prepared and cultured at 2×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum at 37°C in 5% CO_2. Cells were cultured with 100 μg/ml of CII in 96-well plates. After 72 or 96 h, the supernatant was collected and stored at −80°C until determined for cytokine concentration.

2.5. Histologic Analysis. For histological assessment, mice were sacrificed and whole knee and/or ankle joints were removed and fixed with 10% neutral-buffered formalin. The specimens were decalcified with 5% formic acid and embedded in paraffin [33]. Tissue sections (5 μm) were stained with hematoxylin and eosin (HE) or toluidine blue (Sigma-Aldrich). Arthritis was quantified by a "treatment-blind" observer, and each joint was scored according to the degree of inflammation, synovial hyperplasia, and corrosion as described previously [3, 34].

2.6. Determination of Cytokines and Anticollagen Antibody Levels. All cytokines and anticollagen antibody levels were detected by ELISA. IFN-γ, IL-6, IL-10, IL-12, IL-33, sST2, and TNF-α in serum or culture supernatants were determined with ELISA kits (Biosource, Camarillo, CA) according to the manufacturer's instructions. The titers of serum anti-collagen II antibody were determined according to the method previously reported [3].

2.7. Statistical Analysis. Data were expressed as mean ± SEM. Statistical significance was assessed by the two-tailed unpaired Student t-test or two-way ANOVA with Bonferroni's multiple comparison test, as indicated in the figure legends. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Anti-IL-33 Treatment Inhibited Disease Progress of Murine CIA. On day 24 after immunization, mice began to show clinical symptoms of arthritis. From days 25 to 27, mice were injected i.p. daily with anti-IL-33 or normal rabbit IgG, and then once every 3 days until day 42, for a total of 8 doses (Figure 1). The results showed that mice injected with control rabbit IgG reached the peak of arthritis on day 29. In contrast, mice treated with anti-IL-33 significantly attenuated disease in the arthritic index (Figure 2(a)) and number of arthritic paw (Figure 2(b)).

3.2. Anti-IL-33 Treatment Reduced Synovitis and Bone Erosion. To examine whether anti-IL-33 administration reduced joint damage, histological evaluation of the cartilage and bone was performed. In the control group, there were obvious inflammatory cell infiltration, synovial hyperplasia, and adjacent cartilage and bone erosion (Figure 3(a)). However, anti-IL-33 treatment can significantly inhibit these pathological changes. The histological scores were shown in

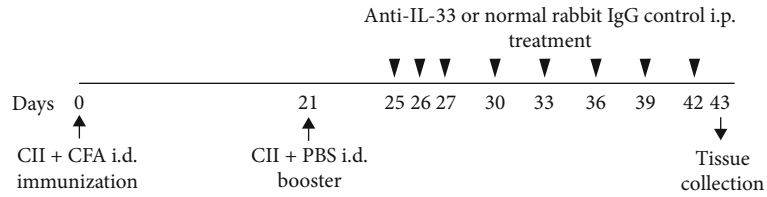


Figure 1: Induction of experimental arthritis and anti-IL-33 treatment. DBA/1 mice were immunized by an intradermal (i.d.) injection of bovine type II collagen (CII) emulsified in complete Freund's adjuvant (CFA) on day 0 and an intraperitoneal (i.p.) booster injection of CII dissolved in phosphate-buffered saline (PBS) on day 21. To evaluate the effect of anti-IL-33 on experimental arthritis, mice ($n = 10$) were injected i.p. with anti-mouse IL-33 (150 μg/mouse) on days 25, 26, 27, 30, 33, 36, 39, and 42. The control group was injected with the same dose of control IgG ($n = 10$). On day 43, mice were killed, blood was collected, and joints were used for histological analysis.

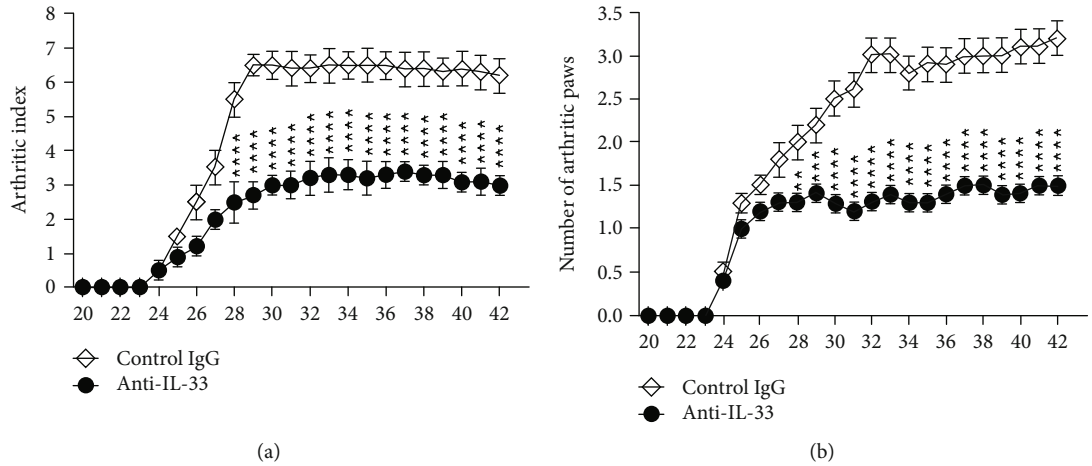


Figure 2: Anti-IL-33 inhibited the development of CIA in mice. From days 25 to 27, CIA mice were injected i.p. with anti-IL-33 or normal rabbit IgG (both 150 μg/dose, $n = 10$) and then injected once every 3 days until day 42 for a total of 8 doses. According to arthritic index (a) and number of arthritic paws (b), the disease progression of mice was analyzed. Compared with the control IgG, anti-IL-33 treatment can significantly reduce the symptoms of arthritis in mice. Data are mean ± SEM ($n = 10$); $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. the control IgG group by two-way ANOVA with Bonferroni's multiple comparison test.

Figure 3(b). These data clearly indicate that anti-IL-33 can suppress the development of CIA and articular destruction.

3.3. Anti-IL-33 Treatment Suppressed Serum Levels of Proinflammatory Cytokines. To investigate the mechanism of action during IL-33 neutralization, serum levels of inflammatory cytokines were measured by ELISA. Compared with the control group, anti-IL-33 treatment significantly reduced serum IFN-γ, IL-6, IL-12, IL-33, and TNF-α levels, but sST2 levels did not change significantly (Figure 4). In addition, serum CII-specific IgG1 and IgG2a levels were similar in the two groups (data not shown).

3.4. Anti-IL-33 Treatment Inhibited CII-Specific Proinflammatory Immune Response In Vitro. We next examined the CII-specific inflammatory cytokine concentration in spleen cells. Compared with the control groups, the production of IFN-γ, IL-6, IL-12, IL-33, and TNF-α in spleen cells obtained at day 43 from anti-IL-33-treated mice was significantly reduced, while sST2 was not significantly changed (Figure 5). Additionally, the concentration of IL-10 in splenocytes of the two groups was similar (data not shown).

4. Discussion

In this study, we demonstrated that anti-IL-33 can reduce the severity of arthritis and the signs of joint injury in CIA mice after the onset of arthritis. Moreover, anti-IL-33 treatment was related to a significant suppression in proinflammatory cytokine (IFN-γ, IL-6, IL-12, IL-33, and TNF-α) production. These findings suggest that the IL-33-neutralizing antibody has a therapeutic effect on the onset of arthritis by inhibiting the activation of inflammatory cells and the production of proinflammatory cytokines.

IL-33 is considered to be an inflammatory cytokine in human RA and some experimental settings including mouse CIA. IL-33 was highly expressed in human RA synovium [35]. The levels of IL-33 were increased in fibroblast-like synoviocytes of RA patients [23]. The level of serum IL-33 was significantly correlated with the number of tender joints, C-reactive protein, disease activity score 28 joints, and leukocyte count and negatively correlated with the level of red blood cell and hemoglobin. At 3 and 6 months after treatment with etanercept (a human TNF antagonist), the average serum IL-33 level in RA patients had decreased significantly [36]. These findings suggest that IL-33 is involved in RA

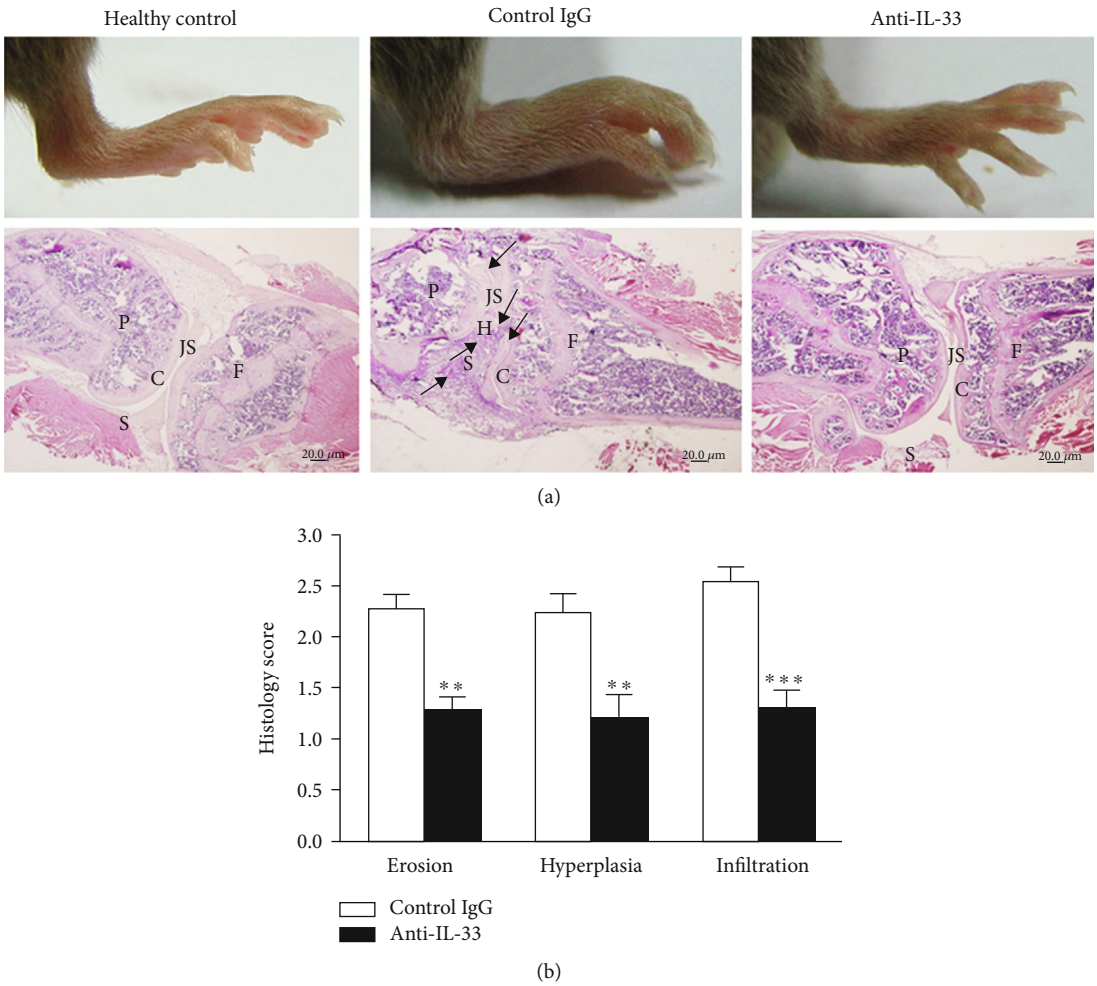


Figure 3: Anti-IL-33 treatment of mice with CIA resulted in reduced joint pathology. (a) On day 43, mice were killed and knee joints were collected and stained with HE. The inflammatory infiltrate, cartilage destruction, and bone erosion were observed in the control IgG-treated mice (arrows). P: patella; S: synovium; H: hyperplasia; JS: joint space; C: cartilage; F: femur. Magnification: ×40. (b) Anti-IL-33 antibody treatment alleviated the joint damage. The histological features of synovial bone erosion, hyperplasia, and cell infiltration were evaluated. Data are mean ± SEM ($n = 8$); $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control IgG group by two-way ANOVA with Bonferroni's multiple comparison test.

pathogenesis. In animal experiments, IL-33 mRNA was detected in the joints of CIA mice and increased in the early stage of the disease [4]. Moreover, IL-33 enhances autoantibody-mediated arthritis by promoting mast cell degranulation and proinflammatory cytokine production [27]. In contrast, the administration of antibodies that block ST2 signaling attenuated the severity of CIA [4]. Similarly, sST2, the decoy receptor for IL-33, significantly reduced the serious cellular infiltration, synovial hyperplasia, and joint erosion in the joints of mice [3]. The mechanism proposed to explain this effect was a direct inhibition of macrophage activation by sST2 via a putative sST2 receptor expressed at the macrophage surface [3]. However, other studies have shown that IL-33 appears to have an opposite effect on experimental arthritis. Palmer of University of Geneva group [28, 37] found that arthritis development is not impaired in absence of endogenous IL-33 in AIA, CIA, and K/BxN serum transfer-induced model of arthritis. Furthermore, Biton et al. [38] recently reported that IL-33 can inhibit the development of experimental arthritis by promoting the expansion of activated Foxp3^{+} regulatory T cells and establishing a type 2 immune response. They found that repeated injections of IL-33 during induction (early) and during development (late) of CIA strongly suppressed clinical and histological signs of arthritis. In contrast, a late IL-33 injection had no effect. These contradictory findings have been associated with environmental factors such as the different microbial colonization of experimental animals in different animal facilities [2]. In addition, a detailed understanding of interactions between ST2 with other signaling pathways seems to be mandatory for rational therapeutic manipulation of this system. These findings indicate that targeting IL-33 or its receptor might lead to radically different outcomes in patients (with arthritis). To the best of our knowledge, there is no report on the treatment of CIA or RA with the IL-33-neutralizing antibody. In the present study, we found that anti-IL-33 treatment can reduce the production of proinflammatory cytokines, thereby inhibiting the disease progress of murine CIA. Anti-IL-33

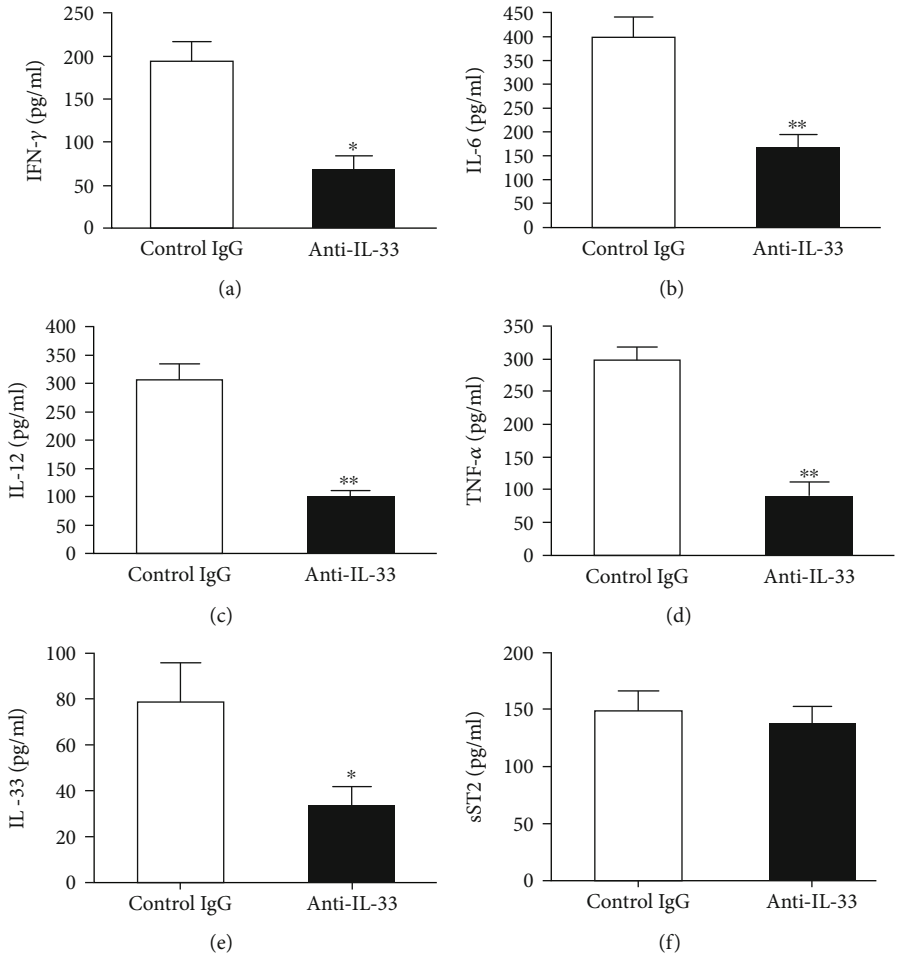


Figure 4: Effect of anti-IL-33 on serum proinflammatory cytokine production. Mice treated with anti-IL-33 or control IgG were killed on day 43, and the serum was collected from eight mice in each group. Levels of IFN-γ (a), IL-6 (b), IL-12 (c), TNF-α (d), IL-33 (e), and sST2 (f) were determined by ELISA. Data are mean ± SEM ($n = 8$); $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control IgG group by two-tailed unpaired Student's t-test.

treatment significantly attenuated disease in the arthritic index and number of arthritic paw in CIA mice. Moreover, anti-IL-33 treatment reduced joint damage, including histological evaluation of the cartilage and bone integrity, infiltration of granulocytes and monocytes into the joint cavity, synovial hyperplasia, and cartilage and bone erosion.

In recent years, there is evidence that IL-33 is involved in the pathogenesis of RA by increasing the production of inflammatory molecules such as proinflammatory cytokines. A number of inflammatory mediators are involved in the inflammatory processes of RA. Some proinflammatory cytokines, including IL-1, IL-6, IL-8, IL-15, and TNF-α, are considered to be key molecules in the formation of RA inflammation.

Hong et al. [35] found that there is a significant positive correlation between IL-33 and IL-6 levels in the RA sera. Palmer et al. [4] reported that an anti-ST2 blocking antibody inhibits IL-33-induced IL-6 secretion. Xu et al. [26] showed that IL-33 increases the levels of IL-6 in vitro by bone marrow-derived mast cells from wild-type DBA/1 mice. Moreover, increased IL-33 leads to the upregulation of inflammatory cytokines such as IL-6 in patients with RA. In CIA mice, IL-33 induced the expression of proinflammatory cytokines such as IL-1β, IL-6, IL-13, granulocyte-macrophage colony-stimulating factor, and chemokines by bone marrow-derived mast cells from DBA/1 mice [26]. TNF-α is widely considered an important target of antirheumatic therapy. Blocking the TNF-α activity has been widely used to improve the progress of RA [39]. TNF-α significantly induced IL-33 mRNA expression and protein synthesis, and overexpression of IL-33 significantly increased TNF-α-induced IL-6, IL-8, and matrix metalloprotease (MMP)-3 in rheumatoid arthritis synovial fibroblast. On the contrary, IL-33 gene silencing significantly reduced the expression of IL-33, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) in TNF-α-induced RA synovial fibroblast [39]. Furthermore, sST2 reduced the production of IFN-γ, IL-17, TNF-α, and IL-10 in draining lymph nodes and decreased the levels of TNF-α and MMP-9 in the ankle joints of CIA mice [4]. IL-33 overexpression significantly increased TNF-α-induced expression of IL-6, IL-8, MCP-1, and MMP-3, while IL-33 gene silencing significantly decreased TNF-α-induced production of IL-6, IL-8, and MCP-1 [40]. The sST2 treatment also downregulated serum levels of IL-6, IL-12, and TNF-α. Spleen cells from the sST2-treated mice produced significantly less IFN-γ, TNF-α, IL-6, and IL-12 compared with cells from the control mice when cultured with collagen in vitro [3]. In

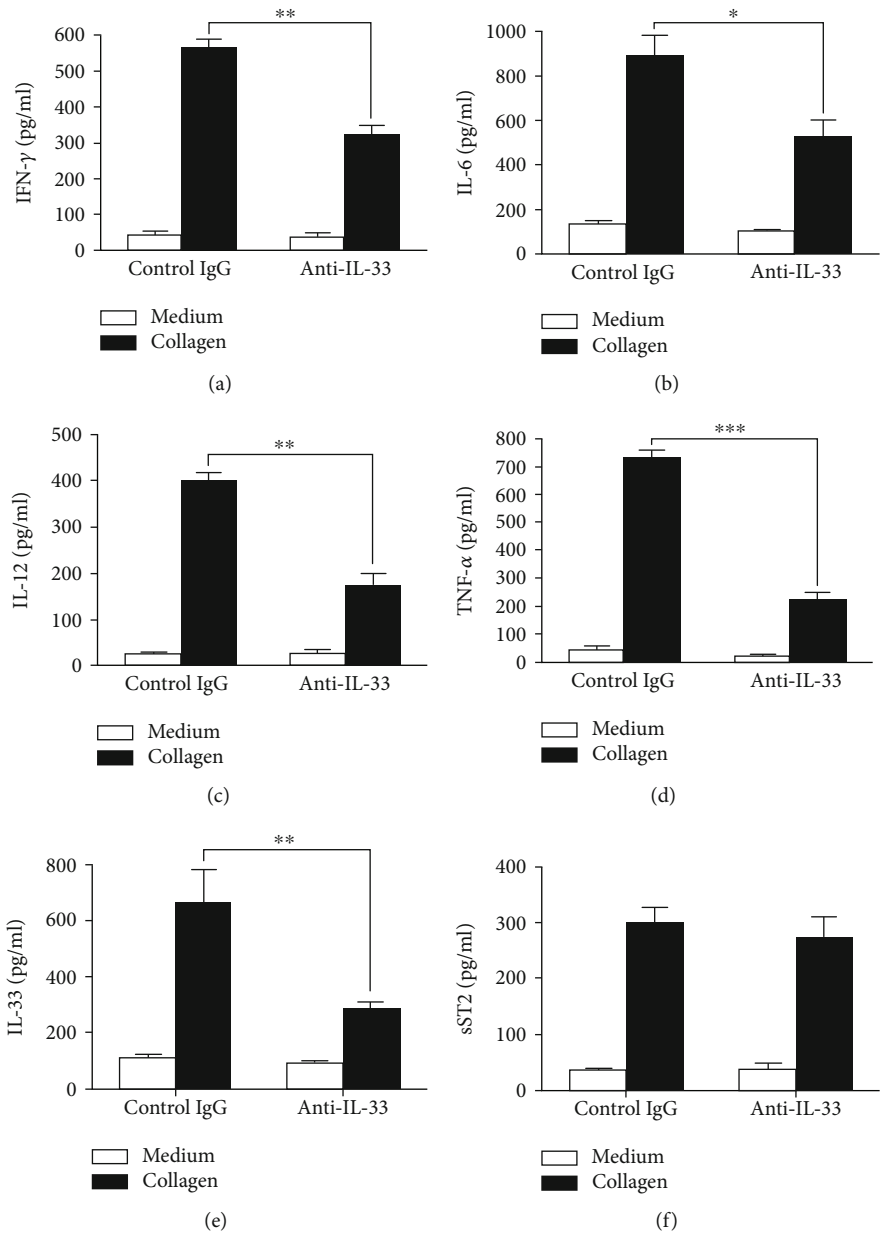


Figure 5: Anti-IL-33 decreases CII-specific proinflammatory cytokine production. Splenocytes ($n = 5$) were prepared from mice on day 43 and cultured with CII for 96 h. The content of cytokines in supernatants (72 h for IL-6, IL-12, IL-33, sST2, and TNF-α; 96 h for IFN-γ and IL-10) was detected by ELISA. Compared with the control group, the production of IFN-γ (a), IL-6 (b), IL-12 (c), TNF-α (d), and IL-33 (e) in spleen cell cultures of mice with anti-IL-33 treatment was significantly inhibited. There was no significant difference in the content of sST2 in spleen cells between the two groups (f). Data are mean ± SEM of triplicate cultures; $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by two-way ANOVA with Bonferroni's multiple comparison test.

this study, we treated CIA mice with the IL-33-neutralizing antibody and obtained similar results of sST2 treatment in CIA mice [3]. We detected that the production of IFN-γ, IL-6, IL-12, IL-33, and TNF-α in spleen cells of CIA mice treated with anti-IL-33 was significantly lower than that of control mice. Compared with the control group, anti-IL-33 treatment also significantly reduced the levels of serum IFN-γ, IL-6, IL-12, IL-33, and TNF-α. However, our data demonstrated that the neutralizing antibody against IL-33 had no significant effect on the production of sST2 in serum and spleen cells of CIA mice, suggesting that the inhibitory effect of anti-IL-33 may be independent of sST2. Altogether, our results showed that anti-IL-33 can inhibit the development of CIA in DBA/1 mice by downregulating the expression of proinflammatory cytokines IFN-γ, IL-6, IL-12, IL-33, and TNF-α.

In conclusion, our findings suggest that IL-33 may be a potential therapeutic target for RA. The IL-33-neutralizing antibody can decrease the joint inflammation and inhibit the development of CIA in mice. However, it is necessary to further investigate the role of the IL-33-neutralizing antibody in human RA. The treatment of the IL-33-neutralizing antibody may provide a new therapeutic approach for human RA.

Data Availability

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

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

YL, YF, HC, and XL performed the experiments. YL and ML analyzed the data and wrote the manuscript. ML designed the research and revised the article. All authors have read and approved the final manuscript.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81970735), Zhejiang Provincial Natural Science Foundation of China (LY18H010003, LY17H010001), Natural Science Foundation of Ningbo (202003N4115, 202003N4118), and K.C. Wong Magna Fund in Ningbo University.

References

[1] J. Schmitz, A. Owyang, E. Oldham et al., "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines," *Immunity*, vol. 23, no. 5, pp. 479–490, 2005.

[2] T. Kamradt and S. Drube, "A complicated liaison: IL-33 and IL-33R in arthritis pathogenesis," *Arthritis Research & Therapy*, vol. 15, no. 3, p. 115, 2013.

[3] B. P. Leung, D. Xu, S. Culshaw, I. B. McInnes, and F. Y. Liew, "A novel therapy of murine collagen-induced arthritis with soluble T1/ST2," *Journal of Immunology*, vol. 173, no. 1, pp. 145–150, 2004.

[4] G. Palmer, D. Talabot-Ayer, C. Lamacchia et al., "Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis," *Arthritis and Rheumatism*, vol. 60, no. 3, pp. 738–749, 2009.

[5] Z. Allakhverdi, D. E. Smith, M. R. Comeau, and G. Delespesse, "Cutting edge: the ST2 ligand IL-33 potently activates and drives maturation of human mast cells," *Journal of Immunology*, vol. 179, no. 4, pp. 2051–2054, 2007.

[6] S. Ali, M. Huber, C. Kollewe, S. C. Bischoff, W. Falk, and M. U. Martin, "IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 47, pp. 18660–18665, 2007.

[7] D. Moulin, O. Donzé, D. Talabot-Ayer, F. Mézin, G. Palmer, and C. Gabay, "Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells," *Cytokine*, vol. 40, no. 3, pp. 216–225, 2007.

[8] F. Rivellese, J. Suurmond, K. Habets et al., "Ability of interleukin-33- and immune complex-triggered activation of human mast cells to down-regulate monocyte-mediated immune responses," *Arthritis & Rhematology*, vol. 67, no. 9, pp. 2343–2353, 2015.

[9] M. Komai-Koma, F. Brombacher, P. N. Pushparaj et al., "Interleukin-33 amplifies IgE synthesis and triggers mast cell degranulation via interleukin-4 in naïve mice," *Allergy*, vol. 67, no. 9, pp. 1118–1126, 2012.

[10] S. Kaieda, J. X. Wang, R. Shnayder et al., "Interleukin-33 primes mast cells for activation by IgG immune complexes," *PLoS ONE*, vol. 7, no. 10, article e47252, 2012.

[11] A. Balato, S. Lembo, M. Mattii et al., "IL-33 is secreted by psoriatic keratinocytes and induces pro-inflammatory cytokines via keratinocyte and mast cell activation," *Experimental Dermatology*, vol. 21, no. 11, pp. 892–894, 2012.

[12] M. Komai-Koma, D. Xu, Y. Li, A. . N. . J. McKenzie, I. . B. McInnes, and F. . Y. Liew, "IL-33 is a chemoattractant for human Th2 cells," *European Journal of Immunology*, vol. 37, no. 10, pp. 2779–2786, 2007.

[13] W. A. Verri, F. O. Souto, S. M. Vieira et al., "IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy," *Annals of the Rheumatic Diseases*, vol. 69, no. 9, pp. 1697–1703, 2010.

[14] M. Suzukawa, M. Iikura, R. Koketsu et al., "An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor," *Journal of Immunology*, vol. 181, no. 9, pp. 5981–5989, 2008.

[15] W. B. Cherry, J. Yoon, K. R. Bartemes, K. Iijima, and H. Kita, "A novel IL-1 family cytokine, IL-33, potently activates human eosinophils," *The Journal of Allergy and Clinical Immunology*, vol. 121, no. 6, pp. 1484–1490, 2008.

[16] M. Suzukawa, R. Koketsu, M. Iikura et al., "Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils," *Laboratory Investigation*, vol. 88, no. 11, pp. 1245–1253, 2008.

[17] Z. Chen, A. Bozec, A. Ramming, and G. Schett, "Anti-inflammatory and immune-regulatory cytokines in rheumatoid arthritis," *Nature Reviews Rheumatology*, vol. 15, no. 1, pp. 9–17, 2019.

[18] Y. Matsuyama, H. Okazaki, H. Tamemoto et al., "Increased levels of interleukin 33 in sera and synovial fluid from patients with active rheumatoid arthritis," *The Journal of Rheumatology*, vol. 37, no. 1, pp. 18–25, 2010.

[19] E. Rivière, J. Sellam, J. Pascaud, P. Ravaud, J. E. Gottenberg, and X. Mariette, "Serum IL-33 level is associated with autoantibodies but not with clinical response to biologic agents in rheumatoid arthritis," *Arthritis Research & Therapy*, vol. 20, no. 1, p. 122, 2018.

[20] I. A. Choi, S. J. Lee, W. Park et al., "Effects of tocilizumab therapy on serum interleukin-33 and interleukin-6 levels in patients with rheumatoid arthritis," *Archives of Rheumatology*, vol. 33, no. 4, pp. 389–394, 2018.

[21] S. Tang, H. Huang, F. Hu et al., "Increased IL-33 in synovial fluid and paired serum is associated with disease activity and autoantibodies in rheumatoid arthritis," *Clinical and Developmental Immunology*, vol. 2013, Article ID 985301, 6 pages, 2013.

[22] R. Mu, H.-Q. Huang, Y.-H. Li, C. Li, H. Ye, and Z.-G. Li, "Elevated serum interleukin 33 is associated with autoantibody production in patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 37, no. 10, pp. 2006–2013, 2010.

[23] E.-J. Lee, M. W. So, S. Hong, Y.-G. Kim, B. Yoo, and C.-K. Lee, "Interleukin-33 acts as a transcriptional repressor and

extracellular cytokine in fibroblast-like synoviocytes in patients with rheumatoid arthritis," *Cytokine*, vol. 77, pp. 35–43, 2015.

[24] Z. Xiangyang, Y. Lutian, Z. Lin, X. Liping, S. Hui, and L. Jing, "Increased levels of interleukin-33 associated with bone erosion and interstitial lung diseases in patients with rheumatoid arthritis," *Cytokine*, vol. 58, no. 1, pp. 6–9, 2012.

[25] L. J. Shi, C. Liu, J. H. Li, X. Y. Zhu, Y. N. Li, and J. T. Li, "Elevated levels of soluble ST2 were associated with rheumatoid arthritis disease activity and ameliorated inflammation in synovial fibroblasts," *Chinese Medical Journal*, vol. 131, no. 3, pp. 316–322, 2018.

[26] D. Xu, H. R. Jiang, P. Kewin et al., "IL-33 exacerbates antigen-induced arthritis by activating mast cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 31, pp. 10913–10918, 2008.

[27] D. Xu, H. R. Jiang, Y. Li et al., "IL-33 exacerbates autoantibody-induced arthritis," *Journal of Immunology*, vol. 184, no. 5, pp. 2620–2626, 2010.

[28] D. Talabot-Ayer, P. Martin, C. A. Seemayer et al., "Immune-mediated experimental arthritis in IL-33 deficient mice," *Cytokine*, vol. 69, no. 1, pp. 68–74, 2014.

[29] M. M. Zaiss, M. Kurowska-Stolarska, C. Böhm et al., "IL-33 shifts the balance from osteoclast to alternatively activated macrophage differentiation and protects from TNF-alpha-mediated bone loss," *Journal of Immunology*, vol. 186, no. 11, pp. 6097–6105, 2011.

[30] C. Qiu, Y. Li, M. Li et al., "Anti-interleukin-33 inhibits cigarette smoke-induced lung inflammation in mice," *Immunology*, vol. 138, no. 1, pp. 76–82, 2013.

[31] X. Liu, M. Li, Y. Wu, Y. Zhou, L. Zeng, and T. Huang, "Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma," *Biochemical and Biophysical Research Communications*, vol. 386, no. 1, pp. 181–185, 2009.

[32] X. Liu, Y. Wu, M. Li, S. Chen, and Y. Zhou, "Production of polyclonal antibody against interleukin-33 and assessment of its distribution in murine liver and lung," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 729197, 6 pages, 2009.

[33] E. Lubberts, M. I. Koenders, B. Oppers-Walgreen et al., "Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion," *Arthritis and Rheumatism*, vol. 50, no. 2, pp. 650–659, 2004.

[34] T. Geiger, H. Towbin, A. Cosenti-Vargas et al., "Neutralization of interleukin-1 beta activity in vivo with a monoclonal antibody alleviates collagen-induced arthritis in DBA/1 mice and prevents the associated acute-phase response," *Clinical and Experimental Rheumatology*, vol. 11, no. 5, pp. 515–522, 1993.

[35] Y. S. Hong, S. J. Moon, Y. B. Joo et al., "Measurement of interleukin-33 (IL-33) and IL-33 receptors (sST2 and ST2L) in patients with rheumatoid arthritis," *Journal of Korean Medical Science*, vol. 26, no. 9, pp. 1132–1139, 2011.

[36] Y. Kageyama, E. Torikai, K. Tsujimura, and M. Kobayashi, "Involvement of IL-33 in the pathogenesis of rheumatoid arthritis: the effect of etanercept on the serum levels of IL-33," *Modern Rheumatology*, vol. 22, no. 1, pp. 89–93, 2014.

[37] P. Martin, D. Talabot-Ayer, C. A. Seemayer et al., "Disease severity in K/BxN serum transfer-induced arthritis is not affected by IL-33 deficiency," *Arthritis research & therapy*, vol. 15, no. 1, p. R13, 2013.

[38] J. Biton, S. Khaleghparast Athari, A. Thiolat et al., "In vivo expansion of activated Foxp3+ regulatory T cells and establishment of a type 2 immune response upon IL-33 treatment protect against experimental arthritis," *Journal of Immunology*, vol. 197, no. 5, pp. 1708–1719, 2016.

[39] A. Y. Gasparyan, A. Sandoo, A. Stavropoulos-Kalinoglou, and G. D. Kitas, "Mean platelet volume in patients with rheumatoid arthritis: the effect of anti-TNF-alpha therapy," *Rheumatology International*, vol. 30, no. 8, pp. 1125–1129, 2010.

[40] E. Kunisch, S. Chakilam, M. Gandesiri, and R. W. Kinne, "IL-33 regulates TNF-α dependent effects in synovial fibroblasts," *International Journal of Molecular Medicine*, vol. 29, no. 4, pp. 530–540, 2012.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 8528976, 11 pages
https://doi.org/10.1155/2020/8528976

Research Article

Association of *NCF2*, *NCF4*, and *CYBA* Gene Polymorphisms with Rheumatoid Arthritis in a Chinese Population

Tian-Ping Zhang,[1] **Rui Li**,[2] **Qian Huang**,[3] **Han-Feng Pan**,[3] **Dong-Qing Ye**,[3] **and Xiao-Mei Li**[1]

[1]*The First Affiliated Hospital of University of Science and Technology of China, Hefei, 230001 Anhui, China*
[2]*Department of Nosocomial Infection Management, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022 Anhui, China*
[3]*School of Public Health, Anhui Medical University, Anhui Provincial Laboratory of Inflammatory and Immune Diseases, Hefei, 230032 Anhui, China*

Correspondence should be addressed to Dong-Qing Ye; anhuiydq@126.com and Xiao-Mei Li; lixiaomei@ustc.edu.cn

Received 27 March 2020; Revised 7 October 2020; Accepted 14 October 2020; Published 21 October 2020

Academic Editor: Lihua Duan

Objective. Recent studies have focused on the special roles of NADPH-oxidase in multiple autoimmune diseases. Nevertheless, the association of genetic variation in NADPH-oxidase genes with rheumatoid arthritis (RA) was not extensively studied in a Chinese population. We performed this study to examine the association of *NCF2*, *NCF4*, and *CYBA* gene polymorphisms with RA susceptibility in a Chinese population. *Methods*. Six single nucleotide polymorphisms (SNPs) (*NCF2* rs10911363, *NCF4* rs1883112, rs4821544, rs729749, *CYBA* rs3794624, and rs4673) were genotyped in a cohort composed of 593 RA patients and 596 normal controls. Improved multiple ligase detection reaction (iMLDR) was used for genotyping. *Results*. We observed that *NCF4* rs4821544 CT genotype and C allele frequencies in RA patients were significantly decreased when compared to controls (CT vs. TT: $P = 0.043$; C vs. T: $P = 0.031$), and rs4821544 polymorphism was significantly associated with an increased RA risk under the dominant model (TT vs. CT+CC: $P = 0.031$). Our results also indicated that rs729749 CT genotype frequency was significantly lower in RA patients than that in controls (CT vs. CC: $P = 0.033$). Moreover, the rs729749 CT genotype frequency was also significantly decreased in RA patients in males (CT vs. CC: $P = 0.024$). No significant association between *NCF2* and *CYBA* gene polymorphisms and RA susceptibility was observed. There were significant associations between rs4821544 TT genotype and T allele frequencies and anti-CCP in male RA patients. *Conclusions*. In summary, *NCF4* rs4821544 and rs729749 polymorphisms might contribute to RA susceptibility, while *NCF2* and *CYBA* gene polymorphisms were not associated with RA susceptibility.

1. Introduction

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease affecting approximately 1-2% of the population worldwide [1]. The disease is characterized by immunologic inadequacy and chronic inflammation and predominantly causes deformity and destruction of the joints [2]. The pathogenesis of RA is not completely identified and generally considered to be related to the interaction between environmental factors and genetic predisposition [3]. Despite many efforts devoted to the studies of predominant genetic markers, only few genetic loci including human leukocyte antigen (*HLA*) locus and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) have been identified to be associated with RA [4, 5]. Therefore, it is necessary to continue to analyze whether immune-modulating gene variations are associated with RA.

Recent studies indicated that elevated levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were found in several autoimmune diseases such as RA and

systemic lupus erythematosus (SLE) and involved in inflammatory processes [6, 7]. The production of ROS was a physiological defense against microbial infection, and ROS had an important antibacterial effect on a variety of pathogens. However, the aberrant generation of ROS in autoimmune inflammation could result in tissue damage [6]. Moreover, ROS also had important regulatory functions in the immune system [8]. The NADPH-oxidase (NOX) complex, which was critical for ROS generation, was composed of gp91phox, p22phox, IM7phox, p67phox, and p40phox encoded by *CYBB*, *CYBA*, *NCFl*, *NCF2*, and *NCF4* genes, respectively [9, 10]. The ability to induce ROS extends to phagocytes and dendritic cells, also implying the key functions of the NOX complex in the immune system [11].

The hypothesis that genetically controlled reduced production of ROS caused by *NOX* gene variations could affect the risk of autoimmune diseases and ROS-regulated chronic autoimmune inflammatory diseases was supported by several studies [12, 13]. Olsson et al. suggested that *NCF1-339* T allele frequency in SLE patients was increased in comparison to controls. In addition, the *NCF1-339* T allele reduced extracellular ROS production in neutrophils and resulted in the elevated expression of type 1 interferon-regulated gene [12]. It was remarkable that there were several researches exploring the association between *NOX* gene polymorphisms and RA susceptibility [13, 14]. Zhao et al. demonstrated that the p.Arg90His variant in *NCF1*, which was observed to cause reduced ROS production, predisposed to RA in a Korean population [13].

Apart from *NCF1*, several single nucleotide polymorphisms (SNPs) in *NCF2*, *NCF4*, and *CYBA* genes were reported to be associated with autoimmune diseases [15–17]. The results by Olsson et al. indicated that the *NCF4* rs729749 variant was involved in the development of RA in a Swedish cohort [16]. However, the association between *NCF2*, *NCF4*, and *CYBA* genetic variants and RA risk in a Chinese population had not been reported. To investigate whether *NCF2*, *NCF4*, and *CYBA* gene polymorphisms are associated with RA susceptibility, we performed this case-control study in a Chinese population.

2. Materials and Methods

2.1. Study Populations. In this study, a total of 593 RA patients including 101 males and 492 females were recruited from Anhui Provincial Laboratory of Inflammatory and Immune Diseases. All patients were diagnosed depending on the 1987 American College of Rheumatology revised criteria [18]. Then, a normal cohort of 97 males and 499 females, which had no history of inflammatory/autoimmune diseases and cancer, was enrolled from the same region. The average ages of RA patients and normal controls were 51.59 ± 6.68 years and 52.32 ± 12.63 years, respectively, and there was no significant difference in age distribution between RA patients and controls. The demographic and clinical data of RA patients were collected from the medical records and reviewed by a rheumatologist, and the clinical data mainly included anticyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF). All RA patients and normal controls were enrolled after their written informed consent had been obtained, and the study protocol was approved by the Medical Ethics Committee of Anhui Medical University.

2.2. SNP Selection. Several previous studies had shown that *NCF2* gene polymorphisms (rs17849502, rs35937854, rs13306575, rs789181, and rs10911363) were significantly associated with the development of autoimmune diseases [16, 19, 20]. However, only the minor allele frequency (MAF) of rs10911363 was greater than 0.05 in the CHB population. Hence, rs10911363 in *NCF2* was included for genotyping in the present study. Because of limited research on the *NCF4* and *CYBA* genetic variants in RA, we utilized genotype data of the CHB from Ensembl genome browser 85 and CHBS_1000g and selected the tagSNPs capturing all the common SNPs located in the chromosome locus transcribed into *NCF4* and *CYBA* and their flanking 2000 bp region by the Haploview 4.0 software (Cambridge, MA, USA). We finally selected rs1883112, rs4821544, and rs729749 in *NCF4* and rs3794624 and rs4673 in *CYBA* for genotyping, and the *NCF4* rs4821544, rs729749, *CYBA* rs3794624, and rs4673 had also been studied in the Swedish population [16]. Above SNPs accorded with MAF ≥ 0.05 in CHB and r^2 threshold > 0.8.

2.3. DNA Extraction and Genotyping. A total of 5 ml peripheral blood sample was collected from all study populations by tubes containing ethylenediaminetetraacetic acid (EDTA). Then, genomic DNA was prepared from the peripheral blood leukocytes according to the standard procedures with the FlexiGene DNA Kit (Qiagen, Valencia, CA).

The genotyping was conducted using improved multiple ligase detection reaction (iMLDR) genotyping assays, with the technical support of Genesky Biotechnologies Inc., Shanghai. Those subjects with 100% genotype success for all SNPs were involved in the final analysis.

2.4. Statistical Analysis. Statistical analysis was done in the SPSS 23.00 (SPSS Inc., IL, USA). The chi-square (χ^2) test was used to analyze the association of the genotype and allele frequencies of above SNPs and RA patients. Odds ratios (OR) and 95% confidence interval (CI) were also evaluated using logistic regression analyses. Two genetic models including the dominant model and the recessive model were also analyzed. The Hardy-Weinberg equilibrium (HWE) test was conducted in the control group. Haplotype was assessed using the SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) [21]. A two-sided $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Association of NCF2, NCF4, and CYBA Gene Polymorphisms with RA Susceptibility. In normal controls, the genotype frequencies of *NCF2* rs10911363, *NCF4* rs1883112, rs4821544, rs729749 and *CYBA* rs3794624, and rs4673 were all in compliance with the HWE (all $P > 0.05$). The allele and genotype frequencies of these SNPs are shown in Table 1. There was no significant difference in the genotype frequencies of *NCF2* rs10911363 between RA patients

Table 1: The association between *NCF2*, *NCF4*, and *CYBA* gene polymorphisms and RA susceptibility among different groups (*n* (%)).

SNP	Analyze model	RA patients	Controls	*P* value	OR (95% CI)
All					
	GG	125 (21.08)	144 (24.16)	0.371	0.863 (0.625, 1.192)
	GT	304 (51.26)	289 (48.49)	0.747	1.045 (0.798, 1.370)
	TT	164 (27.66)	163 (27.35)	Reference	
	G	554 (46.71)	577 (48.41)	0.408	1.070 (0.911, 1.257)
rs10911363	T	632 (53.29)	615 (51.59)	Reference	
	TT	164 (27.66)	163 (27.35)	0.906	1.016 (0.787, 1.310)
	GT+GG	429 (72.34)	433 (72.65)	Reference	
	GG	125 (21.08)	144 (24.16)	0.204	0.838 (0.639, 1.101)
	GT+TT	468 (78.92)	452 (75.84)	Reference	
	AA	14 (2.36)	15 (2.52)	0.929	0.967 (0.461, 2.027)
	GA	160 (26.98)	147 (24.66)	0.368	1.127 (0.868, 1.464)
	GG	419 (70.66)	434 (72.82)	Reference	
	A	188 (15.85)	177 (14.85)	0.498	1.080 (0.864, 1.350)
rs3794624	G	998 (84.15)	1015 (85.15)	Reference	
	GG	419 (70.66)	434 (72.82)	0.408	0.899 (0.698, 1.157)
	GA+AA	174 (29.34)	162 (27.18)	Reference	
	AA	14 (2.36)	15 (2.52)	0.862	0.937 (0.448, 1.958)
	GA+GG	579 (97.64)	581 (97.48)	Reference	
	AA	1 (0.17)	5 (0.84)	0.140	0.198 (0.023, 1.698)
	GA	85 (14.33)	90 (15.10)	0.673	0.933 (0.677, 1.287)
	GG	507 (85.50)	501 (84.06)	Reference	
	A	87 (7.34)	100 (8.39)	0.340	0.864 (0.641, 1.166)
rs4673	G	1099 (92.66)	1092 (91.61)	Reference	
	GG	507 (85.50)	501 (84.06)	0.491	1.118 (0.814, 1.535)
	GA+AA	86 (14.50)	95 (15.94)	Reference	
	AA	1 (0.17)	5 (0.84)	0.142	0.200 (0.023, 1.714)
	GA+GG	592 (99.83)	591 (99.16)	Reference	
	GG	57 (9.61)	56 (9.39)	0.972	1.007 (0.673, 1.508)
	GA	248 (41.82)	255 (42.79)	0.754	0.962 (0.757, 1.223)
	AA	288 (48.57)	285 (47.82)	Reference	
	G	362 (30.52)	367 (30.79)	0.888	0.988 (0.830, 1.176)
rs1883112	A	824 (69.48)	825 (69.21)	Reference	
	AA	288 (48.57)	285 (47.82)	0.796	1.030 (0.821, 1.294)
	GA+GG	305 (52.43)	311 (52.18)	Reference	
	GG	57 (9.61)	56 (9.40)	0.899	1.025 (0.696, 1.511)
	GA+AA	536 (90.39)	540 (90.60)	Reference	
	CC	4 (0.67)	7 (1.17)	0.323	0.536 (0.156, 1.845)
	CT	117 (19.73)	146 (24.50)	0.043	0.752 (0.571, 0.991)
	TT	472 (79.60)	443 (74.33)	Reference	
	C	125 (10.54)	160 (13.42)	0.031	1.316 (1.026, 1.688)
rs4821544	T	1061 (89.46)	1032 (86.58)	Reference	
	TT	472 (79.60)	443 (74.33)	0.031	0.742 (0.566, 0.974)
	CT+CC	121 (20.40)	153 (25.67)	Reference	
	CC	4 (0.67)	7 (1.17)	0.374	0.571 (0.166, 1.962)
	CT+TT	589 (99.33)	589 (98.83)	Reference	
rs729749	TT	104 (17.54)	102 (17.11)	0.445	0.878 (0.628, 1.226)
	CT	266 (44.86)	302 (50.67)	0.033	0.758 (0.588, 0.977)

Table 1: Continued.

SNP	Analyze model	RA patients	Controls	*P* value	OR (95% CI)
	CC	223 (37.61)	192 (32.21)	Reference	
	T	474 (39.97)	506 (42.45)	0.219	0.903 (0.767, 1.063)
	C	712 (60.03)	686 (57.55)	Reference	
	CC	223 (37.61)	192 (32.21)	0.051	1.268 (0.999, 1.611)
	CT+TT	370 (62.39)	404 (67.79)	Reference	
	TT	104 (17.54)	102 (17.11)	0.847	1.030 (0.763, 1.391)
	CT+CC	489 (82.46)	494 (82.89)	Reference	
Female					
	GG	104 (21.14)	117 (23.45)	0.548	0.896 (0.625, 1.283)
	GT	259 (52.64)	252 (50.50)	0.818	1.036 (0.768, 1.397)
	TT	129 (26.22)	130 (26.05)	Reference	
	G	467 (47.46)	486 (48.70)	0.581	1.051 (0.881, 1.253)
rs10911363	T	517 (52.54)	512 (51.30)	Reference	
	TT	129 (26.22)	130 (26.05)	0.952	0.991 (0.747, 1.316)
	GT+GG	363 (73.78)	369 (73.95)	Reference	
	GG	104 (21.14)	117 (23.45)	0.383	1.143 (0.847, 1.542)
	GT+TT	388 (78.86)	382 (76.55)	Reference	
	AA	12 (2.44)	12 (2.40)	0.876	1.067 (0.473, 2.407)
	GA	137 (27.85)	121 (24.25)	0.194	1.208 (0.908, 1.607)
	GG	343 (69.72)	366 (73.35)	Reference	
	A	161 (16.36)	145 (14.53)	0.259	0.869 (0.681, 1.109)
rs3794624	G	823 (83.64)	853 (85.47)	Reference	
	GG	343 (69.72)	366 (73.35)	0.205	1.195 (0.907, 1.576)
	GA+AA	149 (30.28)	133 (26.65)	Reference	
	AA	12 (2.44)	12 (2.40)	0.972	0.986 (0.438, 2.216)
	GA+GG	480 (97.56)	487 (97.60)	Reference	
	AA	1 (0.20)	5 (1.00)	0.140	0.198 (0.023, 1.703)
	GA	70 (14.23)	77 (15.43)	0.558	0.900 (0.634, 1.279)
	GG	421 (85.57)	417 (83.57)	Reference	
	A	72 (7.32)	87 (8.72)	0.252	1.210 (0.874, 1.675)
rs4673	G	912 (92.68)	911 (91.28)	Reference	
	GG	421 (85.57)	417 (83.57)	0.346	0.847 (0.600, 1.196)
	GA+AA	71 (14.43)	83 (16.63)	Reference	
	AA	1 (0.20)	5 (1.00)	0.144	4.970 (0.579, 42.692)
	GA+GG	491 (99.8)	494 (99.00)	Reference	
	GG	41 (8.33)	47 (9.42)	0.630	0.894 (0.567, 1.409)
	GA	211 (42.89)	206 (41.28)	0.715	1.050 (0.808, 1.364)
	AA	240 (48.78)	246 (49.30)	Reference	
	G	293 (29.78)	300 (30.06)	0.890	1.014 (0.836, 1.229)
rs1883112	A	691 (70.22)	698 (69.94)	Reference	
	AA	240 (48.78)	246 (49.30)	0.870	1.021 (0.796, 1.310)
	GA+GG	252 (51.22)	253 (50.70)	Reference	
	GG	41 (8.33)	47 (9.42)	0.548	1.144 (0.738, 1.774)
	GA+AA	451 (91.67)	452 (90.58)	Reference	
	CC	4 (0.81)	6 (1.20)	0.494	0.641 (0.180, 2.290)
rs4821544	CT	95 (19.31)	115 (23.05)	0.141	0.795 (0.585, 1.079)
	TT	393 (79.88)	378 (75.75)	Reference	
	C	103 (10.47)	127 (12.73)	0.117	1.247 (0.946, 1.644)

Table 1: Continued.

SNP	Analyze model	RA patients	Controls	*P* value	OR (95% CI)
	T	881 (89.53)	871 (87.27)	Reference	
	TT	393 (79.88)	378 (75.75)	0.118	0.787 (0.583, 1.063)
	CT+CC	99 (20.12)	121 (24.25)	Reference	
	CC	4 (0.81)	6 (1.20)	0.540	1.485 (0.416, 5.294)
	CT+TT	488 (99.19)	493 (98.80)	Reference	
	TT	84 (17.07)	87 (17.43)	0.469	0.873 (0.604, 1.261)
	CT	231 (46.95)	252 (50.50)	0.186	0.829 (0.627, 1.095)
	CC	177 (35.98)	160 (32.06)	Reference	
	T	399 (40.55)	426 (42.69)	0.335	1.092 (0.913, 1.306)
rs729749	C	585 (59.45)	572 (57.31)	Reference	
	CC	177 (35.98)	160 (32.06)	0.194	0.840 (0.646, 1.093)
	CT+TT	315 (64.02)	339 (67.94)	Reference	
	TT	84 (17.07)	87 (17.43)	0.880	1.026 (0.738, 1.426)
	CT+CC	408 (82.93)	412 (82.57)	Reference	
Male					
	GG	21 (20.79)	27 (27.84)	0.413	0.733 (0.349, 1.541)
	GT	45 (44.55)	37 (38.14)	0.677	1.147 (0.602, 2.184)
	TT	35 (34.65)	33 (34.02)	Reference	
	G	87 (43.07)	91 (46.91)	0.443	1.168 (0.786, 1.736)
rs10911363	T	115 (56.93)	103 (53.09)	Reference	
	TT	35 (34.65)	33 (34.02)	0.925	0.972 (0.541, 1.749)
	GT+GG	66 (65.35)	64 (65.98)	Reference	
	GG	21 (20.79)	27 (27.84)	0.249	1.469 (0.764, 2.827)
	GT+TT	80 (79.21)	70 (72.16)	Reference	
	AA	2 (1.98)	3 (3.09)	0.578	0.596 (0.097, 3.677)
	GA	23 (22.77)	26 (26.8)	0.480	0.791 (0.413, 1.515)
	GG	76 (75.25)	68 (70.1)	Reference	
	A	27 (13.37)	32 (16.49)	0.383	1.280 (0.735, 2.230)
rs3794624	G	175 (86.63)	162 (83.51)	Reference	
	GG	76 (75.25)	68 (70.1)	0.417	0.771 (0.412, 1.444)
	GA+AA	25 (24.75)	29 (29.9)	Reference	
	AA	2 (1.98)	3 (3.09)	0.621	1.580 (0.258, 9.666)
	GA+GG	99 (98.02)	94 (96.91)	Reference	
	AA	0	0	—	—
	GA	15 (14.85)	13 (13.40)	0.770	1.127 (0.506, 2.511)
rs4673	GG	86 (85.15)	84 (86.60)	Reference	
	A	15 (7.43)	13 (6.70)	0.779	0.895 (0.414, 1.934)
	G	187 (92.57)	181 (93.30)	Reference	
	GG	16 (15.84)	9 (9.28)	0.433	1.444 (0.576, 3.623)
	GA	37 (36.63)	49 (50.52)	0.111	0.614 (0.336, 1.119)
	AA	48 (47.52)	39 (40.21)	Reference	
	G	69 (34.16)	67 (34.54)	0.937	1.017 (0.672, 1.540)
rs1883112	A	133 (65.84)	127 (65.46)	Reference	
	AA	48 (47.52)	39 (40.21)	0.300	0.742 (0.423, 1.304)
	GA+GG	53 (52.48)	58 (59.79)	Reference	
	GG	16 (15.84)	9 (9.28)	0.169	0.543 (0.228, 1.296)
	GA+AA	85 (84.16)	88 (90.72)	Reference	
rs4821544	CC	0	1 (1.03)	1.000	—

Table 1: Continued.

SNP	Analyze model	RA patients	Controls	*P* value	OR (95% CI)
	CT	22 (21.78)	31 (31.96)	0.098	0.584 (0.309, 1.104)
	TT	79 (78.22)	65 (67.01)	Reference	
	C	22 (10.89)	33 (17.01)	0.081	1.677 (0.939, 2.995)
	T	180 (89.11)	161 (82.99)	Reference	
	TT	79 (78.22)	65 (67.01)	0.078	0.566 (0.300, 1.067)
	CT+CC	22 (21.78)	32 (32.99)	Reference	
	CC	0	1 (1.03)	1.000	—
	CT+TT	101 (100.00)	96 (98.97)	Reference	
	TT	20 (19.8)	15 (15.46)	0.855	0.928 (0.414, 2.079)
	CT	35 (34.65)	50 (51.55)	0.024	0.487 (0.261, 0.909)
	CC	46 (45.54)	32 (32.99)	Reference	
	T	75 (37.13)	80 (41.24)	0.403	1.188 (0.793, 1.780)
rs729749	C	127 (62.87)	114 (58.76)	Reference	
	CC	46 (45.54)	32 (32.99)	0.072	0.589 (0.331, 1.041)
	CT+TT	55 (54.46)	65 (67.01)	Reference	
	TT	20 (19.80)	15 (15.46)	0.425	0.741 (0.355, 1.547)
	CT+CC	81 (80.20)	82 (84.54)	Reference	

and controls. Similarly, the allele frequencies of rs10911363 in RA patients were comparable to controls. Then, we stratified all subjects by sex and analyzed the association between rs10911363 and RA susceptibility in males and females, respectively. No significant association was found.

Regarding the genotype and allele frequencies of *NCF4* rs1883112, rs4821544, and rs729749, we noted that the CT genotype and C allele frequencies of rs4821544 in RA patients were significantly decreased when compared to controls (CT vs. TT: OR = 0.752, 95% CI: 0.571-0.991, $P = 0.043$; C vs. T: OR = 1.316, 95% CI: 1.026-1.688, $P = 0.031$). Moreover, the rs4821544 polymorphism was significantly associated with an increased RA risk under the dominant model (TT vs. CT+CC: OR = 0.742, 95% CI: 0.566-0.974, $P = 0.031$). However, the rs4821544 polymorphism was not correlated with RA susceptibility in male and female subjects, respectively. The results also indicated that rs729749 CT genotype frequency in RA patients was significantly lower than that in controls (CT vs. CC: OR = 0.758, 95% CI: 0.588-0.977, $P = 0.033$). Moreover, the rs729749 CT genotype frequency was significantly decreased in RA patients in males (CT vs. CC: OR = 0.487, 95% CI: 0.261-0.909, $P = 0.024$) and was not associated with RA susceptibility in females. No significant association between rs1883112 polymorphism and RA susceptibility was observed.

The genotype and allele frequencies of rs3794624 in *CYBA* were not significantly different among RA patients and controls (all $P > 0.05$), and no significant differences have existed in genotype and allele frequencies of *CYBA* rs4673 polymorphism (all $P > 0.05$). Similarly, *CYBA* rs3794624 and rs4673 polymorphisms were also not related to RA susceptibility in males and females, respectively.

3.2. Association of NCF2, NCF4, and CYBA Gene Polymorphisms with Clinical Features in RA Patients. Considering that RA was a heterogeneous disease, and different RF, anti-CCP antibody status might reflect disparate mechanisms of RA patients. We determined to evaluate whether *NCF2*, *NCF4*, and *CYBA* gene polymorphisms were associated with different serotypes of RA in a case-only study (Table 2). No significant differences have existed in *NCF2*, *NCF4*, and *CYBA* gene polymorphisms between RF-positive RA patients and RF-negative RA patients, as well as anti-CCP-positive RA patients and anti-CCP-negative RA patients, among the entire study population (all $P > 0.05$). Nonetheless, we found that the TT genotype and T allele frequencies of rs4821544 were significantly associated with anti-CCP in male RA patients ($P = 0.031$, $P = 0.043$).

3.3. Haplotype Analyses. In the present study, six main haplotypes (ATC, ATT, GCC, GCT, GTC, and GTT) for *NCF4* and three main haplotypes (AG, GA, and GG) for *CYBA* were detected by the SHEsis software. There was no significant difference regarding these haplotype frequencies between RA patients and normal controls (Tables 3 and 4).

4. Discussion

ROS was initially thought to be primarily involved in the chronic inflammation of autoimmune diseases such as SLE and RA. Then, the important regulatory functions of ROS were also observed in the immune system [8]. Additionally, some studies had attempted to explore whether *NOX* gene polymorphisms were related to multiple autoimmune diseases and suggested that *NOX* gene polymorphisms were associated with autoimmune diseases risk, as well as several specific clinical features [16, 22]. However, such studies were limited, especially in the Chinese population. To our knowledge, the present study was the first to analyze the relationship between polymorphisms of rs10911363 in *NCF2*;

Table 2: Association of clinical characteristics with genotype and allele frequencies in *NCF2*, *NCF4*, and *CYBA* genes among different groups (*n* (%)).

SNP	Allele (M/m)	Clinical features	Group	Genotypes *n* (%)			*P* value	Alleles *n* (%)		*P* value
				MM	Mm	mm		M	m	
All										
rs10911363	T/G	Anti-CCP	Positive	128 (26.28)	255 (52.36)	104 (21.35)	0.110	511 (52.46)	463 (47.54)	0.206
			Negative	28 (37.33)	31 (41.33)	16 (21.33)		87 (58.00)	63 (42.00)	
		RF	Positive	126 (26.30)	256 (53.44)	97 (20.25)	0.122	508 (53.03)	450 (46.97)	0.806
			Negative	30 (30.93)	41 (42.27)	26 (26.80)		101 (52.06)	93 (47.94)	
rs3794624	G/A	Anti-CCP	Positive	346 (71.05)	132 (27.10)	9 (1.85)	0.172	824 (84.60)	150 (13.40)	0.416
			Negative	52 (69.33)	19 (25.33)	4 (5.33)		123 (82.00)	27 (18.00)	
		RF	Positive	340 (70.98)	128 (26.72)	11 (2.30)	0.682	808 (84.34)	150 (15.66)	0.946
			Negative	68 (70.10)	28 (28.87)	1 (1.03)		164 (84.54)	30 (15.46)	
rs4673	G/A	Anti-CCP	Positive	421 (86.45)	66 (13.55)	0	0.238	908 (93.22)	66 (6.78)	0.257
			Negative	61 (81.33)	14 (28.67)	0		136 (90.67)	14 (9.33)	
		RF	Positive	409 (85.39)	69 (14.41)	1 (0.21)	0.904	887 (92.59)	71 (7.41)	0.925
			Negative	83 (85.57)	14 (14.43)	0		180 (92.78)	14 (7.22)	
rs1883112	A/G	Anti-CCP	Positive	241 (49.49)	201 (41.27)	45 (9.24)	0.880	683 (70.12)	291 (29.88)	0.976
			Negative	36 (48.00)	33 (44.00)	6 (8.00)		105 (70.00)	45 (30.00)	
		RF	Positive	238 (49.69)	192 (40.08)	49 (10.23)	0.076	668 (69.73)	290 (30.27)	0.697
			Negative	45 (46.39)	48 (49.48)	4 (4.12)		138 (71.13)	56 (28.87)	
rs4821544	T/C	Anti-CCP	Positive	384 (78.85)	99 (20.33)	4 (0.82)	0.598	867 (89.01)	107 (10.99)	0.392
			Negative	62 (82.67)	13 (17.33)	0		137 (91.33)	13 (8.67)	
		RF	Positive	378 (78.91)	97 (20.25)	4 (0.84)	0.609	853 (89.04)	105 (10.96)	0.489
			Negative	79 (81.44)	18 (18.56)	0		176 (90.72)	18 (9.28)	
rs729749	C/T	Anti-CCP	Positive	188 (38.60)	215 (44.15)	84 (17.25)	0.820	591 (60.68)	383 (39.32)	0.754
			Negative	29 (38.67)	31 (41.33)	15 (20.00)		89 (59.33)	61 (40.67)	
		RF	Positive	183 (38.20)	215 (44.89)	81 (16.91)	0.676	581 (60.65)	377 (39.35)	0.533
			Negative	36 (37.11)	41 (42.27)	20 (20.62)		113 (58.25)	81 (41.75)	
Female										
rs10911363	T/G	Anti-CCP	Positive	86 (21.18)	218 (53.69)	102 (25.12)	0.162	390 (48.03)	422 (51.97)	0.265
			Negative	13 (21.31)	26 (42.62)	22 (36.07)		52 (42.62)	70 (57.38)	
		RF	Positive	98 (24.69)	220 (55.42)	79 (19.9)	0.074	416 (52.39)	378 (47.61)	0.680
			Negative	24 (29.63)	34 (41.98)	23 (28.40)		82 (50.62)	80 (49.38)	
rs3794624	G/A	Anti-CCP	Positive	283 (69.70)	115 (28.33)	8 (1.97)	0.360	681 (83.87)	131 (16.13)	0.597
			Negative	42 (68.85)	16 (26.23)	3 (4.92)		100 (81.97)	22 (18.03)	
		RF	Positive	277 (69.77)	110 (27.71)	10 (2.52)	0.750	664 (83.63)	130 (16.37)	0.919
			Negative	56 (69.14)	24 (29.63)	1 (1.23)		136 (83.95)	26 (16.05)	
rs4673	G/A	Anti-CCP	Positive	352 (86.70)	54 (13.30)	0	0.096	109 (89.34)	13 (10.66)	0.110
			Negative	48 (78.69)	13 (21.31)	0		758 (93.35)	54 (6.65)	
		RF	Positive	341 (85.89)	55 (13.85)	1 (0.25)	0.660	737 (92.82)	57 (7.18)	0.517
			Negative	67 (82.72)	14 (17.28)	0		148 (91.36)	14 (8.64)	
rs1883112	A/G	Anti-CCP	Positive	202 (49.75)	171 (42.12)	33 (8.13)	0.844	575 (70.81)	237 (29.19)	0.658
			Negative	28 (45.90)	28 (45.90)	5 (8.20)		84 (68.85)	38 (31.15)	
		RF	Positive	201 (50.63)	162 (40.81)	34 (8.56)	0.151	564 (71.03)	230 (28.97)	0.629
			Negative	35 (43.21)	42 (51.85)	4 (4.94)		112 (69.14)	50 (30.86)	
rs4821544	T/C	Anti-CCP	Positive	324 (79.80)	78 (19.21)	4 (0.99)	0.694	726 (89.41)	86 (10.59)	0.983
			Negative	48 (78.69)	13 (21.31)	0		109 (89.34)	13 (10.66)	
		RF	Positive	317 (79.85)	76 (19.14)	4 (1.01)	0.625	710 (89.42)	84 (10.58)	0.974
			Negative	64 (79.01)	17 (20.99)	0		145 (89.51)	17 (10.49)	
rs729749	C/T	Anti-CCP	Positive	150 (36.95)	187 (46.06)	69 (17.00)	0.978	487 (59.98)	325 (40.02)	0.840

Table 2: Continued.

SNP	Allele (M/m)	Clinical features	Group	Genotypes *n* (%) MM	Mm	mm	*P* value	Alleles *n* (%) M	m	*P* value
			Negative	22 (36.07)	28 (45.90)	11 (18.03)		72 (59.02)	50 (40.98)	
		RF	Positive	148 (37.28)	184 (46.35)	65 (16.37)	0.513	480 (60.45)	314 (39.55)	0.247
			Negative	26 (32.10)	38 (46.91)	17 (20.99)		90 (55.56)	72 (44.44)	
Male										
		Anti-CCP	Positive	26 (32.10)	37 (45.68)	18 (22.22)	0.711	89 (54.94)	73 (45.06)	0.570
rs10911363	T/G		Negative	6 (42.86)	5 (35.71)	3 (21.43)		17 (60.71)	11 (39.29)	
		RF	Positive	28 (34.15)	36 (43.90)	18 (21.95)	0.948	92 (56.10)	72 (43.90)	0.732
			Negative	6 (37.50)	7 (43.75)	3 (18.75)		19 (59.38)	13 (40.63)	
		Anti-CCP	Positive	63 (77.78)	17 (20.99)	1 (1.23)	0.360	143 (88.27)	19 (11.73)	0.367
rs3794624	G/A		Negative	10 (71.43)	3 (21.43)	1 (7.14)		23 (82.14)	5 (17.86)	
		RF	Positive	63 (76.83)	18 (21.95)	1 (1.22)	0.880	144 (87.80)	20 (12.20)	0.962
			Negative	12 (75.00)	4 (25.00)	0		28 (87.50)	4 (12.50)	
		Anti-CCP	Positive	69 (85.19)	12 (14.81)	0	0.441	150 (92.59)	12 (7.41)	0.458
rs4673	G/A		Negative	13 (92.86)	1 (7.14)	0		27 (96.43)	1 (3.57)	
		RF	Positive	68 (82.93)	14 (17.07)	0	0.074	150 (91.46)	14 (8.54)	0.086
			Negative	16 (100.00)	0	0		32 (100.00)	0	
		Anti-CCP	Positive	39 (48.15)	30 (37.04)	12 (14.81)	0.700	108 (66.67)	54 (33.33)	0.383
rs1883112	A/G		Negative	8 (57.14)	5 (35.71)	1 (7.14)		21 (75.00)	7 (25.00)	
		RF	Positive	37 (45.12)	30 (36.59)	15 (18.29)	0.152	104 (63.41)	60 (36.59)	0.051
			Negative	10 (62.50)	6 (37.50)	0		26 (81.25)	6 (18.75)	
		Anti-CCP	Positive	60 (74.07)	21 (25.93)	0	0.031	141 (87.04)	21 (12.96)	0.043
rs4821544	T/C		Negative	14 (100.00)	0	0		28 (100.00)	0	
		RF	Positive	61 (74.39)	21 (25.61)	0	0.090	143 (87.20)	21 (12.80)	0.113
			Negative	15 (93.75)	1 (6.25)	0		31 (96.88)	1 (3.13)	
		Anti-CCP	Positive	38 (46.91)	28 (34.57)	15 (18.52)	0.533	104 (64.20)	58 (35.80)	0.723
rs729749	C/T		Negative	7 (50.00)	3 (21.43)	4 (28.57)		17 (60.71)	11 (39.29)	
		RF	Positive	35 (42.68)	31 (37.80)	16 (19.51)	0.279	101 (61.59)	63 (38.41)	0.269
			Negative	10 (62.50)	3 (18.75)	3 (18.75)		23 (71.88)	9 (28.13)	

M: major alleles; m: minor alleles.

Table 3: Haplotype analysis of three SNPs in the *NCF4* gene in RA patients and controls (*n* (%)).

Haplotype	RA patients	Controls	*P* value	OR (95% CI)
rs1883112-rs4821544-rs729749				
ATC	525.82 (44.3)	502.44 (42.2)	0.282	1.093 (0.929, 1.286)
ATT	298.18 (25.1)	322.56 (27.1)	0.286	0.905 (0.754, 1.087)
GCC	97.99 (8.3)	118.49 (9.9)	0.155	0.816 (0.616, 1.080)
GCT	27.01 (2.3)	41.51 (3.5)	0.079	0.646 (0.395, 1.056)
GTC	88.19 (7.4)	65.06 (5.5)	0.050	1.391 (0.999, 1.937)
GTT	148.81 (12.5)	141.94 (11.9)	0.634	1.061 (0.830, 1.357)

Frequency < 0.03 in both controls and RA patients has been dropped.

rs1883112, rs4821544, and rs729749 in *NCF4*; and rs3794624 and rs4673 in *CYBA* and RA susceptibility in a Chinese population. Since RA was more frequent in females than males, the disease mechanisms might be sex-dependent; hence, we stratified all subjects by sex and analyzed the association between all SNPs and RA susceptibility in males and females, respectively.

As a key component of the multiprotein NOX system, NCF2 was also called p67phox and encoded by the *NCF2* gene. The function of NCF2 was considered to regulate the transfer of electrons from NADPH to flavin, and phagocyte ROS production [23]. NCF2 was recruited to the cell membrane for combination with other components to form the active NOX system through microbial stimuli [24].

Table 4: Haplotype analysis of two SNPs in the *CYBA* gene in RA patients and controls (n (%)).

Haplotype	RA patients	Controls	P value	OR (95% CI)
rs3794624-rs4673				
AG	179.43 (15.1)	173.64 (14.6)	0.667	1.051 (0.838, 1.318)
GA	78.43 (6.6)	96.64 (8.1)	0.172	0.806 (0.592, 1.099)
GG	919.57 (77.5)	918.36 (77.0)	0.625	1.049 (0.865, 1.274)

Frequency < 0.03 in both controls and RA patients has been dropped.

Moreover, mutations in the *NCF2* gene had been reported to affect the risk of a variety of diseases in previous studies [25]. Gateva et al. suggested that *NCF2* rs10911363 polymorphism was associated with SLE risk in the US and Sweden populations [26]. In another study, the *NCF2* rs789181 variant had been found to have a mild association with RA risk in men [16]. Here, we analyzed the possible relationship between rs10911363 polymorphism and RA susceptibility. However, the present result implied that rs10911363 might not be a contributing factor specific to RA susceptibility. Similarly, *NCF2* rs10911363 polymorphism exhibited no significant association with SLE risk in a Chinese population [20]. These interesting observations implied that *NCF2* gene rs10911363 polymorphism might not be involved in the pathogenesis of autoimmune diseases such as RA and SLE in a Chinese population. On the other hand, Yu et al. found that the rs10911363 G allele was positively correlated with several clinical characteristics and laboratory parameters in SLE patients and might influence the severity of this disease [20]. Therefore, the potential role of rs10911363 in RA development should be explored in future studies.

NCF4, known as a component of the NOX complex, could induce the NOX complex to phagosomal membranes through binding to phosphatidylinositol 3-phosphate (PtdIns3P) and had been proven to regulate the production of intracellular ROS [27, 28]. A recent animal experiment had explored the critical role of the NCF4-regulated intracellular ROS level in regulating chronic inflammation and autoimmunity, and the results found a mutation in the PtdIns3P-binding site of the regulatory NOX subunit NCF4/p40phox that could enhance autoimmune responses [29]. In another context, several genetic variants in the *NCF4* gene had been associated with autoimmune diseases including RA and Crohn's disease [16, 30, 31]. Roberts et al. revealed that *NCF4* rs4821544 polymorphism was significantly related to ileal Crohn's disease [30]. Similarly, our results demonstrated that rs4821544 CT genotype and C allele frequencies were significantly decreased in RA patients than controls. Olsson et al. found that rs729749 variant in *NCF4* was significantly associated with RA risk in men and observed a meaningful association for rs729749 in auto-antibody-negative disease, especially RF negative [16]. Consistent with this result, we found a significant association between rs729749 variation and RA risk in males, while our study also suggested that rs729749 polymorphism was significantly associated with RA susceptibility among the entire study population. The results from our study strengthened the hypothesis that the disease pathway affected by the rs729749 variation was specific to men. Moreover, our results also suggested a significant finding that the role of rs729749 variation in the pathogenesis of RA was closely related to race. In another study, the author found rs1883112 GG genotype frequency associated with a higher risk of diffuse large B-cell lymphoma [32]. However, there was no statistical association between rs1883112 variant and RA susceptibility in our study.

The present results suggested that TT genotype and T allele frequencies of rs4821544 were significantly lower in RA patients with anti-CCP-positive when compared to RA patients with anti-CCP-negative in male. This might help to develop a more appropriate therapeutic schedule for RA patients of different genders. However, it was worth noting that the sample of male RA patients with anti-CCP-negative in this study was small, and this result needed further verification. Our results found no association between rs729749 variants between RF and anti-CCP in RA patients. This was inconsistent with the results by Olsson et al. [16], possibly due to the different sample sizes and sources.

As a necessary subunit of NOX, p22phox was encoded by the *CYBA* gene and had an important role in regulating NOX activity. The other NOX subunits were expressed in thyroid and colon cells and paired with p22phox; therefore, p22phox was considered as an essential component in maintaining the function of NOX [33, 34]. A functional SNP (rs4673) had aroused great interest of many researchers, because it resulted in Tyr instead of His at residue 72 of p22phox, which has been suggested to significantly reduce basal and NAD(P)H-stimulated superoxide production [35]. Therefore, many studies had focused on the special role of *CYBA* gene variation in the development of multiple diseases. Lan et al. revealed that rs4673 polymorphism might lead to a high susceptibility of non-Hodgkin lymphoma [36]. Seibold et al. found some evidence for the association of *CYBA* rs3794624 polymorphism with postmenopausal breast cancer susceptibility [37]. Another meta-analysis showed a significant correlation between the rs4673 variant and T2DM susceptibility [38]. However, our data provide the first evidence that *CYBA* rs3794624 and rs4673 polymorphisms might not contribute to RA susceptibility in a Chinese population. Although rs3794624 and rs4673 polymorphisms showed no apparent link to RA risk, the accuracy of our results might be influenced by different genotyping, ethnicity, and environmental factors.

In conclusion, the present study demonstrated that *NCF4* rs4821544 and rs729749 polymorphisms were associated with RA susceptibility, and rs4821544 polymorphism was also related to anti-CCP in male RA patients. Furthermore, our findings strongly supported the viewpoint that the NOX system played an important role in the pathogenesis

of RA. What is worth mentioning is that several limitations existed in the present study. Firstly, our study subjects might be insufficient, which resulted in the low power of this study. Secondly, this study did not detect the protein expression levels of these genes, and we were unable to further analyze the association between gene polymorphism and protein expression levels. Thirdly, special functional verification of relevant SNPs was missing; thus, the mechanism behind the results of genetic association was still unclear. The functional, replication studies with larger sample size, disparate races, and gene-environmental and gene-gene interaction are required.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Ethical Approval

This study was approved by the Ethical Committee of Anhui Medical University.

Consent

All the study subjects provided informed consent to participate in this study.

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Authors' Contributions

Tian-Ping Zhang and Rui Li contributed equally to this work and should be considered co-first authors.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81871271) and the Anhui Key Research and Development Program (1804b06020354).

References

[1] M. Rossini, E. Rossi, D. Bernardi et al., "Prevalence and incidence of rheumatoid arthritis in Italy," *Rheumatology International*, vol. 34, no. 5, pp. 659–664, 2014.

[2] I. B. McInnes and G. Schett, "The pathogenesis of rheumatoid arthritis," *The New England Journal of Medicine*, vol. 365, no. 23, pp. 2205–2219, 2011.

[3] H. F. Pan, R. X. Leng, G. C. Wu, and D. Q. Ye, "Advance in epidemiologic studies on major autoimmune diseases," *Chinese Journal of Disease Control and Prevention*, vol. 22, pp. 1093–1095, 2018.

[4] C. M. Weyand and J. J. Goronzy, "Association of MHC and rheumatoid arthritis: HLA polymorphisms in phenotypic variants of rheumatoid arthritis," *Arthritis Research*, vol. 2, no. 3, pp. 212–216, 2000.

[5] A. B. Begovich, V. E. H. Carlton, L. A. Honigberg et al., "A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis," *American Journal of Human Genetics*, vol. 75, no. 2, pp. 330–337, 2004.

[6] M. J. Smallwood, A. Nissim, A. R. Knight, M. Whiteman, R. Haigh, and P. G. Winyard, "Oxidative stress in autoimmune rheumatic diseases," *Free Radical Biology & Medicine*, vol. 125, pp. 3–14, 2018.

[7] S. G. Sukkar and E. Rossi, "Oxidative stress and nutritional prevention in autoimmune rheumatic diseases," *Autoimmunity Reviews*, vol. 3, no. 3, pp. 199–206, 2004.

[8] R. Holmdahl, O. Sareila, L. M. Olsson, L. Bäckdahl, and K. Wing, "Ncf1 polymorphism reveals oxidative regulation of autoimmune chronic inflammation," *Immunological Reviews*, vol. 269, no. 1, pp. 228–247, 2016.

[9] W. M. Nauseef, "Assembly of the phagocyte NADPH oxidase," *Histochemistry and Cell Biology*, vol. 122, no. 4, pp. 277–291, 2004.

[10] Y. Groemping and K. Rittinger, "Activation and assembly of the NADPH oxidase: a structural perspective," *The Biochemical Journal*, vol. 386, no. 3, pp. 401–416, 2005.

[11] H. Matsue, D. Edelbaum, D. Shalhevet et al., "Generation and function of reactive oxygen species in dendritic cells during antigen presentation," *Journal of Immunology*, vol. 171, no. 6, pp. 3010–3018, 2003.

[12] L. M. Olsson, Å. C. Johansson, B. Gullstrand, A. Jönsen, S. Saevarsdottir, and L. Rönnblom, "A single nucleotide polymorphism in theNCF1gene leading to reduced oxidative burst is associated with systemic lupus erythematosus," *Annals of the Rheumatic Diseases*, vol. 76, no. 9, pp. 1607–1613, 2017.

[13] J. Zhao, J. Ma, Y. Deng et al., "A missense variant in NCF1 is associated with susceptibility to multiple autoimmune diseases," *Nature Genetics*, vol. 49, no. 3, pp. 433–437, 2017.

[14] L. M. Olsson, A. Nerstedt, A. K. Lindqvist et al., "Copy number variation of the gene NCF1 is associated with rheumatoid arthritis," *Antioxidants & Redox Signaling*, vol. 16, no. 1, pp. 71–78, 2012.

[15] C. O. Jacob, M. Eisenstein, M. C. Dinauer et al., "Lupus-associated causal mutation in neutrophil cytosolic factor 2 (NCF2) brings unique insights to the structure and function of NADPH oxidase," *Proceedings of the National Academy of Sciences*, vol. 109, pp. E59–E67, 2012.

[16] L. M. Olsson, A. K. Lindqvist, H. Källberg et al., "A case-control study of rheumatoid arthritis identifies an associated single nucleotide polymorphism in the NCF4 gene, supporting a role for the NADPH-oxidase complex in autoimmunity," *Arthritis Research & Therapy*, vol. 9, no. 5, p. R98, 2007.

[17] E. N. Borisov, T. N. Krasnova, L. M. Samohodskaia, L. V. Ivanitskiĭ, N. V. Nikiforova, and N. A. Mukhin, "Prognostic value of allelic variants affecting the hemostatic system in the development of antiphospholipid syndrome and kidney lesion in patients with systemic lupus erythematosus," *Terapevticheskiĭ Arkhiv*, vol. 86, no. 6, pp. 57–62, 2014.

[18] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[19] X. Kim-Howard, C. Sun, J. E. Molineros et al., "Allelic heterogeneity in NCF2 associated with systemic lupus erythematosus (SLE) susceptibility across four ethnic populations," *Human Molecular Genetics*, vol. 23, no. 6, pp. 1656–1668, 2014.

[20] B. Yu, Y. Chen, Q. Wu et al., "The association between single-nucleotide polymorphisms of NCF2 and systemic lupus erythematosus in Chinese mainland population," *Clinical Rheumatology*, vol. 30, no. 4, pp. 521–527, 2011.

[21] Z. Li, Z. Zhang, Z. He et al., "A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis," *Cell Research*, vol. 19, no. 4, pp. 519–523, 2009, http://analysis.bio-x.cn.

[22] S. M. Vieira, M. B. Monteiro, T. Marques et al., "Association of genetic variants in the promoter region of genes encoding p22phox (CYBA) and glutamate cysteine ligase catalytic subunit (GCLC) and renal disease in patients with type 1 diabetes mellitus," *BMC Medical Genetics*, vol. 12, no. 1, p. 129, 2011.

[23] Y. Nisimoto, S. Motalebi, C. H. Han, and J. D. Lambeth, "The p67(phox) activation domain regulates electron flow from NADPH to flavin in flavocytochrome b(558)," *The Journal of Biological Chemistry*, vol. 274, no. 33, pp. 22999–23005, 1999.

[24] J. El-Benna, P. M. Dang, M. A. Gougerot-Pocidalo, and C. Elbim, "Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses," *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, vol. 53, pp. 199–206, 2005.

[25] L. Jiao, J. Song, L. Ding et al., "A novel genetic variation inNCF2, the core component of NADPH oxidase, contributes to the susceptibility of tuberculosis in western Chinese Han population," *DNA and Cell Biology*, vol. 39, no. 1, pp. 57–62, 2020.

[26] V. Gateva, J. K. Sandling, G. Hom et al., "A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus," *Nature Genetics*, vol. 41, no. 11, pp. 1228–1233, 2009.

[27] K. E. Anderson, T. A. M. Chessa, K. Davidson et al., "PtdIns3P and Rac direct the assembly of the NADPH oxidase on a novel, pre-phagosomal compartment during FcR-mediated phagocytosis in primary mouse neutrophils," *Blood*, vol. 116, no. 23, pp. 4978–4989, 2010.

[28] C. Ellson, K. Davidson, K. Anderson, L. R. Stephens, and P. T. Hawkins, "PtdIns3P binding to the PX domain of p40phox is a physiological signal in NADPH oxidase activation," *The EMBO Journal*, vol. 25, no. 19, pp. 4468–4478, 2006.

[29] S. Winter, M. Hultqvist Hopkins, F. Laulund, and R. Holmdahl, "A reduction in intracellular reactive oxygen species due to a mutation in NCF4 promotes autoimmune arthritis in mice," *Antioxidants & Redox Signaling*, vol. 25, no. 18, pp. 983–996, 2016.

[30] R. L. Roberts, J. E. Hollis-Moffatt, R. B. Gearry, M. A. Kennedy, M. L. Barclay, and T. R. Merriman, "Confirmation of association of IRGM and NCF4 with ileal Crohn's disease in a population-based cohort," *Genes and Immunity*, vol. 9, no. 6, pp. 561–565, 2008.

[31] Y. Mazor, I. Maza, E. Kaufman et al., "Prediction of disease complication occurrence in Crohn's disease using phenotype and genotype parameters at diagnosis," *Journal of Crohn's & Colitis*, vol. 5, no. 6, pp. 592–597, 2011.

[32] D. Liu, N. Wu, H. Sun et al., "ABCG2andNCF4polymorphisms are associated with clinical outcomes in diffuse large B-cell lymphoma patients treated with R-CHOP," *Oncotarget*, vol. 8, no. 35, pp. 58292–58303, 2017.

[33] H. Kikuchi, M. Hikage, H. Miyashita, and M. Fukumoto, "NADPH oxidase subunit, gp91(phox) homologue, preferentially expressed in human colon epithelial cells," *Gene*, vol. 254, no. 1-2, pp. 237–243, 2000.

[34] H. Sumimoto, "Structure, regulation and evolution of Nox—-family NADPH oxidases that produce reactive oxygen species," *The FEBS Journal*, vol. 275, no. 13, pp. 3249–3277, 2008.

[35] T. J. Guzik, N. E. West, E. Black et al., "Functional effect of the C242T polymorphism in the NAD(P)H oxidase p22phox gene on vascular superoxide production in atherosclerosis," *Circulation*, vol. 102, no. 15, pp. 1744–1747, 2000.

[36] Q. Lan, T. Zheng, M. Shen et al., "Genetic polymorphisms in the oxidative stress pathway and susceptibility to non-Hodgkin lymphoma," *Human Genetics*, vol. 121, no. 2, pp. 161–168, 2007.

[37] P. Seibold, P. Hall, N. Schoof et al., "Polymorphisms in oxidative stress-related genes and mortality in breast cancer patients – Potential differential effects by radiotherapy?," *International Journal of Cancer*, vol. 22, no. 5, pp. 817–823, 2013.

[38] Q. Sun, Y. Yin, Z. Zhu, and Z. Yan, "Association of the C242T polymorphism in the NAD(P)H oxidase P22 phox gene with type 2 diabetes mellitus risk: a meta-analysis," *Meta Gene*, vol. 30, no. 3, pp. 415–422, 2014.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 2847316, 7 pages
https://doi.org/10.1155/2020/2847316

Research Article

The Symptoms and Medications of Patients with Inflammatory Bowel Disease in Hubei Province after COVID-19 Epidemic

Huan Wang,[1] Lei Tu,[1] Ying Li,[1] Tao Bai,[1] Kaifang Zou,[1] Fang Xiao,[2] Jin Li,[3] Min Chen,[3] Heng Zhang,[4] Gangqin Li,[5] Yueyue Lu,[6] Kai Wang,[7] Shu Jin,[8] Yuanping Yang,[9] Liangru Zhu,[1] and Xiaohua Hou[1]

[1]*Division of Gastroenterology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China*
[2]*Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China*
[3]*Department of Gastroenterology, Zhongnan Hospital, Wuhan University, Wuhan, Hubei Province, China*
[4]*Department of Gastroenterology, The Central Hospital of Wuhan, Wuhan, Hubei Province, China*
[5]*Department of Gastroenterology, The Eighth Hospital of Wuhan, Wuhan, Hubei Province, China*
[6]*Department of Gastroenterology, The First Affiliated Hospital of Changjiang University, Jingzhou, Hubei Province, China*
[7]*Department of Gastroenterology, Jingzhou Hospital, Tongji Medical College, Huazhong University of Science and Technology, Jingzhou, Hubei Province, China*
[8]*Department of Gastroenterology, Taihe Hospital, Huei University of medicine, Shiyan, Hubei Province, China*
[9]*Department of Gastroenterology, Yichang Central Hospital, Yichang, Hubei, China*

Correspondence should be addressed to Liangru Zhu; zhuliangru@hust.edu.cn and Xiaohua Hou; houxh@medmail.com.cn

Received 8 June 2020; Accepted 13 September 2020; Published 9 October 2020

Academic Editor: Yan Yang

Objectives. The COVID-19 epidemic triggered by coronavirus SARS-CoV-2 is rapidly spreading around the globe. This study is aimed at finding out the suspected or confirmed SARS-CoV-2 infection in patients with inflammatory bowel disease (IBD) in Hubei province, China. We also investigated symptoms, medications, life quality, and psychological issues of IBD patients under the ongoing pandemic. *Methods*. We conducted a self-reported questionnaire survey via an online survey platform. SARS-CoV-2 infection-related data was collected from IBD patients. The status quo of medications and symptoms of the subjects were investigated. Life quality, depression, and anxiety were measured by clinical questionnaires and rated on scoring systems. *Results*. A total of 204 IBD patients from Hubei province were included in this study. No suspected or confirmed SARS-CoV-2 infection case was found in this study. As a result of city shutdown, two-thirds of the patients (138/204) in our series reported difficulty in accessing medicines and nearly half of them (73/138) had to discontinue medications. Apart from gastrointestinal symptoms, systemic symptoms were common while respiratory symptoms were rare in the cohort. Though their quality of life was not significantly lowered, depression and anxiety were problems that seriously affected them during the COVID-19 epidemic. *Conclusions*. Inaccessibility to medications is a serious problem for IBD patients after city shutdown. Efforts have to be made to address the problems of drug withdrawal and psychological issues that IBD patients suffer from during the COVID-19 outbreak.

1. Introduction

Coronavirus disease 2019 (COVID-19), triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), hit Hubei province at the end of 2019 and has turned into a global pandemic [1]. The cases of COVID-19 have been soaring around the globe over the past months. As of June 7th, 2020, 6 896 800 cases have been reportedly confirmed and 397 588 have died in over 200 countries [2].

Both experimental and clinical data showed that people of all ages can fall victim to SARS-CoV-2, especially elderly adults and those with underlying diseases. Inflammatory bowel disease (IBD) is an idiopathic inflammatory condition involving gastrointestinal tract. The use of glucocorticoids, immunosuppressants, and other immunity-weakening medications in IBD patients may increase the risk of opportunistic infections [3]. Surveillance Epidemiology of Coronavirus Under Research Exclusion (SECURE-IBD) reported that 1379 IBD patients were complicated with COVID-19 as of June 7th, mainly from Europe and America but only one case in China [4]. In this study, we conducted a questionnaire survey with an attempt to obtain accurate data concerning the *status quo* of SARS-CoV-2 infection in IBD patients in Hubei.

Naturally, IBD patients alternately go through periods of remission and relapses that entail regular medication and follow-up. However, in areas hit hard by COVID-19, lockdown and travel restrictions are introduced. As a consequence, IBD patients may not be followed up as scheduled and may have limited or no access to medicines they need. The disease may relapse or deteriorate if patients are cut off from their medications. We believe that the data regarding of the current situation of IBD patients is of great value to doctors responsible for the management of IBD patients under the epidemic of COVID-19.

The mounting confirmed cases of COVID-19 infections and deaths, as reported by various media, are posing tremendous psychological pressure on IBD patients. In fact, IBD patients were reported to be associated with mental stress and anxiety [5]. Psychological stress has been identified as a worsening factor for IBD. Adverse life events and chronically perceived stress may contribute to elevated anxiety, aggravation of inflammation, and disease relapse in IBD patients [6]. Therefore, understanding the mental status of IBD patients during COVID-19 epidemic can help physicians take measures to reduce the impact of negative emotions on the patients.

This study comprehensively investigated the current status of IBD patients during the epidemic of COVID-19, including symptoms that may or may not be related to COVID-19, medications, mental health state, and quality of life, with an attempt to help doctors understand the physical and mental conditions of IBD patients and better manage IBD patients under the ongoing pandemic.

2. Methods

2.1. Study Participants. This study included 204 IBD patients admitted to level-A and class one hospitals in Hubei province, China. IBD was diagnosed on the basis of conventional criteria of the disease. All patients were electronically invited to anonymously complete an online questionnaire from February 7th to March 15th, 2020, during the COVID-19 epidemic.

2.2. Study Design. The clinical study was of observational and cross-sectional nature. A self-reported questionnaire survey was conducted via an online survey platform ("SurveyStar," Changsha Ranxing Science and Technology, Shanghai, China). Demographic and social data were collected from IBD patients. Quality of life depression and anxiety were rated against validated clinical questionnaires and scoring systems. This study was approved by the Medical Ethical Review Committee, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China ([2020] No. 0033).

2.3. Questionnaires. Sociodemographic information of IBD patients mainly included age, gender, body weight, education background, marital status, education, occupations, place of residence during the COVID-19 epidemic, and smoking status (smoker and nonsmoker/past smoker).

IBD-related data included subtypes, course, and activity of the condition. The data on treatments of the patients received before the COVID-19 epidemic were also harvested. Digestive symptoms, such as abdominal pain, diarrhea, bloody stools, perianal discomfort, and vomiting were also involved. Due to the home quarantine imposed in most regions, the subjects did not have full access to medicines. Data on drug withdrawal and symptom changes were also collected.

In order to identify the cases suspected of SARS-CoV-2 infection, we used a recent guideline of diagnosis and treatment of COVID-19 (version7) [7], released by the National Health Commission in China. A suspected COVID-19 patient was defined as someone fulfilling both of the following criteria: (1) having at least one of the following symptoms or signs, including fever, respiratory symptoms, such as cough, sore throat, or dyspnea or radiographic evidence of pneumonia, and reduced counts of white blood cell or lymphocytes and (2) recently having lived in or paid visit to or gone to cities where community transmission of SARS-CoV-2 virus was reported, including Wuhan city, or recently having close contact with a confirmed COVID-19 patient or patients with fever/respiratory symptoms from Wuhan within 14 days before illness onset.

A confirmed case was defined as a patient testing positive for SARS-CoV-2 by PCR detection of upper respiratory specimen or for IgM and IgG in the serum. We harvested data on contact history, clinical symptoms, and examination results in the past two weeks. In particular, respiratory symptoms mainly included stuffy nose, nasal discharge, cough, sputum production, and chest pain. Systemic symptoms involved fever, fatigue, debility, muscle pain, headache, and palpitations.

Inflammatory Bowel Disease Questionnaire (IBDQ) is a 32-item, disease-specific health-related quality of life (HRQoL) questionnaire [8]. It consists of 4 domains, i.e., bowel symptoms, systemic symptoms, emotional function, and social function. Responses to each item are scored on a 7-point scale, in which 1 was listed as worst and 7 the best. The total IBDQ points range from 32 to 224, with a higher score indicative of better quality of life [9].

Depression was assessed against the Patient Health Questionnaire (PHQ-9). The PHQ-9 contains nine items that assess, from 9 aspects, if a major depression is present [10]. The total score ranges from 0 to 27 points. It rates depression on four different levels: mild, moderate, moderately severe, and severe, on the basis of cut-off scores of 5,

10, 15, and 20, respectively [11]. 10 was the cut-off score for depression status.

Anxiety was measured by the Generalized Anxiety Disorder Assessment (GAD-7) [12]. GAD-7 includes 7 items, and each is scored in terms of frequency on a scale from 0 to 3. The total scores range from 0 to 21. It categorizes anxiety into three degrees: mild, moderate, and severe, on the basis of cut-off scores of 5, 10, and 15.

2.4. Statistical Analysis. Data was presented as the mean ± standard deviation (SD). Differences between groups were evaluated by using t-test or analysis of variance (ANOVA). Pearson's correlation analysis and multivariate analysis were employed to determine the structural relationship between the measured variables. Statistical analysis was performed using SPSS statistical package (version 17).

3. Results

3.1. Subjects. A total of 204 IBD patients were found in Hubei province, and they were aged 35.14 ± 11.50 years, with a BMI of 22.57 ± 8.01 kg/m^2 on average. There were 123 men and 81 women. 41 patients were from Wuhan, and 163 from other cities of Hubei province (Table 1).

3.2. Status Quo of IBD Patients. In our series, 116 patients had Crohn's disease (CD); 85 had ulcerative colitis (UC); and 3 had undefined IBD (IBDU). 43.14% (88/204) of the IBD patients remained in remission while 26.47% (54/204) of them were in active stage. The other 62 patients were uncertain of their disease activity. The main drugs the IBD patients took were aminosalicylic acid in 48.53% (99/204), glucocorticoid in 9.31% (19/204), immunosuppressants in 22.55% (46/204), biological agents in 31.37% (64/204), and enteral nutrition drugs in 11.76% (24/204).

3.3. Inaccessibility to Medications as a Serious Problem for IBD Patients. More than two-thirds of patients (138/204) reported difficulty in accessing medicines as a consequence of city shutdown during the COVID-19 epidemic. Drug withdrawal occurred in half of them (70/138). Biological agents, a major medication used by CD patients, topped the list as a drug hard to procure. Half of the patients (32/60) discontinued the biological agents they had been on. Came next are immunosuppressants and 5-aminosalicylic acid (Figure 1(a)). In UC patients, 23.26% (20/86) of patients had to stop taking 5-aminosalicylic acid (Figure 1(b)).

54.29% (38/70) of patients who discontinued medication reported recurrence and exacerbation of symptoms, such as abdominal pain (71.05%), diarrhea (50%), bloody stools (31.58%), perianal discomfort (21.05%), and vomiting (10.53%)

3.4. Gastrointestinal, Respiratory, and Systemic Symptoms in IBD Patients. More than half of patients (106/204) with IBD had gastrointestinal symptoms in the last two weeks during the COVID-19 epidemic. Abdominal pain (24.51%) and diarrhea (25.00%) remained predominant symptoms, followed by perianal discomfort (16.18%) and bloody stools (15.69%). Nausea and vomiting (3.43%) were extremely rare.

Table 1: Characteristics of the patients with IBD responding to the questionnaire.

Numbers of patients, n (%)	204 (100%)
Gender, n (%)	
Male	123 (60.29%)
Female	81 (29.71%)
Age (mean ± SD)	35.14 ± 11.50
BMI (mean ± SD), kg/m^2	22.57 ± 8.01
Time since diagnosis (mean ± SD)	3.93 ± 3.40
Habitat, n (%)	
Wuhan	41 (20.10%)
Outside of Wuhan in Hubei province	163 (79.90%)
Smoking, n (%)	
Nonsmoker	163 (79.90%)
Current smoker	12 (5.88%)
Past smoker	29 (14.22%)
Marital status, n (%)	
Unmarried	70 (34.31%)
Married	130 (63.73%)
Divorced	4 (1.96%)
Education level, n (%)	
Primary education	109 (53.43%)
University educated	95 (46.57%)
Occupation, n (%)	
Mental worker	62 (30.39%)
Manual worker	30 (14.71%)
Mental-physical worker	48 (23.53%)
Unoccupied	64 (31.37%)
Diagnosis, n (%)	
Ulcerative colitis	85 (41.67%)
Crohn's disease	116 (56.86%)
Inflammatory bowel disease unclassified	3 (1.47%)
Disease activity, n (%)	
Active stage	54 (26.47%)
Inactive stage	150 (73.53%)

A few IBD patients (8.82%) reported respiratory symptoms in the last two weeks, including stuffy nose (4.90%), nasal discharge (3.92%), cough (1.96%), sputum production (1.47%), and chest pain (0.98%).

Constitutional or systemic symptoms were very common in patients with IBD, which could easily be confused with SARS-CoV-2 infection. 26.24% of the patients had general symptoms in the last two weeks, such as listlessness (16.29%), fatigue (14.03%), muscle pain (6.79%), headache (4.52%), and palpitations (2.71%). Nine patients developed fever, which was mostly mild or moderate (Table 2).

We used diagnostic criteria of COVID-19, as a reference, in combination with epidemiological data and clinical manifestations. No suspected SARS-CoV-2 infection case was found in our cohort. In this study, four patients had a definite contact history with the confirmed cases of COVID-19. One

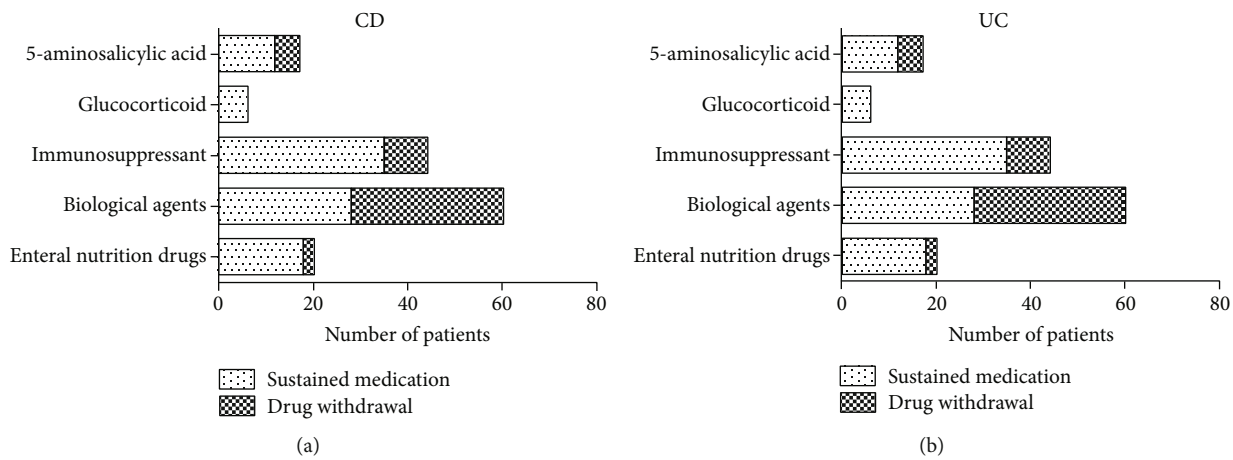


Figure 1: Continuation and discontinuation of medications in IBD patients during the COVID-19 epidemic. (a) The number of CD patients who continued and discontinued their medications (5-aminosalicylic acid, glucocorticoid, immunosuppressants, biological agents, and enteral nutrition drugs). (b) The number of UC patients who continued and discontinued their medications.

Table 2: Gastrointestinal, respiratory, and systemic symptoms in IBD patients.

	CD	UC	IBDU	Total	%
Gastrointestinal symptoms					
Abdominal pain	28	22	0	50	24.51%
Diarrhea	29	22	0	51	25.00%
Nausea and vomiting	3	4	0	7	3.43%
Bloody stools	4	28	0	32	15.69%
Perianal discomfort	16	17	0	33	16.18%
Total	54	52	0	106	51.96%
Systemic symptoms					
Fever	6	4	0	10	4.90%
Fatigue	18	12	0	30	14.71%
Listlessness	19	16	0	35	17.16%
Headache	4	5	0	9	4.41%
Muscle pain	8	7	0	15	7.35%
Palpitations	2	4	0	6	2.94%
Total	30	26	0	56	27.45%
Respiratory symptoms					
Stuffy nose	5	4	1	10	8.82%
Nasal discharge	5	2	1	8	4.90%
Cough	2	2	0	4	3.92%
Sputum production	2	1	0	3	1.96%
Chest pain	0	1	1	2	1.47%
Total	11	5	2	18	8.82%

The numbers of patients with gastrointestinal, respiratory, or systemic symptoms were showed in different subtypes of IBD. Total numbers and percentage were displayed in the last two columns.

of them had mild fever, and the highest body temperature was lower than 38°C, presenting no respiratory symptoms. Two other patients had cough and sputum production. The last one only had listlessness, headache, and diarrhea, but without fever or respiratory symptoms. However, the four patients did not seek medical attention or receive any SARS-CoV-2-related tests or examinations. Besides, there were nine patients with mild or moderate fever and most of them were free of respiratory symptoms.

3.5. HRQoL Scores in IBD Patients during COVID-19 Epidemic. In order to find out the change in quality of life in IBD patients during the COVID-19 epidemic, we collected IBDQ score data that had been collected before the outbreak of COVID-19, from Jan 1st to Oct 31st, 2019. In CD patients, the total score after COVID-19 outbreak (171.16 ± 33.80, $n = 113$) was not significantly different from that before the epidemic (163.73 ± 30.77, $n = 73$). Moreover, the IBDQ scores in UC patients were similar to those in CD patients. The total score after COVID-19 outbreak (166.07 ± 37.87, $n = 85$) was not significantly different from that before the epidemic (153.36 ± 36.78, $n = 33$).

3.6. PHQ-9 and GAD-7 Scores of IBD Patients during COVID-19 Outbreak. Depression could be assessed for 201 patients and anxiety for 203 patients, and missing data is due to lack of or incomplete questionnaires. In our series, 21.39% (43/201) patients scored over 10 points and were considered to have depression. Furthermore, two patients suffered from severe depression. 22.39% (45/201) of them reported borderline depression symptoms (Table 3). 30.54% (62/203) of the IBD patients were found to have anxiety of various degrees. 21.57% (44/203) of the patients were mildly anxious. A few patients (7.35%) developed moderate anxiety. Three patients were considered to suffer from severe anxiety. The level of depression and anxiety between CD and UC seemed similar (Table 3).

3.7. Factors Associated with the Quality of Life, Depression, and Anxiety. The quality of life was found to be associated with the disease activity, travel restraint, or restriction and drug withdrawal during COVID-19 epidemic, but not with gender, marital status, smoking, occupations, and subtypes of IBD.

Table 3: Psychological status in IBD patients during the COVID-19 epidemic.

	IBD		CD		UC	
	n	%	*n*	%	*n*	%
Level of depression						
No depression	113	56.22%	67	57.76%	45	52.94%
Mild depression	45	22.39%	23	19.83%	21	24.71%
Moderate depression	27	13.43%	16	13.79%	11	12.94%
Moderately severe depression	14	6.97%	9	7.76%	5	5.89%
Moderately severe depression	2	1.00%	0	0.00%	2	2.35%
Level of anxiety						
No anxiety	141	69.12%	84	72.41%	54	63.53%
Mild anxiety	44	21.57%	24	20.69%	20	23.53%
Moderate anxiety	15	7.35%	6	5.17%	9	10.59%
Severe anxiety	3	1.47%	2	1.72%	1	1.18%

The numbers and percentage of different levels of depression and anxiety were displayed in IBD patients.

The level of depression and anxiety bore a correlation with education, disease activity, and travel restriction. Besides, mental workers (white-collar workers) were more anxious than their manual and mental-physical counterparts (Table 4).

4. Discussion

In this study, all of IBD patients were from Hubei province, the so-called "epicenter" of the COVID-19 outbreak. As a result of city shutdown, two-thirds of the patients in our series reported difficulty in accessing medications and drug discontinuation became a serious problem in patients with IBD. Apart from gastrointestinal symptoms, systemic symptoms were common in IBD patients while respiratory symptoms were rare. However, no suspected or confirmed SARS-CoV-2 infection cases were found in our series. Though the quality of life was not significantly decreased, depression and anxiety were serious problems that plagued the patients during the COVID-19 epidemic.

IBD is considered to be a group of immune disorders characterized by chronic gut inflammation [13, 14]. Since IBD patients were usually on biologics and immunosuppressive agents, they appeared to be vulnerable to serious and/or opportunistic infections [3] by such viruses as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and herpes simplex virus (HSV) [15]. However, no increased risk of developing SARS-CoV-2 infection in IBD patients had been previously reported by other researchers [16]. In this study, no suspected SARS-CoV-2 infection case was found since the outbreak of the COVID-19 epidemic in Hubei province. Moreover, no IBD patient with SARS-CoV-2 infection has been reported from the tertiary IBD centers in Wuhan. Until now, only one patient was reportedly infected with SARS-CoV-2 in China [4]. However, in the United States, Spain, France, and Italy, in all there were more than nine hundred IBD patients suffering SARS-CoV-2 infection [4]. Many factors might be attributed to the discrepancy in the incidence rate of SARS-CoV-2 infection in IBD patients at home and abroad. Firstly, the incidence of IBD in China was considerably lower than in European and American. Second, the sample might be relatively small in our study. More importantly, several guidelines for managing IBD were released by the Chinese IBD Society in early February 2020 [17] and they covered home quarantine, dieting, personal protection, use of immunosuppressive agents, biologics, and cytokine blockers under IBD doctor's suggestions to prevent COVID-19 [16]. After completion of this survey, one UC patient coming to our hospital was found to be positive for IgG to while negative for IgM to SARS-CoV-2 before IFX injection; although SARS-CoV-2 RNA test showed negative, the positive for IgG demonstrated that he was previously infected with the virus. This patient had a fever of 38°C a month and half ago, but without respiratory symptoms. As a result, he did not seek medical attention or receive any SARS-Cov-2-related tests. Without special treatment, his symptoms subsided spontaneously after the home quarantine.

Gastrointestinal (GI) symptoms had been previously reported in a significant portion of COVID-19 patients, including diarrhea, nausea, vomiting, and abdominal pain [18–20]. Moreover, diarrhea was reportedly the most common GI symptom [21]. GI symptoms could even be the initial clinical manifestation of COVID-19. A study reported that 23.3% of mild COVID-19 patients did not present with respiratory symptoms but had only GI symptoms [22]. In our study, more than half of the IBD patients had GI symptoms in the last two weeks during the COVID-19 epidemic, especially abdominal pain and diarrhea. On the other hand, systemic symptoms, even fever, were very common in patients with IBD in our series. A few of them also developed respiratory symptoms that might be confused with COIVID-19. Therefore, IBD patients, particularly those in Hubei province, were extremely worried about SARS-CoV-2 infection and tended to seek medical attention, which might substantially increase their risk of infection. Given this, since Jan 29, 2020, China Crohn's & Colitis Foundation enlisted a great many IBD specialists, as volunteers, to offer online consultancy to IBD patients. Moreover, some IBD centers set up online IBD clinics. All these measures greatly avoided unnecessary visits to hospital or clinic and thereby minimized the risk of SARS-CoV-2 infection in IBD patients during the epidemic of COVID-19.

Duration of treatments is crucial to the management of IBD patients. In our study, two-thirds of the patients reported difficulty in accessing medicines and half of them discontinued their medications. A significant portion of patients reported recurrence and exacerbation of symptoms after drug withdrawal. Two IBD patients admitted to our hospital developed severe complications, including intestinal obstruction and perforation after drug withdrawal. It is extremely difficult for doctors to manage IBD patients during the city shutdown and under travel ban. We advise that all patients should continue their medications and avoid drug withdrawal whenever possible. Mesalamine and biologic agent treatments are important, and dose reduction is risky

Table 4: The multiple-factor analysis of the quality of life, depression, and anxiety.

	IBDQ		Depression		Anxiety	
	AR^2	p	AR^2	p	AR^2	p
Gender	-0.001	0.408	-0.004	0.852	-0.005	0.983
BMI	0.01	0.071	0.02	0.022*	0.015	0.913
Smoking	-0.003	0.557	-0.005	0.956	-0.001	0.365
Marital status	0	0.325	0.006	0.125	-0.004	0.898
Education	0.025	0.011*	0.011	0.065	0.033	0.004**
Occupation	-0.001	0.38	0.014	0.044*	0.031	0.005**
Subtypes of IBD	0	0.312	-0.004	0.7	0.001	0.281
Disease activity	0.008	<0.001*	0.058	<0.001**	0.062	<0.001**
Residence area	-0.005	0.988	-0.003	0.589	0.006	0.133
Traffic restraint	0.044	0.001**	0.028	0.008**	0.015	0.038*
Drug withdrawal	0.032	0.016*	0.012	0.097	0	0.322

*$p < 0.05$; **$p < 0.01$.

[23]. If intravenous infusion is not feasible, patients could use adalimumab as an alternative for infliximab since adalimumab allows for subcutaneous injection. However, any change in medication must be made in strict accordance with the directions of IBD specialists. New assessments, including self-reported questionnaire, online consultancy, and treatment are recommended [24]. Travel restrictions and city shutdown have forced IBD units and IBD patients to drastically change and restructure the way they handle IBD.

Our study showed that the quality of life in IBD patients was not significantly impacted after the outbreak of COVID-19. This might largely be ascribed to the relatively small sample size of this study. The strengths of the study are its relatively large sample size, and that it was conducted in the early phases of the outbreak. Moreover, the network questionnaire approach we used might produce, to some extent, information bias. Cases of IBD patients with less education or older age are less likely to participate in the network research. Therefore, cohort studies with larger samples are warranted to further investigate the change in the quality of life in IBD patients.

We found that the COVID-19 epidemic heavily inflicted depression and anxiety on the IBD patients in our series. The findings showed that 22.39% of the patients were considered to be depressed, and 30.54% of the patients experienced anxiety of varying degrees. A previous survey showed the prevalence of anxiety (21.2%) and depression (25.8%) in IBD patients by the same questionnaire of PHQ-9 and GAD-7 [25]. The prevalence of anxiety and depression during the COVID-19 epidemic in our study appeared higher than the previous, but the variation in the rates of depression and anxiety was likely due to differences in populations studied, methods used to evaluate depression and anxiety, and study designs. Nevertheless, the high prevalence of mental disorders found in our study is an important problem that requires further attention. IBD units and IBD specialists should pay more attention to the psychological issues of IBD patients during the COVID-19 epidemic. Furthermore, patient education, psychological counseling, and appropriate mental health support should be given. Antidepressant and antianxiety drugs could be applied to the patients with severe mental disorders whenever appropriate.

5. Conclusions

Inaccessibility to medications and status of depression and anxiety are serious problems in IBD patients after COVID-19 epidemic. This study helps doctors understand the physical and mental conditions of IBD patients and better manage IBD patients under the ongoing pandemic.

Data Availability

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

Conflicts of Interest

All authors declare no conflicts of interest.

Authors' Contributions

Huan Wang and Lei Tu contributed equally to this work.

Acknowledgments

This work is supported by the National Natural Foundation Project of China (No. 81873558, 81700490, and 81500415) and the National Key Research and Development Program of China (No. 2018YFC0114604). We thank Troy Gharibani at the University of Maryland, USA, for editing and providing feedback on this article.

References

[1] World Health Organization Coronavirus2020, https://www.who.int/health-topics/coronavirus#tab=tab_1.

[2] World Health Organization Coronavirus disease2019, https://www.who.int/emergencies/diseases/novel-coronavirus-2019.

[3] J. Kirchgesner, M. Lemaitre, F. Carrat, M. Zureik, F. Carbonnel, and R. Dray-Spira, "Risk of serious and opportunistic infections associated with treatment of inflammatory bowel diseases," *Gastroenterology*, vol. 155, no. 2, pp. 337–346.e10, 2018.

[4] "Surveillance epidemiology of coronavirus under research exclusion," https://covidibd.org/current-data/.

[5] J. R. Goodhand, M. Wahed, J. E. Mawdsley, A. D. Farmer, Q. Aziz, and D. S. Rampton, "Mood disorders in inflammatory bowel disease: relation to diagnosis, disease activity, perceived stress, and other factors," *Inflammatory Bowel Diseases*, vol. 18, no. 12, pp. 2301–2309, 2012.

[6] R. Maunder and S. Levenstein, "The role of stress in the development and clinical course of inflammatory bowel disease: epidemiological evidence," *Current Molecular Medicine*, vol. 8, no. 4, pp. 247–252, 2008.

[7] "National Health Commission. Diagnosis and treatment of COVID-19 (version7)," http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml.

[8] C. Marinelli, E. Savarino, M. Inferrera et al., "Factors influencing disability and quality of life during treatment: a cross-sectional study on IBD patients," *Gastroenterology Research and Practice*, vol. 2019, Article ID 5354320, 10 pages, 2019.

[9] R. W. L. Leong, Y. T. Lee, J. Y. L. Ching, and J. J. Y. Sung, "Quality of life in Chinese patients with inflammatory bowel disease: validation of the Chinese translation of the Inflammatory Bowel Disease Questionnaire," *Alimentary Pharmacology & Therapeutics*, vol. 17, no. 5, pp. 711–718, 2003.

[10] K. Kroenke, R. L. Spitzer, J. B. W. Williams, and B. Löwe, "The patient health questionnaire somatic, anxiety, and depressive symptom scales: a systematic review," *General Hospital Psychiatry*, vol. 32, no. 4, pp. 345–359, 2010.

[11] K. Kroenke, R. L. Spitzer, and J. B. W. Williams, "The PHQ-9," *Journal of General Internal Medicine*, vol. 16, no. 9, pp. 606–613, 2001.

[12] C. N. Bernstein, L. Zhang, L. M. Lix et al., "The validity and reliability of screening measures for depression and anxiety disorders in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 24, no. 9, pp. 1867–1875, 2018.

[13] B. Khor, A. Gardet, and R. J. Xavier, "Genetics and pathogenesis of inflammatory bowel disease," *Nature*, vol. 474, no. 7351, pp. 307–317, 2011.

[14] M. F. Neurath, "Cytokines in inflammatory bowel disease," *Nature Reviews Immunology*, vol. 14, no. 5, pp. 329–342, 2014.

[15] A. Wisniewski, J. Kirchgesner, P. Seksik et al., "Increased incidence of systemic serious viral infections in patients with inflammatory bowel disease associates with active disease and use of thiopurines," *United European Gastroenterology Journal*, vol. 8, no. 3, pp. 303–313, 2020.

[16] M. Giovanni and S. Ardizzone, "Are patients with inflammatory bowel disease at increased risk for Covid-19 infection?," *Journal of Crohn's and Colitis*, vol. 14, no. 9, pp. 1334–1336, 2020.

[17] Chinse Society of IBD, "Managing IBD patients during the outbreak of COVID-19," *Chinese Journal of Digestive Diseases*, vol. 40, article E001, 2020.

[18] F. Xiao, M. Tang, X. Zheng, Y. Liu, X. Li, and H. Shan, "Evidence for gastrointestinal infection of SARS-CoV-2," *Gastroenterology*, vol. 158, no. 6, pp. 1831–1833.e3, 2020.

[19] X. Jin, J. S. Lian, J. H. Hu et al., "Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms," *Gut*, vol. 69, no. 6, pp. 1002–1009, 2020.

[20] G. Jinyang, B. Han, and J. Wang, "COVID-19: Gastrointestinal Manifestations and Potential Fecal-Oral Transmission," *Gastroenterology*, vol. 158, no. 6, pp. 1518-1519, 2020.

[21] Y. Tian, L. Rong, W. Nian, and Y. He, "Review article: gastrointestinal features in COVID-19 and the possibility of faecal transmission," *Alimentary Pharmacology & Therapeutics*, vol. 51, no. 9, pp. 843–851, 2020.

[22] C. Han, C. Duan, S. Zhang et al., "Digestive symptoms in COVID-19 patients with mild disease severity: clinical presentation, stool viral RNA testing, and outcomes," *The American journal of Gastroenterology*, vol. 115, no. 6, pp. 916–923, 2020.

[23] S. Danese, M. Cecconi, and A. Spinelli, "Management of IBD during the COVID-19 outbreak: resetting clinical priorities," *Nature Reviews Gastroenterology & Hepatology*, vol. 17, no. 5, pp. 253–255, 2020.

[24] G. Fiorino, et al.M. Allocca, F. Furfaro et al., "Inflammatory bowel disease care in the COVID-19 pandemic era: the Humanitas, Milan, experience," *Journal of Crohn's and Colitis*, vol. 14, no. 9, pp. 1330–1333, 2020.

[25] G. Byrne, G. Rosenfeld, Y. Leung et al., "Prevalence of anxiety and depression in patients with inflammatory bowel disease," *Canadian Journal of Gastroenterology & Hepatology*, vol. 2017, article 6496727, pp. 1–6, 2017.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 8146502, 8 pages
https://doi.org/10.1155/2020/8146502

Research Article

Cytokine Profiling in Chinese SLE Patients: Correlations with Renal Dysfunction

Chen Yan, Le Yu, Xiu-Ling Zhang, Jing-Jing Shang, Jie Ren, Jie Fan, Xue-Qin Feng, Rong-Wei Zhang, Zhong-Bin Xia, and Xin-Wang Duan

Department of Rheumatology, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China

Correspondence should be addressed to Xin-Wang Duan; dxw_efyfsmyk@163.com

Received 14 April 2020; Revised 23 September 2020; Accepted 24 September 2020; Published 9 October 2020

Academic Editor: Yan Yang

Background. Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease that commonly causes kidney damage. Therefore, we measured plasma levels of cytokines that may be related to renal dysfunction in SLE patients. *Methods*. To explore the differences between SLE patients with renal dysfunction and healthy volunteers, the levels of cytokines in plasma were screened using a human cytokine antibody array. Then, we chose fourteen of the elevated cytokines for verification with an expanded sample size by a human magnetic Luminex assay. Plasma samples were isolated from SLE patients ($n = 72$) and healthy volunteers ($n = 8$). *Results*. Cytokine antibody array data showed elevated plasma cytokines in SLE patients with renal dysfunction compared with healthy volunteers. By using the human magnetic Luminex assay, we found that plasma levels of CHI3L1, GDF-15, IGFBP-2, MIF, ST2, TFF3, and uPAR were significantly higher in SLE patients than in healthy volunteers. Plasma levels of CXCL4 were significantly lower in the active group than in the inactive group, and plasma levels of CHI3L1, IGFBP-2, MIF, and MPO were significantly higher in the active group than in the inactive group. We also analyzed the correlation between plasma cytokine levels and the SLEDAI-2K, and our results showed that the plasma levels of the fourteen selected cytokines were weakly correlated or not correlated with the SLEDAI-2K. We further analyzed the correlation between cytokines and renal dysfunction. Plasma levels of GDF-15 and TFF3 were highly positively correlated with serum creatinine levels and 24-hour urine protein levels. *Conclusion*. Our data suggest that plasma levels of GDF-15 and TFF3 are potential renal dysfunction markers in SLE patients, but plasma levels of these cytokines are not correlated with the SLEDAI-2K. Further study is warranted to determine how these cytokines regulate inflammatory responses and renal dysfunction in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease that causes damage to multiple organ systems and is characterized by antinuclear antibody (ANA) production [1, 2]. The prevalence of SLE in the Chinese population was estimated to be approximately 40 cases per 100,000 persons [3]. Considering the huge population of China, this would be the largest cluster of cases in the world. During the last decade, there has been an increased understanding of the underlying autoimmune process in SLE, including the dysregulation of cytokines, increased expression of type I interferon- (IFN-) regulated genes, and activation of autoreactive B cells [2, 4]. It was also suggested that immune dysregulation precedes the development of clinical disease in SLE [4, 5].

Lupus nephritis (LN) significantly impacts the quality of life and longevity of SLE patients [6]. Renal dysfunction is the second leading cause of death in Chinese SLE patients [7]. However, traditional clinical parameters are not sensitive or specific enough to detect activity and early relapse of LN [8]. It has been reported that several cytokines in patients with SLE, such as IFN-α, B lymphocyte stimulator (BLyS), IL-17, and IL-1β, are related to disease activity and organ involvement [9–11]. To identify the potential biomarkers of LN, it is critical to know the roles of cytokines in renal dysfunction in SLE patients.

In this study, we investigated the correlation between plasma cytokine levels and renal dysfunction in Chinese

SLE patients. We observed that plasma levels of GDF-15 and TFF3 are potential renal dysfunction markers in Chinese SLE patients. Furthermore, it should be noted that the expression and function of cytokines in SLE were significantly affected by different races/ethnicities and environmental exposures.

2. Materials and Methods

2.1. Patients and Samples. Patients with SLE (scoring ≥ 4 in the 1997 updated American College of Rheumatology (ACR) classification criteria) were recruited from the Second Affiliated Hospital of Nanchang University between 2017 and 2019. Seventy-two SLE patients and eight healthy volunteers were recruited. All patients provided informed written consent, and the study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University (No. 201620). Patients' disease activity and concurrent medication were assessed by a physician, and fasting blood samples were collected. Clinical features and serological data relevant to the ACR classification criteria were collected retrospectively from hospital records. Serum autoantibodies and complement levels were measured using standard laboratory assays. SLE disease activity was measured using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K). Active disease was defined as SLEDAI-2K > 4. All plasma samples were stored at -80°C until analysis.

2.2. Human XL Cytokine Array. To explore the differences in the levels of cytokines between SLE patients with renal dysfunction ($n = 3$, SLE group) and healthy volunteers ($n = 3$, control group), the average levels of cytokines in plasma were compared among the groups using a human XL cytokine Proteome Profiler™ array (ARY022b, R&D Systems, United States) per the manufacturer's instructions. Briefly, premixed plasmas were incubated with blocked membranes overnight at 4°C. The membranes were washed, incubated with a detection antibody cocktail for 1 hour, and washed three times, followed by incubation with streptavidin conjugated to horseradish peroxidase for 30 minutes. Expression was visualized using an enhanced chemiluminescence detection kit (#RPN2106, GE Healthcare Life Sciences, United States). Semi-quantitative analysis was performed by measuring the density of the bands using an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Life Sciences, United States).

2.3. Quantification of Plasma Cytokines. To detect the cytokine levels in plasma, we performed a human magnetic Luminex assay (LXSAHM-14) using a Luminex X-200. Fourteen cytokines were detected, including BLyS, C-C chemokine ligand 5 (CCL5), chitinase 3 like 1 (CHI3L1), C-X-C motif ligand 4 (CXCL4), growth differentiation factor 15 (GDF-15), intercellular cell adhesion molecule-1 (ICAM-1), insulin-like growth factor binding protein-2 (IGFBP-2), macrophage migration inhibitory factor (MIF), myeloperoxidase (MPO), resistin, serpin E1, suppression of tumorigenicity 2 (ST2), trefoil factor 3 (TFF3), and urokinase-type plasminogen activator receptor (uPAR). Briefly, 50 μL of the standard or sample and 50 μL of the microparticle cocktail were added to each well of the microplate. The plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. After the plate was washed, 50 μL of the diluted biotin-antibody cocktail was added to each well and incubated for 1 hour at room temperature on a shaker. After the plate was washed, 50 μL of diluted streptavidin-PE was added to each well. The plate was incubated for 30 minutes at room temperature on the shaker. After the plate was washed, the samples were read within 90 minutes using a Luminex X-200 analyzer.

Table 1: Characteristics of the study participants in the human XL cytokine array.

Characteristics	SLE patient with renal dysfunction ($n = 3$)	Healthy volunteers ($n = 3$)
Age (±SD) (years)	34.00 ± 7.55	29.33 ± 9.24
Gender (female)	3	3
Disease duration (±SD) (months)	22.67 ± 22.03	—
SLEDAI-2K (±SD)	6.67 ± 4.16	—
Cr (±SD) (μmol/L)	185.7 ± 33.16	—
24-hour urine protein (±SD) (mg/24 h)	2933 ± 1955	—

2.4. Statistical Analyses. All data are presented as the mean ± SD. Whether the variables in each group were normally distributed was determined. To analyze the data that were not normally distributed, logarithmic transformation was performed. Statistical analysis was performed by the unpaired t-test between two groups, and correlations between variables were assessed using the Pearson test (GraphPad Prism 7, GraphPad Software, Inc.). A p value less than 0.05 was accepted as significant.

3. Results

3.1. Elevated Cytokine Levels in SLE Patients Compared with Healthy Volunteers. Three SLE patients and three healthy volunteers were recruited for the human XL cytokine Proteome Profiler™ array, of whom 3/3 (100%) and 3/3 (100.0%) were female, with mean (±SD) ages of 34.00 ± 7.55 and 29.33 ± 9.24 years, respectively (Table 1). Cytokine array data showed elevated cytokine levels in the plasma of SLE patients compared with healthy volunteers (Figure 1(a)). Then, we chose fourteen of the elevated cytokines for verification with an expanded sample size (Figure 1(b)).

Seventy-two SLE patients and eight healthy volunteers were recruited, of whom 65/72 (90.3%) and 8/8 (100.0%) were female, with mean (±SD) ages of 32.83 ± 10.53 and 31 ± 6.44 years, respectively (Table 2). Plasma levels of CHI3L1, GDF-15, IGFBP-2, MIF, ST2, TFF3, and uPAR were significantly higher in SLE patients compared with healthy volunteers (Figure 1(c)). There were no significant differences in the other 7 cytokines (Supplementary Fig. 1).

3.2. Differential Expression of Cytokines in Active and Inactive SLE Patients. In the SLE patient group, 48/72 patients

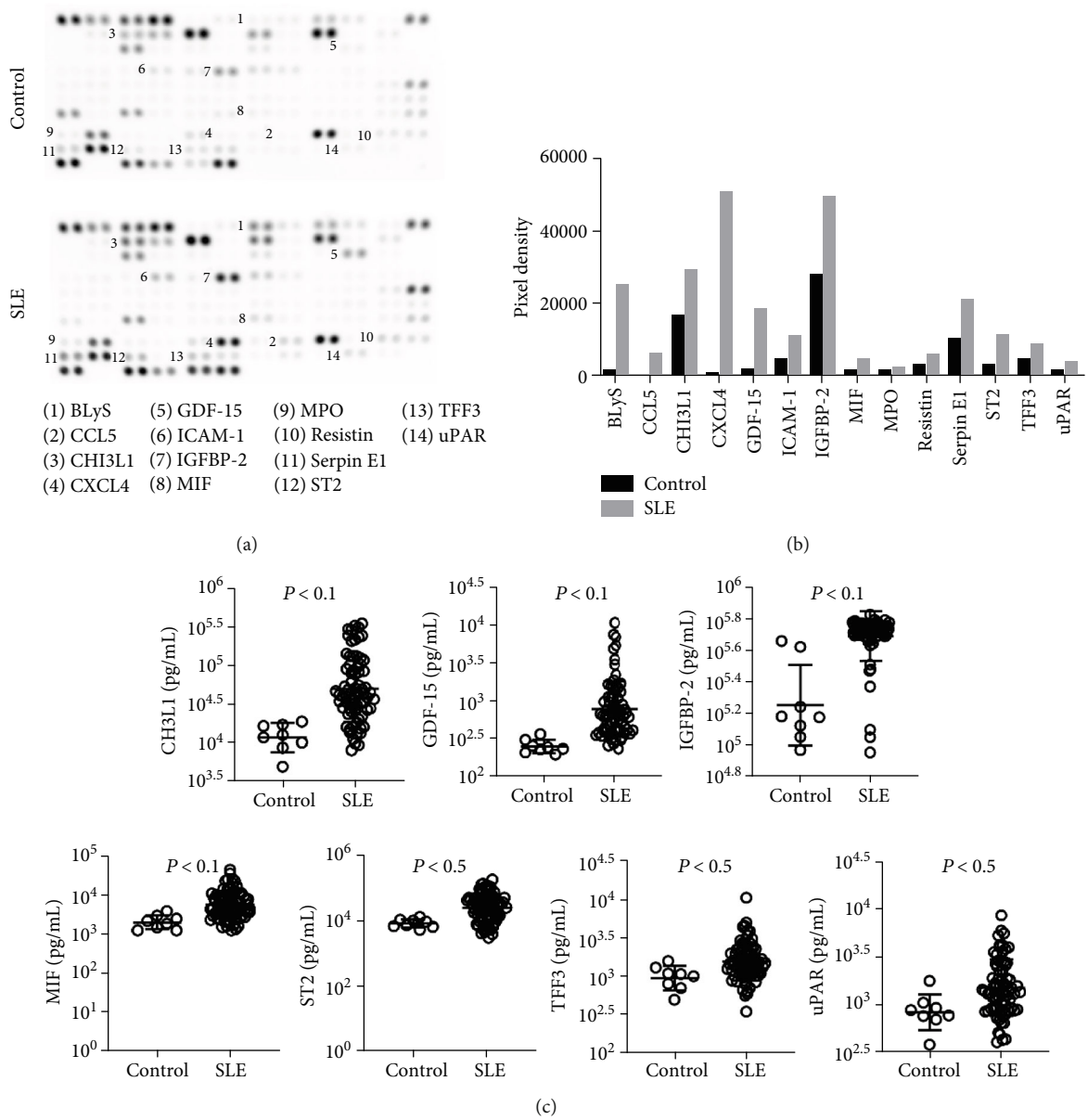


Figure 1: Elevated cytokine levels in SLE patients compared with healthy volunteers. (a) Raw images of the cytokine array. (b) Pixel densities of the fourteen selected cytokines. (c) Quantitative analysis of the level of cytokines in SLE patients and healthy volunteers.

(66.7%) had active disease (SLEDAI-2K > 4). The active group was younger than the inactive group (32.04 ± 10.52 vs. 34.42 ± 10.58 years) (Table 2). Plasma levels of CXCL4 were significantly lower in the active group than in the inactive group, and plasma levels of CHI3L1, IGFBP-2, MIF, and MPO were significantly higher in the active group compared with the inactive group (Figure 2). There were no significant differences in the other 9 cytokines (Supplementary Fig. 2). We also analyzed the correlation between plasma cytokine levels and the SLEDAI-2K, and our results showed that the plasma levels of these cytokines were weakly correlated or uncorrelated with the SLEDAI-2K (Figure 3).

3.3. Correlation of Plasma Cytokine Levels with Clinical Parameters of Renal Dysfunction. We further analyzed the correlation between cytokines and renal dysfunction. Plasma levels of CHI3L1, GDF-15, IGFBP-2, resistin, and TFF3 were significantly higher in LN patients than in SLE patients without renal involvement (Figure 4). There were no significant differences in the other 9 cytokines (Supplementary Fig. 3). Plasma levels of GDF-15 and TFF3 were highly positively correlated with serum creatinine levels and 24-hour urine protein levels ($r > 0.5$, $p < 0.01$, Figures 5(a) and 5(b)). There was no obvious correlation between renal dysfunction and the other 11 cytokines (Supplementary Fig. 4 and Supplementary Fig. 5).

4. Discussion

Cytokine dysregulation is a characteristic of SLE [2, 12]. Increased plasma cytokine levels have been found in SLE

Table 2: Characteristics of the study participants.

Characteristics	SLE ($n = 72$)	Healthy volunteers ($n = 8$)	Active SLE ($n = 48$)	Inactive SLE ($n = 24$)	LN ($n = 34$)	Non-LN ($n = 38$)
Age (±SD) (years)	32.83 ± 10.53	31 ± 6.44	32.04 ± 10.52	34.42 ± 10.58	32.26 ± 10.80	33.34 ± 10.40
Gender (female)	65	8	44	21	30	35
Disease duration (±SD) (months)	25.4 ± 44.22	—	18.31 ± 38.72	39.58 ± 51.55	24.11 ± 47.81	26.56 ± 41.35
SLEDAI-2K (±SD)	8.042 ± 5.476	—	10.75 ± 4.72	2.625 ± 1.056	10.82 ± 5.82	5.55 ± 3.73
Cr (±SD) (μmol/L)	77.98 ± 74.31	—	78.55 ± 53.99	76.86 ± 104.8	105.9 ± 99.60	52.33 ± 15.24
24-hour urine protein (±SD) (mg/24 h)	1511 ± 2626	—	1875 ± 2846	126.7 ± 136.2	2514 ± 3045	138.7 ± 118.5

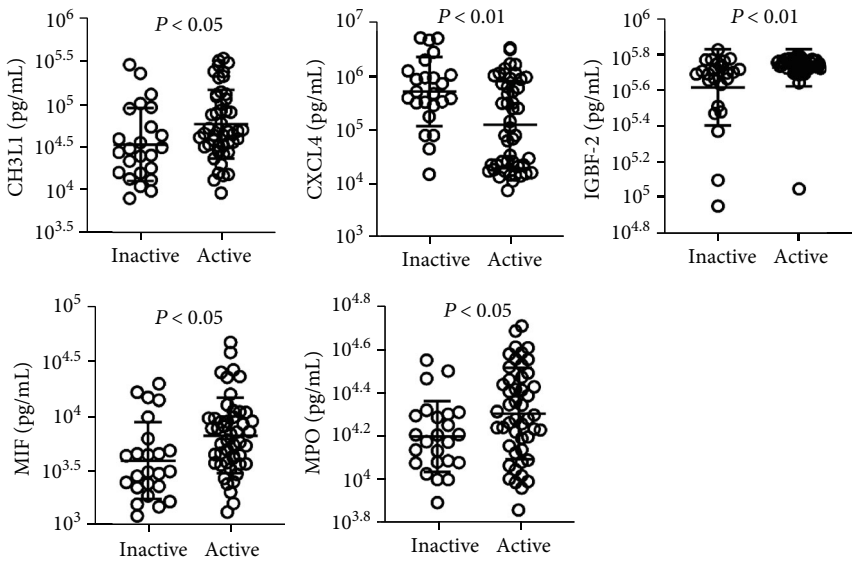


Figure 2: Differential expression of cytokines in inactive and active SLE patients.

patients. These cytokines, in turn, have a number of effects that drive lupus pathophysiology, as well as the increased organ damage. In our study, plasma levels of CHI3L1, GDF-15, IGFBP-2, MIF, ST2, TFF3, and uPAR were significantly higher in SLE patients than in healthy volunteers. Consistent with our study, it has been reported that plasma levels of IGFBP-2, MIF, ST2, and uPAR were increased in SLE patients compared with healthy volunteers [13–19]. Previous studies suggested MIF as a therapeutic target for SLE, as *in vivo* miRNA inhibition of MIF decreased downstream cytokine production and ameliorated murine lupus nephritis [13, 14]. However, the role of elevated plasma CHI3L1, GDF-15, and TFF3 in SLE patients is still unknown.

Moreover, it has been reported that the disease activity of SLE is related to cytokine levels, such as pentraxin-related protein (PTX3) and C-X-C motif ligand 10 (CXCL10) [9]. We found decreased CXCL4 levels and elevated CHI3L1, IGFBP-2, MIF, and MPO levels in active SLE patients compared with inactive SLE patients. In addition, plasma levels of these cytokines were weakly or not correlated with the SLEDAI-2K. It has been reported that serum IGFBP-2 levels were significantly higher in patients with active SLE than in those with inactive SLE or in healthy volunteers in a cohort of Chinese patients with SLE [17]. Increased plasma MPO levels in SLE have also been reported in Brazil [20]. However, there was no significant difference in plasma MPO levels between active SLE patients and inactive SLE patients, and there was no correlation between plasma MPO levels and SLEDAI-2K ($r = 0.07$, $p = 0.58$) [20]. A previous study from Australia also reported decreased serum MPO levels in SLE patients compared with healthy volunteers [21]. However, we found higher plasma MPO levels in active SLE patients than in inactive SLE patients, and plasma MPO levels were weakly correlated with the SLEDAI-2K ($r = 0.317$, $p = 0.007$). In contrast, a study in Greece showed that plasma levels of CXCL4 were comparable between SLE patients and healthy volunteers [22]. It is not clear why the research results in different countries are inconsistent. It seems that environmental exposure and race/ethnicity may affect the expression and function of cytokines in SLE [23, 24].

Most organs can be involved in SLE, and the typical major organ manifestations are in the kidney and central nervous system [25]. It has been found that more lupus nephritis cases and fewer neuropsychiatric lupus cases

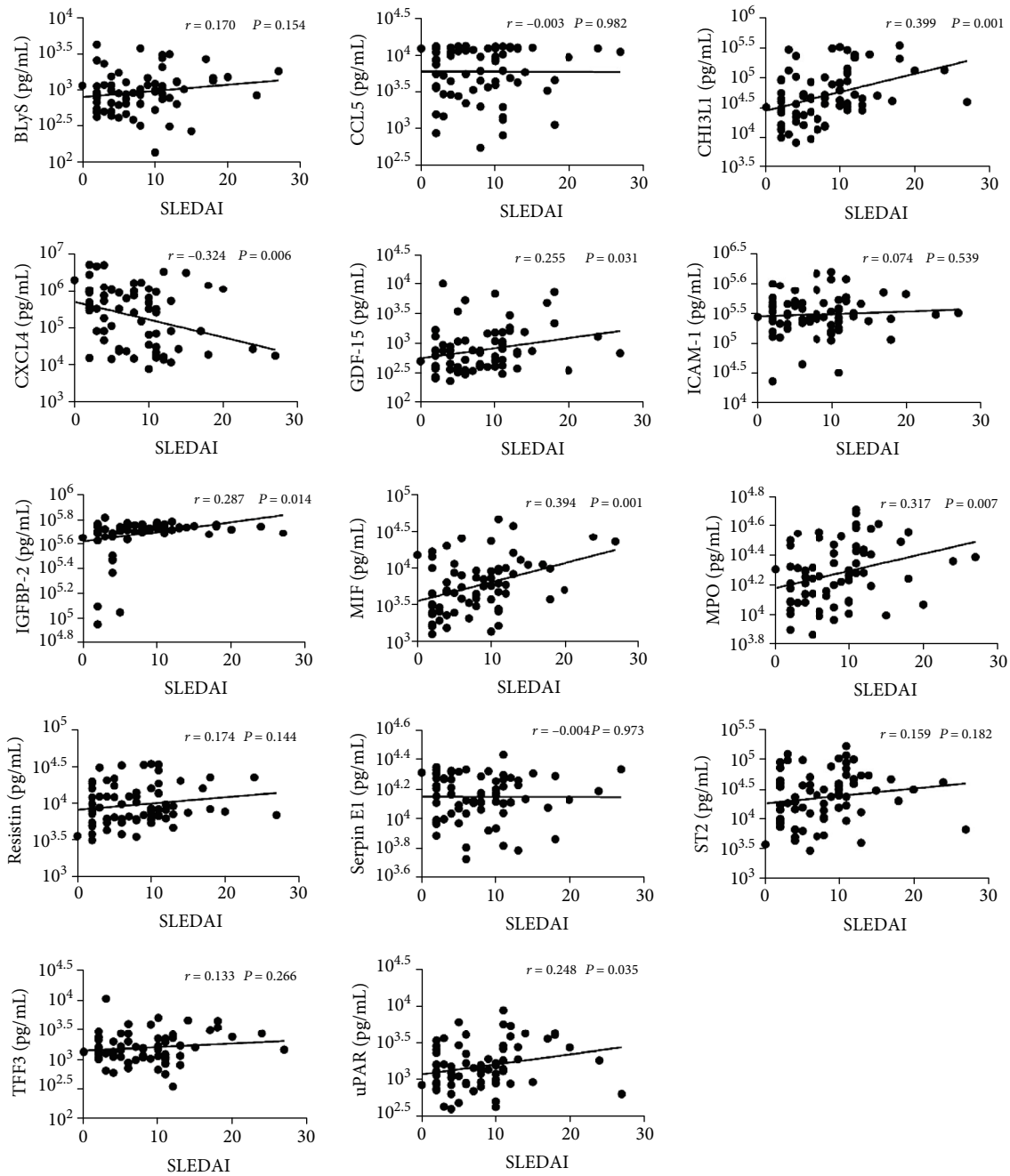


Figure 3: Correlation of plasma cytokine levels with clinical parameters of disease activity.

were detected in Chinese patients with SLE than in SLE patients from other countries [26]. A higher percentage of Chinese patients presented with nephropathy (47.4%) than European patients (27.9%) [26]. It is necessary to pay close attention to the diagnosis and treatment of renal dysfunction in Chinese SLE patients. Novel serum and urinary biomarkers, such as cytokines and chemokines, have been evaluated for detecting early flares in LN [8]. In our results, plasma levels of CHI3L1, GDF-15, IGFBP-2, resistin, and TFF3 were significantly higher in LN patients than in SLE patients without renal involvement. Moreover, plasma levels of GDF-15 and TFF3 were highly positively correlated with clinical parameters of renal dysfunction. IGFBP-2 may differentiate active renal SLE from active nonrenal or inactive SLE [17]. However, our results showed that the level of IGFBP-2 was not correlated with clinical parameters of renal dysfunction ($p > 0.05$, Supplementary Fig. 3 and Supplementary Fig. 4). It has been reported that GDF-15 and TFF3 are related to renal dysfunction diseases. GDF-15 is suggested to be a marker of cardiac injury and renal dysfunction in patients undergoing cardiac surgery [27, 28]. Circulating GDF-15 levels are significantly associated with an increased risk of CKD progression [29]. TFF3 has been suggested to be a highly

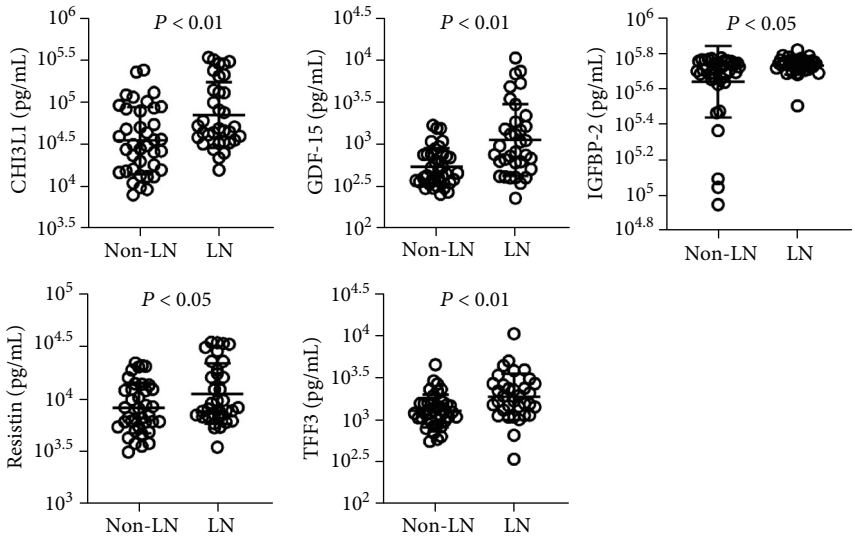


Figure 4: Differential expression of cytokines in LN and non-LN patients.

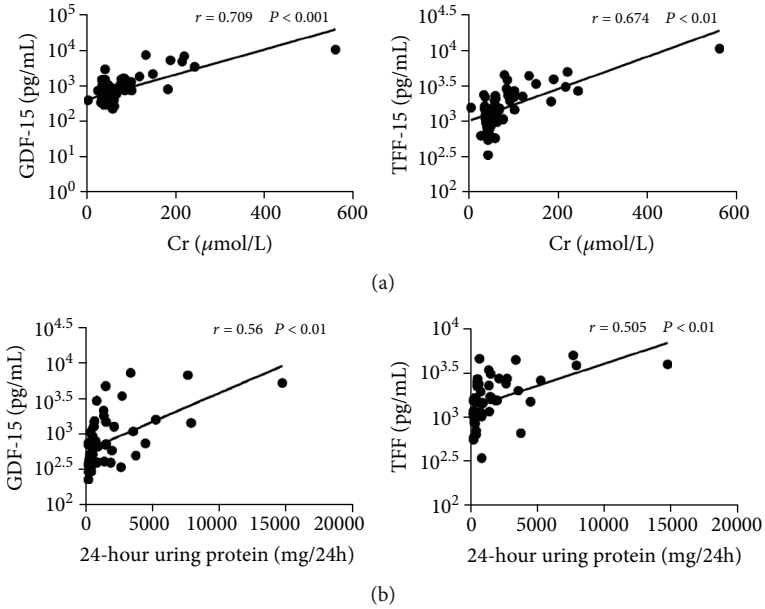


Figure 5: Correlation of plasma cytokine levels with clinical parameters of renal dysfunction. (a) Correlation between plasma cytokine levels and serum creatinine levels. (b) Correlation between plasma cytokine levels and 24-hour urine protein levels.

sensitive and specific urinary biomarker to monitor drug-induced kidney injury in clinical trials [30]. In addition, serum and urine concentrations of TFF3 were correlated with the stage of CKD severity and these increased concentrations of TFF3 may be due to secretion from renal tubular epithelial cells in damaged kidneys [31]. Although the roles of GDF-15 and TFF3 in SLE were not clear, our results suggest that the plasma levels of GDF-15 and TFF3 reflect the severity of renal dysfunction in SLE.

In conclusion, our data suggest that plasma levels of GDF-15 and TFF3 are potential renal dysfunction markers in SLE patients, but plasma levels of these cytokines were weakly or not correlated with the SLEDAI-2K. Furthermore, it should be noted that the expression and function of cytokines in SLE were significantly affected by different races/ethnicities and environmental exposures. This study is largely limited by the relatively small sample size, and we did not investigate the relevant mechanisms of these cytokines in regulating SLE disease progression. Further study is warranted to expand the sample size and determine how these cytokines regulate inflammatory responses and renal dysfunction in SLE.

Data Availability

The datasets generated and analyzed for the present study are available from the corresponding author on reasonable request.

Disclosure

The founders did not participate in this study.

Conflicts of Interest

The authors indicate that there are no potential conflicts of interest.

Authors' Contributions

C. Y. and X.-W. D. conceived and designed the experiments. J.-J. S., J. R., J. F., X.-Q. F., R.-W. Z., and Z.-B. X. collected plasma samples and clinical data. C. Y., J. R., L. Y., and X.-L. Z. performed the experiments and analyzed the data. C. Y. and X.-W. D. wrote the main manuscript text. All authors reviewed the manuscript. Chen Yan, Le Yu, and Xiu-Ling Zhang contributed equally to this work.

Acknowledgments

We would like to acknowledge the Biobank Centre staff in our hospital for their hard work and dedication to the study participants. This study was supported by a grant from the project of Science and Technology Department of Jiangxi Province (NO. 20181BAB215019), the Science and Technology Research Program of Department of Education of Jiangxi Province (NO. GJJ180148 and NO. GJJ190148), and the National Natural Science Foundation of China (NO. 81860425).

Supplementary Materials

Supplementary Fig. 1: quantitative analysis of the level of cytokines in SLE patients and healthy volunteers. Supplementary Fig. 2: differential expression of cytokines in the inactive and active SLE patients. Supplementary Fig. 3: differential expression of cytokines in LN patients compared with non-LN patients. Supplementary Fig. 4: correlation between plasma cytokine levels and serum creatinine levels. Supplementary Fig. 5: correlation between plasma cytokine levels and 24-hour urine protein levels. *(Supplementary Materials)*

References

[1] D. S. Pisetsky, "Evolving story of autoantibodies in systemic lupus erythematosus," *Journal of autoimmunity*, vol. 110, p. 102356, 2020.

[2] M. D. Catalina, K. A. Owen, A. C. Labonte, A. C. Grammer, and P. E. Lipsky, "The pathogenesis of systemic lupus erythematosus: harnessing big data to understand the molecular basis of lupus," *Journal of autoimmunity*, vol. 110, p. 102359, 2020.

[3] Q. Y. Zeng, R. Chen, J. Darmawan et al., "Rheumatic diseases in China," *Arthritis research & therapy*, vol. 10, no. 1, p. R17, 2008.

[4] M. E. Munroe, R. Lu, Y. D. Zhao et al., "Altered type II interferon precedes autoantibody accrual and elevated type I interferon activity prior to systemic lupus erythematosus classification," *Annals of the rheumatic diseases*, vol. 75, no. 11, pp. 2014–2021, 2016.

[5] R. Lu, M. E. Munroe, J. M. Guthridge et al., "Dysregulation of innate and adaptive serum mediators precedes systemic lupus erythematosus classification and improves prognostic accuracy of autoantibodies," *Journal of autoimmunity*, vol. 74, pp. 182–193, 2016.

[6] S. Almaani, A. Meara, and B. H. Rovin, "Update on lupus nephritis," *Clin J Am Soc Nephrol*, vol. 12, no. 5, pp. 825–835, 2017.

[7] Z. Wang, Y. Wang, R. Zhu et al., "Long-term survival and death causes of systemic lupus erythematosus in China: a systemic review of observational studies," *Medicine (Baltimore)*, vol. 94, no. 17, p. e794, 2015.

[8] S. Soliman and C. Mohan, "Lupus nephritis biomarkers," *Clinical immunology (Orlando, Fla)*, vol. 185, pp. 10–20, 2017.

[9] J. A. Reynolds, E. M. McCarthy, S. Haque et al., "Cytokine profiling in active and quiescent SLE reveals distinct patient subpopulations," *Arthritis research & therapy*, vol. 20, no. 1, p. 173, 2018.

[10] H. Zhou, B. Li, J. Li et al., "Dysregulated T cell activation and aberrant cytokine expression profile in systemic lupus erythematosus," *Mediators Inflamm*, vol. 2019, 11 pages, 2019.

[11] F. B. Vincent, M. Northcott, A. Hoi, F. Mackay, and E. F. Morand, "Clinical associations of serum interleukin-17 in systemic lupus erythematosus," *Arthritis research & therapy*, vol. 15, no. 4, p. R97, 2013.

[12] C. C. Berthier, M. Kretzler, and A. Davidson, "A systems approach to renal inflammation in SLE," *Clinical immunology (Orlando, Fla)*, vol. 185, pp. 109–118, 2017.

[13] Y. Tu, R. Guo, J. Li et al., "MiRNA regulation of MIF in SLE and attenuation of murine lupus nephritis with miR-654," *Frontiers in immunology*, vol. 10, 2019.

[14] J. B. Bilsborrow, E. Doherty, P. V. Tilstam, and R. Bucala, "Macrophage migration inhibitory factor (MIF) as a therapeutic target for rheumatoid arthritis and systemic lupus erythematosus," *Expert Opin Ther Targets*, vol. 23, no. 9, pp. 733–744, 2019.

[15] M. Y. Mok, F. P. Huang, W. K. Ip et al., "Serum levels of IL-33 and soluble ST2 and their association with disease activity in systemic lupus erythematosus," *Rheumatology (Oxford, England)*, vol. 49, no. 3, pp. 520–527, 2010.

[16] P. Italiani, M. L. Manca, F. Angelotti et al., "IL-1 family cytokines and soluble receptors in systemic lupus erythematosus," *Arthritis research & therapy*, vol. 20, no. 1, p. 27, 2018.

[17] C. C. Mok, H. H. Ding, M. Kharboutli, and C. Mohan, "Axl, ferritin, insulin-like growth factor binding protein 2, and tumor necrosis factor receptor type II as biomarkers in systemic lupus erythematosus," *Arthritis care & research*, vol. 68, no. 9, pp. 1303–1309, 2016.

[18] H. Ding, M. Kharboutli, R. Saxena, and T. Wu, "Insulin-like growth factor binding protein-2 as a novel biomarker for disease activity and renal pathology changes in lupus nephritis," *Clinical and experimental immunology*, vol. 184, no. 1, pp. 11–18, 2016.

[19] M. Usami, M. Shimizu, M. Mizuta et al., "Extensive serum biomarker analysis in patients with macrophage activation syndrome associated with systemic lupus erythematosus," *Clinical immunology (Orlando, Fla)*, vol. 208, p. 108255, 2019.

[20] R. W. Telles, G. A. Ferreira, N. P. Silva, and E. I. Sato, "Increased plasma myeloperoxidase levels in systemic lupus erythematosus," *Rheumatology international*, vol. 30, no. 6, pp. 779–784, 2010.

[21] P. E. Morgan, A. D. Sturgess, and M. J. Davies, "Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus," *Arthritis and rheumatism*, vol. 52, no. 7, pp. 2069–2079, 2005.

[22] M. D. Patsouras, M. P. Sikara, E. P. Grika, H. M. Moutsopoulos, A. G. Tzioufas, and P. G. Vlachoyiannopoulos, "Elevated expression of platelet-derived chemokines in patients with antiphospholipid syndrome," *Journal of autoimmunity*, vol. 65, pp. 30–37, 2015.

[23] C. G. Parks, A. de Souza Espindola Santos, M. Barbhaiya, and K. H. Costenbader, "Understanding the role of environmental factors in the development of systemic lupus erythematosus," *Best Pract Res Clin Rheumatol*, vol. 31, no. 3, pp. 306–320, 2017.

[24] K. L. Connelly, R. Kandane-Rathnayake, A. Hoi, M. Nikpour, and E. F. Morand, "Association of MIF, but not type I interferon-induced chemokines, with increased disease activity in Asian patients with systemic lupus erythematosus," *Scientific reports*, vol. 6, no. 1, 2016.

[25] L. Durcan, T. O'Dwyer, and M. Petri, "Management strategies and future directions for systemic lupus erythematosus in adults," *Lancet*, vol. 393, no. 10188, pp. 2332–2343, 2019.

[26] M. Li, W. Zhang, X. Leng et al., "Chinese SLE Treatment and Research group (CSTAR) registry: I. Major clinical characteristics of Chinese patients with systemic lupus erythematosus," *Lupus*, vol. 22, no. 11, pp. 1192–1199, 2013.

[27] T. Nakajima, I. Shibasaki, T. Sawaguchi et al., "Growth differentiation factor-15 (GDF-15) is a biomarker of muscle wasting and renal dysfunction in preoperative cardiovascular surgery patients," *Journal of Clinical Medicine*, vol. 8, no. 10, p. 1576, 2019.

[28] A. Kahli, C. Guenancia, M. Zeller et al., "Growth differentiation factor-15 (GDF-15) levels are associated with cardiac and renal injury in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass," *Plo S one*, vol. 9, no. 8, 2014.

[29] V. Nair, C. Robinson-Cohen, M. R. Smith et al., "Growth differentiation factor-15 and risk of CKD progression," *Journal of the American Society of Nephrology*, vol. 28, no. 7, pp. 2233–2240, 2017.

[30] B. R. Griffin, S. Faubel, and C. L. Edelstein, "Biomarkers of drug-induced kidney toxicity," *Ther Drug Monit*, vol. 41, no. 2, pp. 213–226, 2019.

[31] T.-y. Du, H.-m. Luo, H.-c. Qin et al., "Circulating serum trefoil factor 3 (TFF3) is dramatically increased in chronic kidney disease," *PloS one*, vol. 8, no. 11, p. e80271, 2013.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 1025857, 10 pages
https://doi.org/10.1155/2020/1025857

Research Article

Integrative Analyses of Genes Associated with Fulminant Type 1 Diabetes

Xiaofeng Ye,[1,2] **Tianshu Zeng**,[1,2] **Wen Kong**,[1,2] **and Lu-lu Chen**[1,2]

[1]*Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*
[2]*Hubei Provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan 430022, China*

Correspondence should be addressed to Wen Kong; wenly-kong@163.com and Lu-lu Chen; cheria_chen@126.com

Received 23 April 2020; Revised 25 August 2020; Accepted 15 September 2020; Published 6 October 2020

Academic Editor: Lihua Duan

Objective. Fulminant type 1 diabetes (FT1D) is a type of type 1 diabetes, which is characterized by rapid onset of disease and severe metabolic disorders. We intend to screen for crucial genes and potential molecular mechanisms in FT1D in this study. *Method*. We downloaded GSE44314, which includes six healthy controls and five patients with FT1D, from the GEO database. Identification of differentially expressed genes (DEGs) was performed by NetworkAnalyst. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs were screened by an online tool—Database for Annotation, Visualization, and Integration Discovery (DAVID). Protein-protein interaction (PPI) network and hub genes among DEGs were analyzed by NetworkAnalyst. And we also use NetworkAnalyst to find out the microRNAs (miRNAs) and transcription factors (TFs) which regulate the expression of DEGs. *Result*. We identified 130 DEGs (60 upregulated and 70 downregulated DEGs) between healthy controls and FT1D patients. GO analysis results revealed that DEGs were mostly enriched in generation of precursor metabolites and energy, neurohypophyseal hormone activity, and mitochondrial inner membrane. KEGG pathway analysis demonstrated that DEGs were mostly involved in nonalcoholic fatty liver disease. Results indicated that NCOA1, SRF, ERBB3, EST1, TOP1, UBE2S, INO80, COX7C, ITGAV, and COX6C were the top hub genes in the PPI network. Furthermore, we recognized that LDLR, POTEM, IFNAR2, BAZ2A, and SRF were the top hub genes in the miRNA-target gene network, and SRF, TSPAN4, CD59, ETS1, and SLC25A25 were the top hub genes in the TF-target gene network. *Conclusion*. Our study pinpoints key genes and pathways associated with FT1D by a sequence of bioinformatics analysis on DEGs. These identified genes and pathways provide more detailed molecular mechanisms of FT1D and may provide novel therapeutic targets.

1. Introduction

Fulminant type 1 diabetes (FT1D) is a novel type of type 1 diabetes (T1DM) raised by Imagawa et al. in 2000 [1], which is featured by abrupt disease onset, no C-peptide secretion, negative islet-related autoantibodies, and elevated pancreatic enzymes. At first, FT1D was identified as idiopathic T1DM because patients with FT1D lack autoimmune markers such as protein tyrosine phosphatase antibody or glutamic acid decarboxylase autoantibody. Over the past 20 years, the understanding of FT1D has increased. And a sequence of studies indicated that the immunity has a role in the occurrence and development of FT1D, which convinced that FT1D is possibly an autoimmune disease [2–4].

There are studies that reported that genetic and environmental factors take part in the initiation and progression of FT1D. Numbers of studies indicated that CTLA-4, HLA-B, and HLA DR-DQ are related with FT1D [5–7]. Many studies advocate that in FT1D, immune response against viral infection in islets caused the β cell destruction [8–10]. Numerous virus infections were covered in FT1D patients, including coxsackievirus, enterovirus, and human cytomegalovirus [11–13]. Genes such as lymphocyte cytosolic protein 1, melanoma differentiation-associated protein 5, DEAD box helicase 5, and C-X-C motif chemokine 10, which take part in the virus infection, have been proved to be associated with the pathogenesis of FT1D [3, 11, 14]. To further reveal the mechanism of FT1D, a microarray data

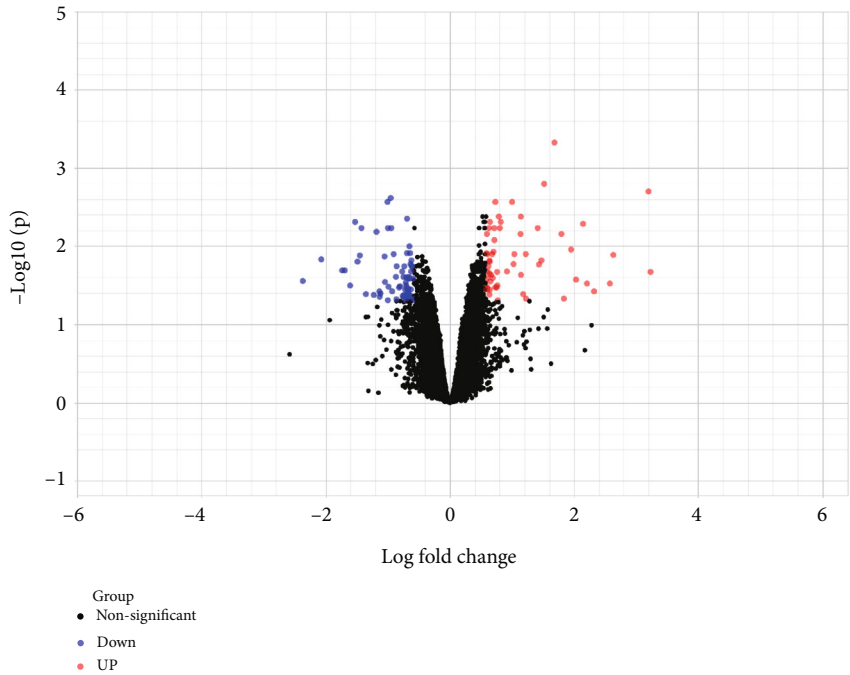


Figure 1: Volcano plot of differentially expressed genes. Genes with a significant change of more than 1.5-fold were selected.

numbered GSE44314 was deposited by Nakata et al., and it has reported that NKG2E-CD94 were significantly reduced in FT1D, indicating that the reduced expression of NK activating receptor gene and low proportion of NK cells are probably involved in the progression of FT1D [15]. However, there are no studies that had reported the possible regulatory mechanisms of transcription factors (TFs) and microRNAs (miRNAs) related to the development of FT1D.

In our study, we reanalyzed the dataset of GSE44314 by the method of bioinformatics, which includes screening differentially expressed genes (DEGs), functional enrichment analysis, protein-protein interaction (PPI) analysis, and the regulatory TFs/miRNAs related to DEG prediction. Through these analyses, we expect to determine novel insights for the knowledge of FT1D and provide more detailed molecular mechanisms underlying the development of FT1D.

2. Materials and Methods

2.1. Microarray Data. We downloaded the gene expression profile data of GSE44314 from the Gene Expression Omnibus (GEO) database in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/geo/). The microarray data was based on the platform of GPL6480 (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F). The datasets available in this analysis were uploaded by Nakata et al. [15], which include 11 samples, containing 6 healthy controls and 5 patients with FT1D.

2.2. Identification of Differentially Expressed Genes. NetworkAnalyst [16, 17] (https://www.networkanalyst.ca), a website for integrative statistical and visualizing tool, was used to determine the DEGs between healthy controls and FT1D patients. The cutoff of the P value was adjusted to 0.05, and $|\log \text{ fold change}|$ $(|\log \text{ FC}|) > 0.585$ for the DEG discrimination, using the false discovery rate (FDR) found on the Benjamini-Hochberg program and moderated t-test based on the Limma algorithm.

2.3. Functional and Pathway Enrichment Analysis. We used an online tool named DAVID [18] (https://david.ncifcrf.gov/) in conducting the Gene Ontology (GO) term [19] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [20] pathway enrichment analyses of DEGs, with the thresholds of count ≥ 2 and P value < 0.05.

2.4. Protein-Protein Interaction (PPI) Network Analysis and Hub Gene Searching. Based on the analyzed DEGs, NetworkAnalyst [21] was used to perform the PPI Network identification with a hypergeometric algorithm, and $P < 0.05$ was identified as having statistically significant differences. Besides, we used NetworkAnalyst to recognize the most significant modules of hub genes using the "module explorer tool," found on the random walk-dependent Walktrap algorithm.

2.5. Prediction of Target Gene-MicroRNA Network. The gene expression was affected by microRNAs in a disease condition through posttranscriptional control. In the present study, the online tool NetworkAnalyst [17] was used to search the miRNAs associated with DEGs, which integrates microRNA databases miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php) [22] and TarBase (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index) [23].

2.6. Prediction of Target Gene-Transcription Factor Network. The gene expression was influenced by TFs in a disease

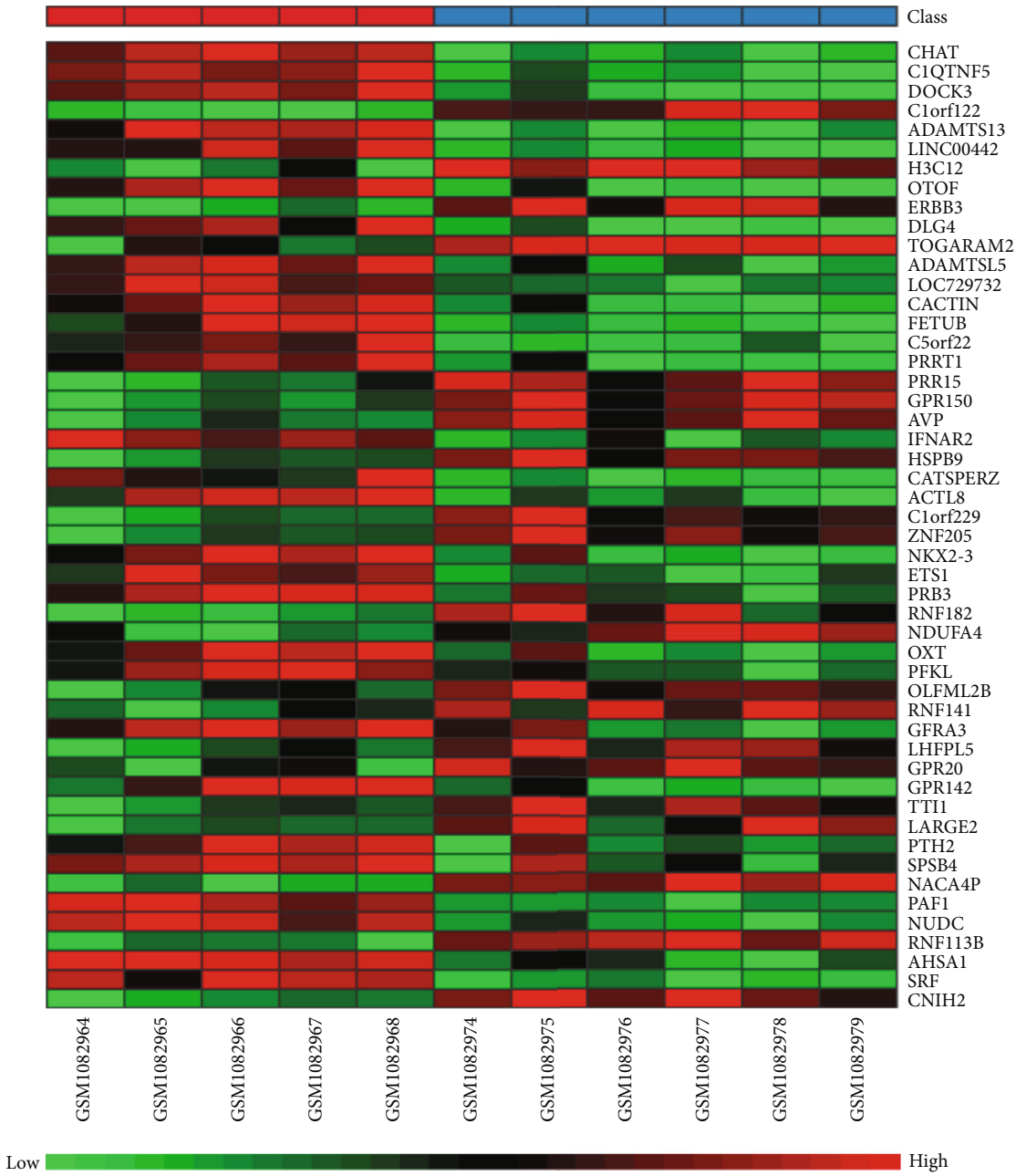


Figure 2: Heat map of differentially expressed genes. The abscissa represents different samples, and the ordinate represents different genes. The red boxes indicate upregulated genes, and the green boxes indicate downregulated genes.

condition by transcriptional control. In our study, NetworkAnalyst [17] was used for recognizing the TFs associated with DEGs, which combines TF database JASPAR (http://jaspar.genereg.net/) [24].

3. Results

3.1. Identification of Differentially Expressed Genes in Fulminant Type 1 Diabetes. We identified 130 DEGs in FT1D patients compared to healthy controls in total, including 60 upregulated genes and 70 downregulated genes (Supplementary Table 1). We draw a volcano plot of the DEGs (Figure 1) and a hierarchical clustering heat map of DEGs (Figure 2). It turned out that these DEGs were well distinguished between the FT1D group and the healthy control group. NK2 homeobox 3 (NKX2-3) and Ring finger protein 182 (RNF182) were, respectively, identified as the most significantly upregulated and downregulated genes in FT1D patients.

3.2. Functional Enrichment Analysis. We recognized 21 Gene Ontology terms (Table 1) and 5 KEGG pathways (Table 2) when analyzed with DAVID. The DEGs were mainly focused on the generation of precursor metabolites and energy, hydrogen ion transmembrane transport, and mitochondrial electron transport, cytochrome c to oxygen by biological

Table 1: The results of Gene Ontology (GO) of DEGs ranked by *P* value.

Term	Count	*P* value	Genes
GO-BPs			
Generation of precursor metabolites and energy	4	0.004	AVP, UQCR11, COX7C, COX6C
Hydrogen ion transmembrane transport	4	0.006	NDUFA4, UQCR11, COX7C, COX6C
Mitochondrial electron transport, cytochrome c to oxygen	3	0.006	NDUFA4, COX7C, COX6C
Extrinsic apoptotic signaling pathway in the absence of ligand	3	0.017	MOAP1, ERBB3, ITGAV
Positive regulation of female receptivity	2	0.018	NCOA1, OXT
Positive regulation of gene expression	6	0.02	AMH, ATF4, AVP, LDLR, ERBB3, GPER1
Maternal aggressive behavior	2	0.024	AVP, OXT
Hyperosmotic salinity response	2	0.029	AVP, OXT
Cellular response to lipopolysaccharide	4	0.03	TNFRSF1B, ADAMTS13, PAF1, CACTIN
Social behavior	3	0.033	AVP, OXT, DLG4
Positive regulation of apoptotic process	6	0.034	MOAP1, ATF4, NCOA1, ARHGEF6, GPER1, PDCD1
Male mating behavior	2	0.035	NCOA1, OXT
Positive regulation of uterine smooth muscle contraction	2	0.041	OXT, GPER1
Drinking behavior	2	0.041	HTR1B, OXT
Positive regulation of cytosolic calcium ion concentration	4	0.046	AVP, OXT, DLG4, GPER1
GO-MFs			
Neurohypophyseal hormone activity	2	0.011	AVP, OXT
Cytochrome c oxidase activity	3	0.013	NDUFA4, COX7C, COX6C
Neuregulin binding	2	0.028	ERBB3, ITGAV
GO-CCs			
Mitochondrial inner membrane	8	0.014	NDUFA4, UQCR11, SLC25A25, COX7C, ROMO1, MRPL30, NDUFB1, COX6C
Extracellular space	14	0.046	INA, AVP, CXCL5, ERBB3, ADAMTS13, OXT, FETUB, AMH, IFNAR2, C1QTNF5, CLEC3B, CD59, SEMA4D, PRSS33
Cell junction	7	0.05	CNIH2, OTOF, PRRT1, DLG4, PAF1, GPER1, GPR142

Table 2: The results of Kyoto Encyclopedia of Genes and Genomes (KEGG) of DEGs ranked by *P* value.

Term	Count	*P* value	Genes
Nonalcoholic fatty liver disease (NAFLD)	6	0.0017	NDUFA4, ATF4, UQCR11, COX7C, NDUFB1, COX6C
Huntington's disease	6	0.0048	NDUFA4, UQCR11, DLG4, COX7C, NDUFB1, COX6C
Oxidative phosphorylation	5	0.0071	NDUFA4, UQCR11, COX7C, NDUFB1, COX6C
Parkinson's disease	5	0.0089	NDUFA4, UQCR11, COX7C, NDUFB1, COX6C
Alzheimer's disease	5	0.0158	NDUFA4, UQCR11, COX7C, NDUFB1, COX6C

process (BP) analysis. For the cellular component (CC) group, mitochondrial inner membrane, extracellular space, and cell junction were the enriched terms. Molecular function (MF) analysis showed that the DEGs were remarkably focused on neurohypophyseal hormone activity, cytochrome c oxidase activity, and neuregulin binding. Moreover, the KEGG pathway analysis indicated that the DEGs were significantly involved in nonalcoholic fatty liver disease, Huntington's disease, Alzheimer's disease, and Parkinson's disease as well as oxidative phosphorylation.

3.3. PPI Network and Hub Gene Identification. There were 363 nodes and 409 edges in the PPI network (Figure 3). In this PPI network, sixteen genes with degrees > 10 were found as key genes (Table 3). The node size is influenced by the fold change between FT1D patients and healthy controls, and the red or orange color nodes indicate that they have a higher score. The core of the whole PPI network was the most key genes in this cluster, including NCOA1, SRF, ERBB3, ETS1, TOP1, UBE2S, INO80, COX7C, ITGAV, COX6C, ATF4, PAF1, YARS, TTI1, UBC, EEF1B2, and AHSA1. Thence, the seventeen genes were recognized as the hub genes.

3.4. miRNA-DEG and TF-DEG Regulating Network Analysis. The miRNAs and TFs for DEGs are displayed in Figures 4 and 5, respectively. The top five targeted genes regulated by miRNA are shown in Supplementary Table 2. It turned out that 167 miRNAs regulate LDLR, 124 miRNAs regulate

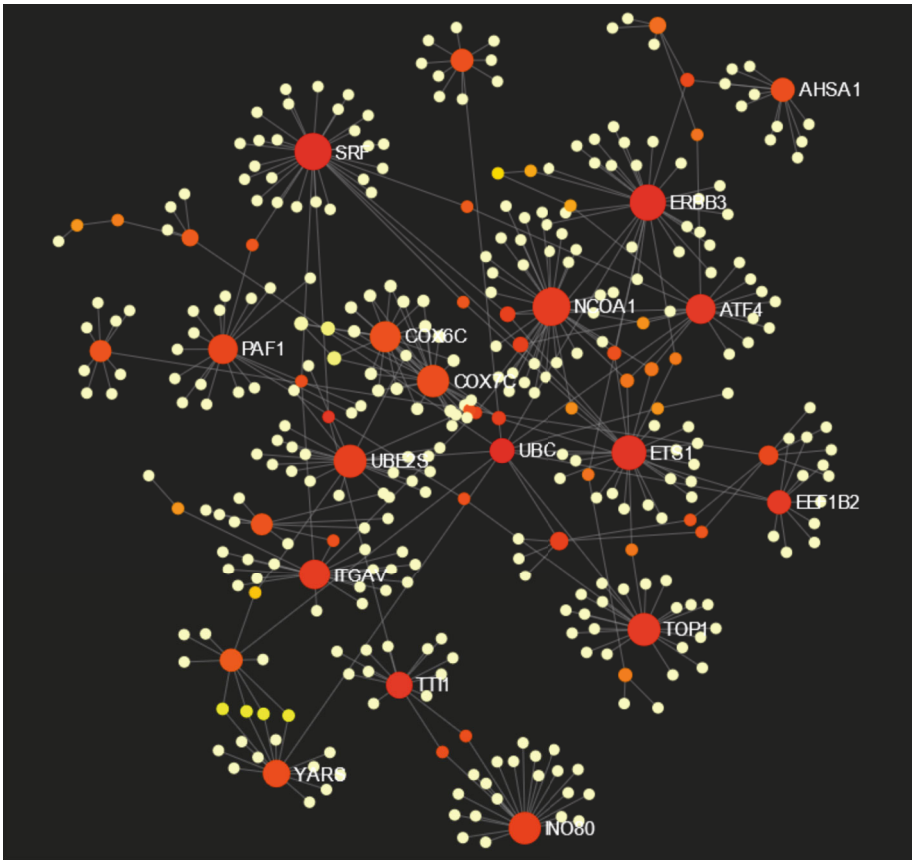


Figure 3: Protein-protein interaction network of the differentially expressed genes. Red and orange nodes stand for hub genes.

Table 3: Sixteen genes with degrees < 10 in the protein-protein interaction network of the differentially expressed genes.

Gene	Regulation	Degree	Betweenness	Expression
ETS1	Up	26	15103.54	1.145
AHSA1	Up	11	3565	0.82
TOP1	Up	23	10312.37	0.764
NCOA1	Up	34	9967.16	0.752
PAF1	Up	18	5908.12	0.732
SRF	Up	31	22498.06	0.647
YARS	Up	15	4237.33	0.644
INO80	Up	22	7030.5	0.606
ITGAV	Down	20	9973.24	-0.603
ATF4	Down	18	11878.52	-0.705
COX6C	Down	20	3314.17	-0.759
COX7C	Down	22	4037.83	-0.801
EEF1B2	Down	11	11109	-0.817
UBE2S	Down	23	7532.83	-0.858
TTI1	Down	14	11460.5	-1.226
ERBB3	Down	29	19037.55	-1.422

POTEM, 109 miRNAs regulate IFNAR2, 107 miRNAs regulate BAZ2A, and 92 miRNAs regulate SRF. The top five targeted genes regulated by TFs are shown in Supplementary Table 3. It turned out that 25 TFs regulate SRF, 18 TFs regulate TSPAN4, 16 TFs regulate CD59, 16 TFs regulate ETS1, and 15 TFs regulate SLC25A25.

4. Discussion

FT1D is a disease with a state of insulin dependency due to the rapid destruction of almost all pancreatic β cells, which causes the radical onset of ketoacidosis in a few days after the appearance of hyperglycemic symptoms [25–27]. It has been reported that most of the patients with FT1D are found in East Asia, but recently, Western countries also reported this disease [8, 28, 29]. FT1D makes up about 20% of abrupt-onset T1DM cases in Japan [8]. It is important to understand the molecular mechanisms of FT1D. We downloaded and analyzed a dataset (GSE44314) that contains five FT1D patients and six healthy controls from the GEO database. We identified 130 DEGs in total, including 60 upregulated DEGs and 70 downregulated DEGs. Among the 130 DEGs, we noticed that programmed cell death-1 (PD-1) was downregulated in FT1D patients. PD-1 is a critical member of the B7-CD28 family and is one of the important costimulatory molecules [30]. PD-1 can regulate the T cell response and keep maintaining peripheral tolerance by delivering critical inhibitory signals [30]. Inhibiting the PD-1 pathway would bring about excessive T cell proliferation, failure of tolerance, and autoimmune activation [31].

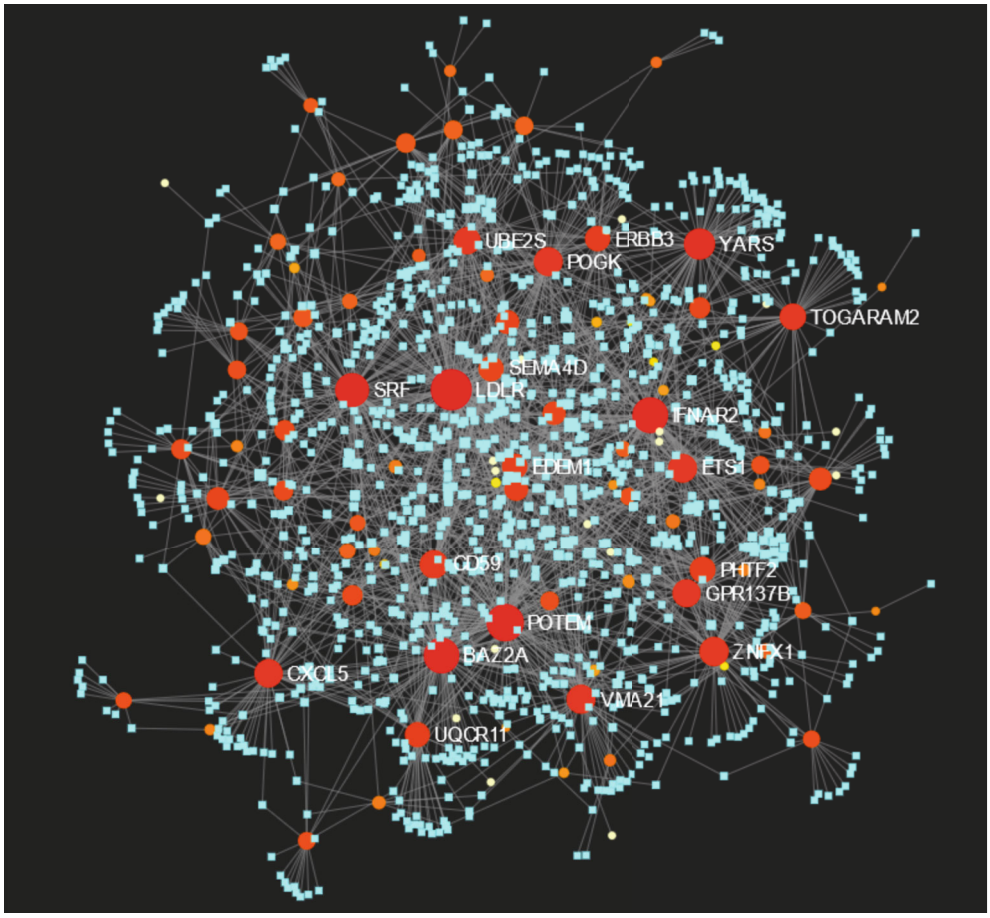


Figure 4: Target gene-miRNA regulatory network. Red and orange nodes stand for differentially expressed genes; blue diamonds stand for miRNA.

Therefore, PD-1 has gained popularity in the treatment of several advanced cancers [32, 33]. Studies have proved that treatment with PD-1 inhibitors can cause FT1D [34–36]. And the termination of anti-PD1 antibody therapy may preserve inherent insulin secretion capacity in "anti-PD1 antibody-induced" FT1D [37]. It seems that PD-1 should be upregulated in FT1D, which is totally opposite to our result. Various researchers have identified that cellular immunity, especially T cell, played a crucial role in β cell destruction in FT1D [38–40]. However, a Japanese study that compares PD-1 expression in peripheral CD4+ T cells between type 1A diabetes (classical type 1 diabetes), FT1D, and healthy controls found that there is no difference between FT1D and healthy controls in PD-1 expression and that there is lower PD-1 expression in CD4+ T cells in patients with type 1A diabetes [41]. Different studies have different conclusions in PD-1 expression in FT1D, which need further studies to confer this question and explore how PD-1 take part in the occurrence and progression of FT1D. Among the increased DEGs, NK2 homeobox 3 (NKX2-3) is the most upregulated gene in FT1D, and an animal study has indicated that NKX2-3 is related to T1DM [42], but further study is needed to figure out how NKX2-3 acts in FT1D.

In the current study, the most significant GO BP term for DEGs is generation of precursor metabolites. UQCR11, COX7C, and COX6C are the new biomarkers for the progression of FT1D. The most significant GO MF term for DEGs is neurohypophyseal hormone activity. Arginine vasopressin (AVP) and oxytocin are associated with type 2 diabetes but are new biomarkers for the progression of FT1D. The most significant GO CC term for DEGs is mitochondrial inner membrane. NDUFA4, SLC25A25, ROMO1, MRPL30, and NDUFB1 are novel biomarkers for the development of FT1D. Nonalcoholic fatty liver disease is the most significant KEGG pathway for DEGs. Activation of activating transcription factor 4 (ATF4) contributes to diabetic hepatotoxicity by ER stress [43]. Besides, ATF4 is a transcription factor implicated in β cell survival and susceptibility to stress [44]. ATF4 is a new biomarker for the progression of FT1D. Parkinson's disease, Alzheimer's disease, and Huntington's disease also are significant KEGG pathways for DEGs. Diabetes mellitus (DM) adversely affects multiple organ systems, including the brain [45]. These evidences suggest that FT1D may also lead to neurodegenerative diseases and adversely affect cognition. Discs large MAGUK scaffold protein 4 (DLG4) is related to neurological disorders and type 2 diabetes [46–48]; DLG4 is a new biomarker for the progression of FT1D.

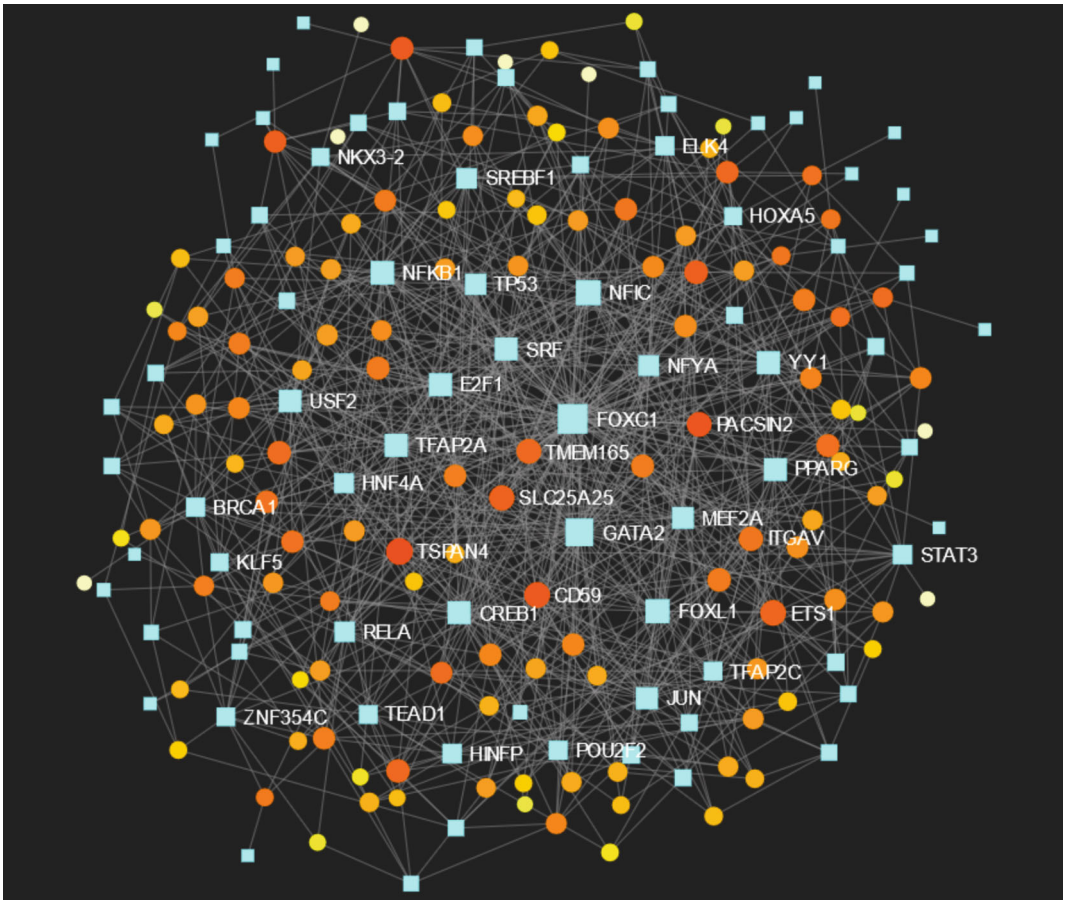


Figure 5: Transcription factor-target DEG regulatory network. Orange and yellow nodes stand for differentially expressed genes; blue diamonds stand for transcription factors.

In the present study, NCOA1, SRF, ERBB3, ETS1, TOP1, UBE2S, INO80, COX7C, ITGAV, and COX6C were recognized as top 10 hub genes in the PPI network. A genome-wide meta-analysis study confirmed that nuclear receptor coactivator 1 (NCOA1) is a T1DM susceptibility gene [49]. An animal study suggests that serum response factor (SRF) is decreased in diabetic nephropathy compared to healthy controls [50]. Many studies confirmed that ERBB3 was the most important T1DM association locus in the non-HLA gene [51–53]. ETS proto-oncogene 1 (EST1) was found associated with T1DM in the NOD mouse and then confirmed in human population [54–56]. Tissues derived from the T1DM animals show that DNA topoisomerase I (TOP1) activity and enzyme protein level decreased, whereas the enzyme mRNA level was not altered, which demonstrates that TOP1 activity is regulated by high glucose levels and may lead to the pathogenesis of diabetic complications [57]. Ubiquitin-conjugating enzyme E2 (UBE2S) takes part in T1DM by enhancing M2 macrophage polarization [58]. Jin et al. compared integrin subunit alpha V (ITGAV) expression between diabetic nephropathy and normal human kidney and found that ITGAV is higher in diabetic nephropathy [59]. Although there are evidences that the hub genes are contacted with T1DM, they are novel biomarkers for the development of FT1D.

LDLR, POTEM, IFNAR2, BAZ2A, and SRF were identified as top five targeted genes in the miRNA-target gene regulatory network. Low-density lipoprotein receptor (LDLR) is increased in a NOD mouse compared with a nondiabetic mouse [60]. A study in Ins2(Akita)Ldlr^{-}/$^{-}$ mice revealed that lack of LDLR will accelerate atherosclerosis in T1DM animals [61]. When lacking the r type II interferon receptor (IFNAR2), diabetes happened only in female NOD mice [62]. POTEM and BAZ2A are novel biomarkers for the development of FT1D. SRF, TSPAN4, CD59, ETS1, and SLC25A25 were identified as top five targeted genes in the TF-DEG regulatory network. Due to the lack of complement regulatory protein CD59, the development of diabetes-induced atherosclerosis in mice is accelerated [63]. Besides, CD59 is reduced in diabetic subjects compared with healthy controls [64]. Tetraspanin 4 (TSPAN4) is a new biomarker for the progression of FT1D.

We noticed that there are two bioinformatics analysis of type 1 diabetes, and there are some the same conclusions between our study and theirs [65, 66]. Fang et al. reported that programmed cell death ligand 1 (PD-L1) was upregulated in the new-onset T1DM samples [66]. This is identical with our result. PD-1/PD-L1 is a negative modulatory signaling pathway for activation of T cell. The upregulated PD-L1 and downregulated PD-l cause the same result, which are

the inactivation of T cell and the progression of immune tolerance, which play a protective role in the pathogenesis of T1DM. Liu et al. found that HLA-DQA1 and HLA-DRB4 might be targets for the treatment of T1D, and IL8 is likely to be a new marker for the diagnosis of T1D [65]. These results indicated that T1DM is an autoimmune disease, which is in accordance with our result.

5. Conclusions

Our data provide a comprehensive bioinformatics analysis of DEGs to search molecular mechanisms related to the progression of FT1D. We found a set of useful genes for future research into the molecular mechanisms of FT1D progression, while further molecular biological experiments are needed to confirm the effect of these DEGs in the progression of FT1D.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interests associated with the manuscript.

Acknowledgments

The authors thank Nakata and his colleges for depositing the dataset of GSE44314. This study is supported by grants from the National Natural Science Foundation of China (Grant Numbers 81770843 and 81974107).

Supplementary Materials

Supplementary Table 1: the list of all differentially expressed genes. Supplementary Table 2: the top five targeted genes regulated by miRNA. Supplementary Table 3: the top five targeted genes regulated by transcription factor. *(Supplementary Materials)*

References

[1] A. Imagawa, T. Hanafusa, J. Miyagawa, and Y. Matsuzawa, "A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies," *The New England Journal of Medicine*, vol. 342, no. 5, pp. 301–307, 2000.

[2] S. Luo, X. Ma, X. Li, Z. Xie, and Z. Zhou, "Fulminant type 1 diabetes: a comprehensive review of an autoimmune condition," *Diabetes/Metabolism Research and Reviews*, vol. 36, article e3317, 2020.

[3] Y. Hosokawa, T. Hanafusa, and A. Imagawa, "Pathogenesis of fulminant type 1 diabetes: genes, viruses and the immune mechanism, and usefulness of patient-derived induced pluripotent stem cells for future research," *Journal of Diabetes Investigation*, vol. 10, no. 5, pp. 1158–1164, 2019.

[4] L. Liu, L. Zeng, D. Sang, Z. Lu, and J. Shen, "Recent findings on fulminant type 1 diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 34, no. 1, 2018.

[5] A. Imagawa, T. Hanafusa, Y. Uchigata et al., "Different contribution of class II HLA in fulminant and typical autoimmune type 1 diabetes mellitus," *Diabetologia*, vol. 48, no. 2, pp. 294–300, 2005.

[6] E. Kawasaki, A. Imagawa, H. Makino et al., "Differences in the contribution of the CTLA4 gene to susceptibility to fulminant and type 1A diabetes in Japanese patients," *Diabetes Care*, vol. 31, no. 8, pp. 1608–1610, 2008.

[7] Y. Kawabata, on behalf of the Committee on Type 1 Diabetes, J. D. Society et al., "Differential association of HLA with three subtypes of type 1 diabetes: fulminant, slowly progressive and acute-onset," *Diabetologia*, vol. 52, no. 12, pp. 2513–2521, 2009.

[8] A. Imagawa, T. Hanafusa, Y. Uchigata et al., "Fulminant type 1 diabetes: a nationwide survey in Japan," *Diabetes Care*, vol. 26, no. 8, pp. 2345–2352, 2003.

[9] A. Imagawa, T. Hanafusa, H. Makino, J. I. Miyagawa, and P. Juto, "High titres of IgA antibodies to enterovirus in fulminant type-1 diabetes," *Diabetologia*, vol. 48, no. 2, pp. 290–293, 2005.

[10] S. Shibasaki, A. Imagawa, S. Tauriainen et al., "Expression of toll-like receptors in the pancreas of recent-onset fulminant type 1 diabetes," *Endocrine Journal*, vol. 57, no. 3, pp. 211–219, 2010.

[11] S. Tanaka, Y. Nishida, K. Aida et al., "Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes," *Diabetes*, vol. 58, no. 10, pp. 2285–2291, 2009.

[12] N. Ohara, M. Kaneko, T. Nishibori et al., "Fulminant type 1 diabetes mellitus associated with Coxsackie virus type A2 infection: a case report and literature review," *Internal Medicine*, vol. 55, no. 6, pp. 643–646, 2016.

[13] S. Yoneda, A. Imagawa, K. Fukui et al., "A histological study of fulminant type 1 diabetes mellitus related to human cytomegalovirus reactivation," *The Journal of Clinical Endocrinology and Metabolism*, vol. 102, no. 7, pp. 2394–2400, 2017.

[14] K. Aida, Y. Nishida, S. Tanaka et al., "RIG-I- and MDA5-initiated innate immunity linked with adaptive immunity accelerates beta-cell death in fulminant type 1 diabetes," *Diabetes*, vol. 60, no. 3, pp. 884–889, 2011.

[15] S. Nakata, A. Imagawa, Y. Miyata et al., "Low gene expression levels of activating receptors of natural killer cells (NKG2E and CD94) in patients with fulminant type 1 diabetes," *Immunology Letters*, vol. 156, no. 1-2, pp. 149–155, 2013.

[16] J. Xia, C. D. Fjell, M. L. Mayer, O. M. Pena, D. S. Wishart, and R. E. Hancock, "INMEX–a web-based tool for integrative meta-analysis of expression data," *Nucleic Acids Research*, vol. 41, no. W1, pp. W63–W70, 2013.

[17] J. Xia, E. E. Gill, and R. E. Hancock, "NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data," *Nature Protocols*, vol. 10, no. 6, pp. 823–844, 2015.

[18] W. H. Da, B. T. Sherman, and R. A. Lempicki, "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources," *Nature Protocols*, vol. 4, no. 1, pp. 44–57, 2009.

[19] M. Ashburner, C. A. Ball, J. A. Blake et al., "Gene ontology: tool for the unification of biology," *Nature Genetics*, vol. 25, no. 1, pp. 25–29, 2000.

[20] M. Kanehisa and S. Goto, "KEGG: Kyoto Encyclopedia of Genes and Genomes," *Nucleic Acids Research*, vol. 28, no. 1, pp. 27–30, 2000.

[21] J. Xia, M. J. Benner, and R. E. Hancock, "NetworkAnalyst–integrative approaches for protein-protein interaction network analysis and visual exploration," *Nucleic Acids Research*, vol. 42, no. W1, pp. W167–W174, 2014.

[22] C. H. Chou, S. Shrestha, C. D. Yang et al., "miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions," *Nucleic Acids Research*, vol. 46, no. D1, pp. D296–D302, 2018.

[23] I. S. Vlachos, M. D. Paraskevopoulou, D. Karagkouni et al., "DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions," *Nucleic Acids Research*, vol. 43, no. D1, pp. D153–D159, 2015.

[24] A. Khan, O. Fornes, A. Stigliani et al., "JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework," *Nucleic Acids Research*, vol. 46, no. D1, p. D1284, 2018.

[25] T. Hanafusa and A. Imagawa, "Fulminant type 1 diabetes: a novel clinical entity requiring special attention by all medical practitioners," *Nature Clinical Practice, Endocrinology & Metabolism*, vol. 3, no. 1, pp. 36–45, 2007.

[26] A. Imagawa and T. Hanafusa, "Fulminant type 1 diabetes–an important subtype in East Asia," *Diabetes/Metabolism Research and Reviews*, vol. 27, no. 8, pp. 959–964, 2011.

[27] A. Imagawa, T. Hanafusa, T. Awata et al., "Report of the Committee of the Japan Diabetes Society on the research of fulminant and acute-onset type 1 diabetes mellitus: new diagnostic criteria of fulminant type 1 diabetes mellitus (2012)," *Journal of Diabetes Investigation*, vol. 3, no. 6, pp. 536–539, 2012.

[28] T. S. Jung, S. I. Chung, M. A. Kim et al., "A Korean patient with fulminant autoantibody-negative type 1 diabetes," *Diabetes Care*, vol. 27, no. 12, pp. 3023-3024, 2004.

[29] C. Moreau, D. Drui, G. Arnault-Ouary, B. Charbonnel, L. Chaillous, and B. Cariou, "Fulminant type 1 diabetes in Caucasians: a report of three cases," *Diabetes & Metabolism*, vol. 34, no. 5, pp. 529–532, 2008.

[30] M. E. Keir, Y. E. Latchman, G. J. Freeman, and A. H. Sharpe, "Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes," *Journal of Immunology*, vol. 175, no. 11, pp. 7372–7379, 2005.

[31] E. Lazar-Molnar, B. Chen, K. A. Sweeney et al., "Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 30, pp. 13402–13407, 2010.

[32] K. C. Ohaegbulam, A. Assal, E. Lazar-Molnar, Y. Yao, and X. Zang, "Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway," *Trends in Molecular Medicine*, vol. 21, no. 1, pp. 24–33, 2015.

[33] A. V. Balar and J. S. Weber, "PD-1 and PD-L1 antibodies in cancer: current status and future directions," *Cancer Immunology, Immunotherapy : CII*, vol. 66, no. 5, pp. 551–564, 2017.

[34] L. Marchand, A. Thivolet, S. Dalle et al., "Diabetes mellitus induced by PD-1 and PD-L1 inhibitors: description of pancreatic endocrine and exocrine phenotype," *Acta Diabetologica*, vol. 56, no. 4, pp. 441–448, 2019.

[35] M. Okamoto, M. Okamoto, K. Gotoh et al., "Fulminant type 1 diabetes mellitus with anti-programmed cell death-1 therapy," *Journal of Diabetes Investigation*, vol. 7, no. 6, pp. 915–918, 2016.

[36] M. Araujo, D. Ligeiro, L. Costa et al., "A case of fulminant type 1 diabetes following anti-PD1 immunotherapy in a genetically susceptible patient," *Immunotherapy*, vol. 9, no. 7, pp. 531–535, 2017.

[37] G. Sakai, D. Saito, R. Nakajima et al., "Intrinsic insulin secretion capacity might be preserved by discontinuing anti-programmed cell death protein 1 antibody treatment in 'anti-programmed cell death protein 1 antibody-induced' fulminant type 1 diabetes," *Journal of Diabetes Investigation*, vol. 9, no. 2, pp. 448-449, 2018.

[38] A. Shimada, J. Morimoto, K. Kodama et al., "T-cell-mediated autoimmunity may be involved in fulminant type 1 diabetes," *Diabetes Care*, vol. 25, no. 3, pp. 635-636, 2002.

[39] A. Shimada, Y. Oikawa, T. Shigihara, T. Senda, and K. Kodama, "A case of fulminant type 1 diabetes with strong evidence of autoimmunity," *Diabetes Care*, vol. 25, no. 8, pp. 1482-1483, 2002.

[40] K. Aoki, M. Taniyama, C. Nagayama, Y. Oikawa, and A. Shimada, "T cell immunity to glutamic acid decarboxylase in fulminant type 1 diabetes without significant elevation of serum amylase," *Annals of the New York Academy of Sciences*, vol. 1079, no. 1, pp. 181–185, 2006.

[41] R. Fujisawa, F. Haseda, C. Tsutsumi et al., "Low programmed cell death-1 (PD-1) expression in peripheral CD4(+) T cells in Japanese patients with autoimmune type 1 diabetes," *Clinical and Experimental Immunology*, vol. 180, no. 3, pp. 452–457, 2015.

[42] H. Weiss, A. Bleich, H. J. Hedrich et al., "Genetic analysis of the LEW.1AR1-iddm rat: an animal model for spontaneous diabetes mellitus," *Mammalian Genome*, vol. 16, no. 6, pp. 432–441, 2005.

[43] V. K. Pandey, A. Mathur, M. F. Khan, and P. Kakkar, "Activation of PERK-eIF2α-ATF4 pathway contributes to diabetic hepatotoxicity: attenuation of ER stress by Morin," *Cellular Signalling*, vol. 59, pp. 41–52, 2019.

[44] C. A. Juliana, J. Yang, C. E. Cannon, A. L. Good, M. W. Haemmerle, and D. A. Stoffers, "A PDX1-ATF transcriptional complex governs β cell survival during stress," *Molecular Metabolism*, vol. 17, pp. 39–48, 2018.

[45] J. Chen, L. Liang, L. Zhan et al., "ZiBuPiYin recipe protects db/db mice from diabetes-associated cognitive decline through improving multiple pathological changes," *PLoS One*, vol. 9, no. 3, article e91680, 2014.

[46] G. H. Kim, E. C. Park, S. H. Yun et al., "Proteomic and bioinformatic analysis of membrane proteome in type 2 diabetic mouse liver," *Proteomics*, vol. 13, no. 7, pp. 1164–1179, 2013.

[47] Y. Matsunaga, T. Negishi, A. Hatakeyama, Y. Kawagoe, E. Sawano, and T. Tashiro, "Impairment of synaptic development in the hippocampus of diabetic Goto-Kakizaki rats," *International Journal of Developmental Neuroscience*, vol. 53, no. 1, pp. 58–67, 2016.

[48] Z. Tucsek, M. Noa Valcarcel-Ares, S. Tarantini et al., "Hypertension-induced synapse loss and impairment in synaptic plasticity in the mouse hippocampus mimics the aging phenotype: implications for the pathogenesis of vascular cognitive impairment," *GeroScience*, vol. 39, no. 4, pp. 385–406, 2017.

[49] J. P. Bradfield, H. Q. Qu, K. Wang et al., "A genome-wide meta-analysis of six type 1 diabetes cohorts identifies multiple associated loci," *PLoS Genetics*, vol. 7, no. 9, article e1002293, 2011.

[50] S. Kostic, B. Williams, S. Ksouri et al., "Changes in snail and SRF expression in the kidneys of diabetic rats during ageing," *Acta Histochemica*, vol. 122, no. 1, p. 151460, 2020.

[51] C. Sun, H. Wei, X. Chen et al., "ERBB3-rs2292239 as primary type 1 diabetes association locus among non-HLA genes in Chinese," *Meta Gene*, vol. 9, pp. 120–123, 2016.

[52] B. I. Frohnert, M. Laimighofer, J. Krumsiek et al., "Prediction of type 1 diabetes using a genetic risk model in the Diabetes Autoimmunity Study in the Young," *Pediatric Diabetes*, vol. 19, no. 2, pp. 277–283, 2018.

[53] D. Wang and G. Pan, "The association between rs2292239 Polymorphism in ERBB3 Gene and type 1 diabetes: a meta-analysis," *BioMed Research International*, vol. 2019, Article ID 7689642, 7 pages, 2019.

[54] M. Prochazka, D. V. Serreze, S. M. Worthen, and E. H. Leiter, "Genetic control of diabetogenesis in NOD/Lt mice. Development and analysis of congenic stocks," *Diabetes*, vol. 38, no. 11, pp. 1446–1455, 1989.

[55] J. M. Aparicio, A. Wakisaka, A. Takada, N. Matsuura, and T. Yoshiki, "Non-HLA genetic factors and insulin dependent diabetes mellitus in the Japanese: TCRA, TCRB and TCRG, INS, THY1, CD3D and ETS1," *Disease Markers*, vol. 8, no. 5, 294 pages, 1990.

[56] J. M. Aparicio, "HLA and non-HLA genetic factors in Japanese IDDM, [Hokkaido igaku zasshi]," *The Hokkaido Journal of Medical Science*, vol. 66, no. 6, pp. 780–793, 1991.

[57] I. Levi, Y. Segev, and E. Priel, "Type 1 diabetes affects topoisomerase I activity and GlcNAcylation in rat organs: kidney, liver and pancreas," *Glycobiology*, vol. 22, no. 5, pp. 704–713, 2012.

[58] F. Wang, F. Sun, J. Luo et al., "Loss of ubiquitin-conjugating enzyme E2 (Ubc9) in macrophages exacerbates multiple low-dose streptozotocin-induced diabetes by attenuating M2 macrophage polarization," *Cell Death & Disease*, vol. 10, no. 12, p. 892, 2019.

[59] D. K. Jin, A. J. Fish, E. A. Wayner et al., "Distribution of integrin subunits in human diabetic kidneys," *Journal of the American Society of Nephrology: JASN*, vol. 7, no. 12, pp. 2636–2645, 1996.

[60] L. M. Aaron-Brooks, T. Sasaki, R. E. Vickman et al., "Hyperglycemia and T cell infiltration are associated with stromal and epithelial prostatic hyperplasia in the nonobese diabetic mouse," *The Prostate*, vol. 79, no. 9, pp. 980–993, 2019.

[61] C. Zhou, B. Pridgen, N. King, J. Xu, and J. L. Breslow, "Hyperglycemic Ins2AkitaLdlr(-)/(-) mice show severely elevated lipid levels and increased atherosclerosis: a model of type 1 diabetic macrovascular disease," *Journal of Lipid Research*, vol. 52, no. 8, pp. 1483–1493, 2011.

[62] J. A. Carrero, N. D. Benshoff, K. Nalley, and E. R. Unanue, "Type I and II interferon receptors differentially regulate type 1 diabetes susceptibility in male versus female NOD mice," *Diabetes*, vol. 67, no. 9, pp. 1830–1835, 2018.

[63] F. Liu, R. Sahoo, X. Ge et al., "Deficiency of the complement regulatory protein CD59 accelerates the development of diabetes-induced atherosclerosis in mice," *Journal of Diabetes and its Complications*, vol. 31, no. 2, pp. 311–317, 2017.

[64] A. Soggiu, C. Piras, L. Bonizzi, H. A. Hussein, S. Pisanu, and P. Roncada, "A discovery-phase urine proteomics investigation in type 1 diabetes," *Acta Diabetologica*, vol. 49, no. 6, pp. 453–464, 2012.

[65] H. Liu, R. Xu, X. Liu, R. Sun, and Q. Wang, "Bioinformatics analysis of gene expression in peripheral blood mononuclear cells from children with type 1 diabetes in 3 periods," *Experimental and Clinical Endocrinology & Diabetes*, vol. 122, no. 8, pp. 477–483, 2014.

[66] C. Fang, Y. Huang, Y. Pei et al., "Genome-wide gene expression profiling reveals that CD274 is up-regulated new-onset type 1 diabetes mellitus," *Acta Diabetologica*, vol. 54, no. 8, pp. 757–767, 2017.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 5093493, 11 pages
https://doi.org/10.1155/2020/5093493

Research Article

Functional Crosstalk between CB and TRPV1 Receptors Protects Nigrostriatal Dopaminergic Neurons in the MPTP Model of Parkinson's Disease

Rayul Wi,[1] Young Cheul Chung,[2] and Byung Kwan Jin[1,2]

[1]*Department of Neuroscience, Graduate School, School of Medicine, Kyung Hee University, Seoul 02447, Republic of Korea*
[2]*Department of Biochemistry & Molecular Biology, School of Medicine, Kyung Hee University, Seoul 02447, Republic of Korea*

Correspondence should be addressed to Young Cheul Chung; ychung01@khu.ac.kr and Byung Kwan Jin; bkjin@khu.ac.kr

Received 29 May 2020; Revised 31 August 2020; Accepted 8 September 2020; Published 28 September 2020

Academic Editor: Lihua Duan

The present study examined whether crosstalk between cannabinoid (CB) and transient potential receptor vanilloid type 1 (TRPV1) could contribute to the survival of nigrostriatal dopamine neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD). MPTP induced a significant loss of nigrostriatal dopamine neurons and glial activation in the substantia nigra (SN) and striatum (STR) as visualized by tyrosine hydroxylase (TH) or macrophage antigen complex-1 (MAC-1) or glial fibrillary acidic protein (GFAP) immunocytochemistry, respectively. RT-PCR analysis shows the upregulation of inducible nitric oxide synthase, interleukin-1β, and tumor necrosis factor-α in microglia in the SN in vivo, indicating the activation of the inflammatory system. By contrast, treatment with capsaicin (a specific TRPV1 agonist) increased the survival of dopamine neurons in the SN and their fibers and dopamine levels in the STR in MPTP mice. Capsaicin neuroprotection is accompanied by inhibiting MPTP-induced glial activation and production of inflammatory cytokines. Treatment with AM251 and AM630 (CB1/2 antagonists) abolished capsaicin-induced beneficial effects, indicating the existence of a functional crosstalk between CB and TRPV1. Moreover, treatment with anandamide (an endogenous agonist for both CB and TRVP1) rescued nigrostriatal dopamine neurons and reduced gliosis-derived neuroinflammatory responses in MPTP mice. These results suggest that the cannabinoid and vanilloid system may be beneficial for the treatment of neurodegenerative diseases, such as PD, that are associated with neuroinflammation.

1. Introduction

Parkinson's disease (PD) is a well-known neurodegenerative disorder that is characterized by the degeneration of dopamine neurons in the substantia nigra pars compacta (SNpc) and dopamine deficiency in the striatum (STR), consequently resulting in motor dysfunction [1, 2]. Several lines of clinical and experimental evidence suggest that proteinopathy related to α-synuclein, environmental toxins, mitochondrial dysfunction, and oxidative stress is associated with the molecular mechanisms of PD etiology [3–5]. Among them, gliosis-derived neuroinflammation is a considerable part of PD development [3, 5]. Recent evidence has shown that reactive microglia and astrocytes are known to play a crucial role in the production of proinflammatory mediators such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), myeloperoxidase, and proinflammatory cytokines [5–7]. These inflammatory mediators are attributable to the degeneration of nigrostriatal dopamine neurons in animal models of PD and PD patients [3, 5, 7]. Current experimental studies, such as the development of neuroprotective agents on dopamine neurons through regulating glial activation and preventing production of neurotoxic inflammatory molecules, have provided opportunities to develop innovative strategies for PD therapy [5].

Transient receptor potential vanilloid subtype 1 (TRPV1), a polymodal and nonselective cation channel, is activated by a number of endogenous and exogenous stimuli, including natural vanilloids (capsaicin and resiniferatoxin), heat, acids, and endocannabinoids such as anandamide

(AEA) [8, 9]. TRPV1 is widely present in various neurons and glial cells (microglia and astrocytes) in the nigrostriatal pathway *in vivo* [10–12]. Many experimental studies demonstrated that TRPV1 activation by capsaicin (CAP) prevents the degeneration of nigrostriatal dopamine neurons in the 1-methyl-4-phenylpyridinium- (MPP^+-) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) or 6-hydroxy dopamine (6-OHDA-) lesioned rodent model of PD via inhibiting glial-derived inflammatory responses and producing ciliary neurotrophic factor (CNTF) [11–13]. We recently demonstrated that TRPV1 activation by CAP increased the survival of nigral dopamine neurons by modulating the M1/M2 microglia/macrophage phenotype in lipopolysaccharide- (LPS-) injected SN [10], indicating that TRPV1 is a possible therapeutic target to treat PD.

Due to the presence of an intracellular binding site for AEA [8] and colocalization of TRPV1 and CB receptors *in vivo* and *in vitro* [14, 15], TRPV1 is thought to be a possible ionotropic receptor counterpart for cannabinoid (CB) receptors. A pharmacological study has reported that both TRPV1 and CB1 are involved with AEA-mediated neuroprotection in the *in vivo* rat model of ouabain-induced excitotoxicity [16] and pentylenetetrazole-induced seizures [17]. However, crosstalk between TRPV1 and CB receptors that possibly influence the fate of dopamine neurons in PD was not determined yet. The present study investigates functional interactions between these two receptors, which might contribute to the survival of dopamine neurons by regulating gliosis-derived neuroinflammation in the MPTP-intoxicated PD mouse model *in vivo*.

2. Materials and Methods

2.1. Chemicals. The following chemicals were purchased from the following companies: AEA, AM251, and AM630 were purchased from Tocris Bioscience, Bristol, UK, and CAP and MPTP were purchased from Sigma-Aldrich, St. Louis, MO, USA. AEA, AM251, and AM630 were dissolved in 1% dimethyl sulfoxide and then diluted with sterile phosphate-buffered saline (PBS). CAP were dissolved in ethanol and Tween-80 and then diluted with phosphate-buffered saline (PBS; 1 : 1 : 8, ethanol : Tween-80 : PBS).

2.2. Animals and Drug Treatment. All experiments were conducted in accordance with the approved animal protocols and guidelines established by Kyung Hee University (KHUASP(SE)-10-030). As previously described [11, 18–21], C57BL/6 mice (eight-ten-week-old male mice) received four intraperitoneal injections of PBS or MPTP (20 mg/kg, free base; Sigma-Aldrich) dissolved in saline at 2-hour intervals. For the experimental group, mice received a single injection of CAP (0.5 mg/kg) into the peritoneum 30 min before the MPTP injections. To inhibit activation of CB receptors, animals received AM251 (CB1 receptor antagonist, 0.1 mg/kg) and AM630 (CB2 receptor antagonist, 0.1 mg/kg) into the peritoneum 30 min before treatment with CAP each day for 7 days (Figure 1(a)). For another experimental group, mice received a single injection of PBS as a control or AEA (0.5 mg/kg) after 12 hours from the last MPTP injection (Figure 2(a)).

2.3. Tissue Preparation and Immunohistochemistry. As previously described [11, 18, 19], animals were perfused with saline solution (0.9%) containing heparin (1,000 units/l) and fixed with 4% paraformaldehyde solution in 0.1 M phosphate-buffered solution. Brain tissues were cryoprotected, and then, 30 μm sections were cut. Tissues were rinsed with PBS and then incubated with PBS containing 0.5% BSA and each of the following primary antibodies: rabbit anti-tyrosine hydroxylase (TH; 1 : 2000, Pel-Freez, Brown Deer, WI, USA) for dopamine neuron, rabbit anti-glial fibrillary acidic protein (GFAP; 1 : 5000, Neuromics, Edina, MN, USA) for astrocytes, and rat anti-CD11b (CD11b; 1 : 200, AbD Serotec, Oxford, UK) for microglia/macrophages. The following day, the brain sections were incubated with the appropriate secondary antibodies (biotinylated anti-rabbit or anti-rat antibody (1 : 400, KPL, Milford, MA, USA)) and developed with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) and 0.003% hydrogen peroxide in 0.1 M PBS.

2.4. Stereological Cell Counts. As described previously [11, 18, 19], midbrain sections were stained with TH and the total number of TH-positive neurons in the SNpc was counted in each animal group at 7-day postinjection (MPTP or saline) using the optical fractionator method performed on an Olympus Computer-Assisted Stereological Toolbox System version 2.1.4 (Olympus Danmark A/S, Ballerup, Denmark). To estimate the distribution of TH^+ cells in the region of interest, the region of interest was defined and then, the area of interest was precisely marked at low magnification. The total number of neurons was estimated according to the optical fractionator equation. More than 300 points over all sections of each specimen were analyzed.

2.5. Densitometric Analysis. The measurement of the optical density (OD) of the TH^+ fiber in the STR was performed with the Image-Pro Plus system (Version 4.0; Media Cybernetics Inc., Silver Spring, MD) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany), as previously described [11, 18, 19]. To control variations in background illumination, the average of background density readings from the corpus callosum was subtracted from that of the density of the STR for each section (an average of 6 coronal sections of STR in each group). The average of all sections in each animal group was calculated separately, and then, it was statistically processed.

2.6. Real-Time PCR. Animals in each experimental group were sacrificed at the indicated time point (Figures 1(a) and 2(a)), and the bilateral SN regions were immediately isolated. The SN tissues were processed for real-time PCR, as previously described [11, 18, 19]. Total RNA was prepared with RNAzol B (Tel-Test Inc., Friendwood, TX, USA), and reverse transcription was carried out with the SuperScript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The primer sequences used in this study were as follows: 5′-TGGACC

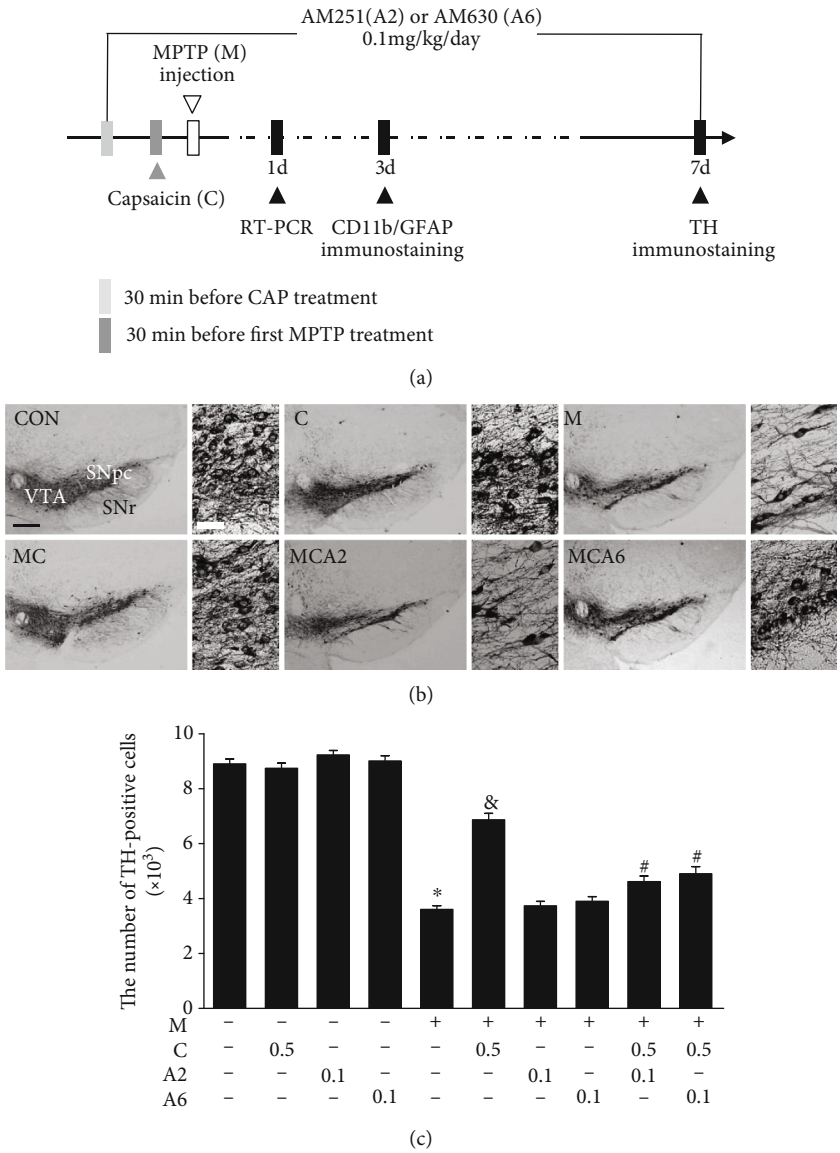


Figure 1: The CB receptor is associated with TRPV1-activated neuroprotection in the SN of MPTP-treated mice in vivo. (a) Diagram of the experimental design. Mice were intraperitoneally given an injection of PBS or MPTP. All mice intraperitoneally received PBS as a control or cannabinoid (CB) antagonist (AM251 (A2) or AM630 (A6); 0.1 mg/kg/day) for 7 days at 30 min before capsaicin (C) and 1 hour before MPTP and a single injection of capsaicin (0.5 mg/kg) at 30 min before MPTP. Mice that received PBS as a control (CON); capsaicin (C); MPTP (M); MPTP and capsaicin (MC); MPTP, capsaicin, and AM251 (MCA2); or MPTP, capsaicin, and AM630 (MCA6) were sacrificed at 7 days after the last MPTP injection. Brains were removed, and coronal sections (30 μm) were cut using a sliding microtome. Every sixth serial section was selected and processed for TH immunohistochemical staining. (b) Photomicrographs of TH^+ neurons in the SN. Higher magnification of each group for TH staining, respectively. These data are representative of five to six animals per group. (c) TH^+ neurons were counted using a stereological technique in the SN. Bars represent the means ± SEM of five to six animals per group. $^*P < 0.001$, significantly different from control. $^{\&}P < 0.001$, significantly different from MPTP. $^{\#}P < 0.001$, significantly different from MPTP and capsaicin (one-way ANOVA with the Neuman-Keuls post hoc test). SNpc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area. Scale bars: 250 μm (left panel for (b)). Scale bars: 50 μm (right panel for (b)).

TTCCAGGATGAGGACA-3′ (forward) and 5′-TTCATC TCGGAGCCTGTAGTG-3′ (reverse) for IL-1β; 5′-GCGA CGTGGAACTGGCAGAAGAG-3′ (forward) and 5′-TGAG AGGGAGGCCATTTGGGAAC-3′ (reverse) for TNF-α; 5′ -GAGACAGGGAAGTCTGAAGCAC-3′ (forward) and 5′ -CCAGCAGTAGTTGCTCCTCTTC-3′ (reverse) for iNOS; and 5′-CATCACTGCCACCCAGAAGACTG-3′ (forward) and 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′ (reverse) for GAPDH. Real-time PCRs were performed in a reaction volume of 20 ml, which included 1 ml of a reverse transcription product as a template, 10 ml of the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), and

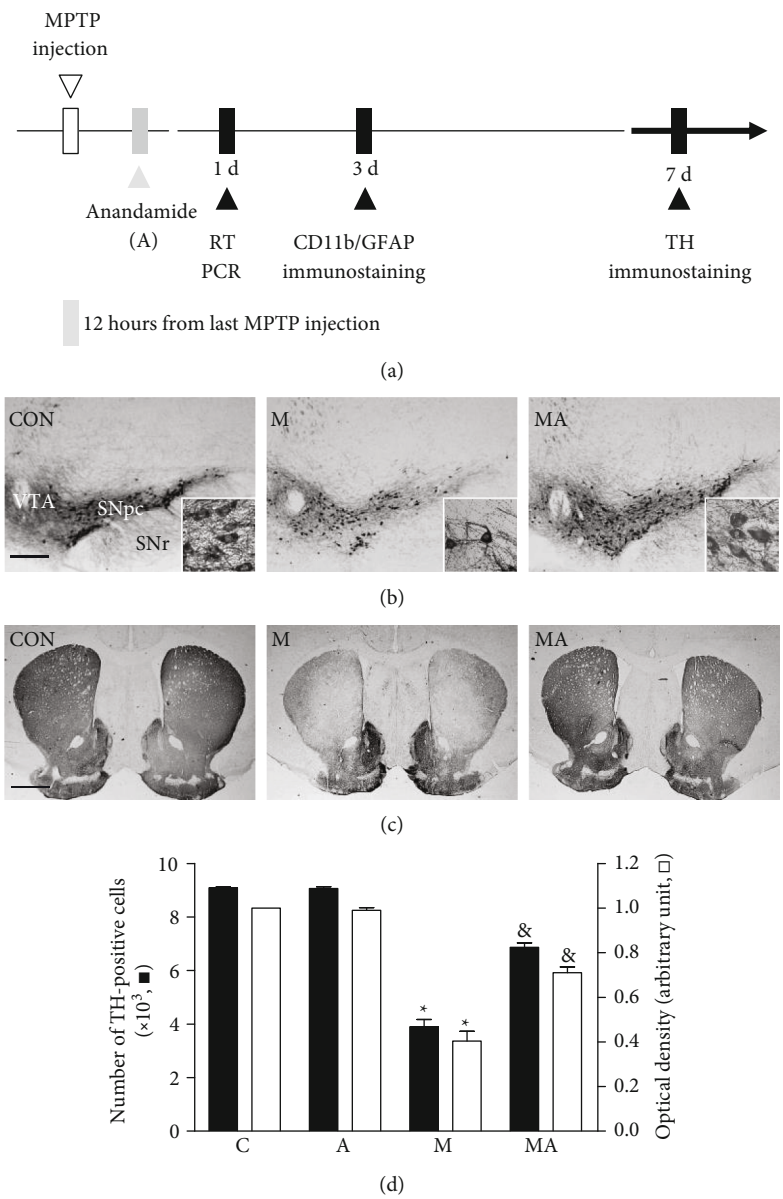


Figure 2: Anandamide prevents MPTP-induced degeneration of dopamine neurons in vivo. (a) Diagram of the experimental design. Mice were intraperitoneally given an injection of PBS as a control (CON) or MPTP only (M) or MPTP + anandamine (0.5 mg/kg) 12 hours after the last injection of MPTP (MA) and were sacrificed 7 days after the last MPTP injection. (b, c) Photomicrographs of TH^+ neurons in the SN (b) and TH^+ fibers in the striatum (c). Insets show higher magnifications of (b) and (c), respectively. (d) Number of TH^+ neurons in the SN pars compacta (SNpc) and optical density of TH^+ fibers in the striatum. Data are presented as means ± SEM of six animals per group. C: PBS-treated control; A: anandamide; M: MPTP; MA: MPTP and anandamide; SNpc: substantia nigra pars compacta; VTA: ventral tegmental area. $^*P < 0.001$, significantly different from control. $^{\&}P < 0.001$, significantly different from MPTP only (one-way ANOVA with the Neuman-Keuls post hoc test). Scale bars: (b) 300 μm; (c) 500 μm.

20 pmol of each primer described above. The PCR amplifications were performed with 40 cycles of 95°C for 30 s and 60°C for 60 s using ABI 7500 (Applied Biosystems). Average threshold cycle (C_T) values of IL-1β and TNF-α from triplicate PCR reactions were normalized from average C_T values of GAPDH.

2.7. Analysis for Microglia Morphology. To analyze the degree of microglial activation in MPTP-treated SN with the absence or presence of CAP and/or CB1/2 antagonists, images were obtained from the same area in each tissue sample using a light microscope (Zeiss Axioskop, Oberkochen, Germany) with 40x magnification (1024 × 1024 pixels). As previously described [22], all original images were collected, converted to 8-bit grayscale, filtered to further increase the contrast of images, and skeletonized using ImageJ. Then, the skeletonized images were selected, and the information for the branch length signal was analyzed using the plugin AnalyzeSkeleton (2D/3D).

2.8. Statistics. All values are expressed as the mean ± s.e.m.. Statistical significance ($P < 0.05$ for all analysis) was assessed

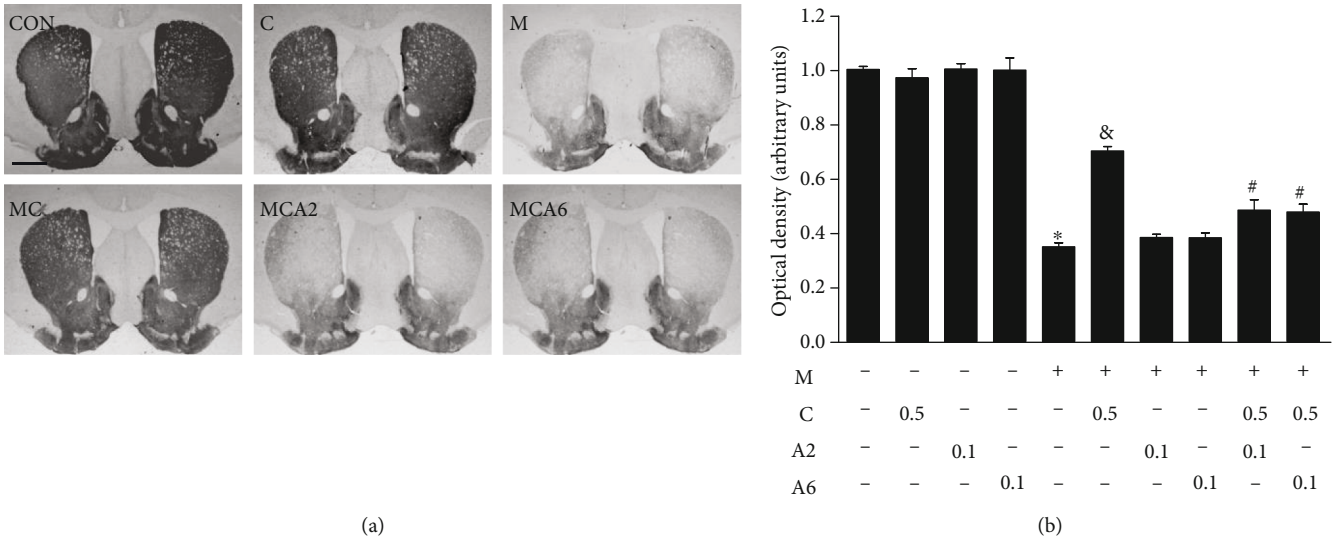


Figure 3: CB receptor is associated with TRPV1-activated neuroprotection in the striatum of MPTP-treated mice in vivo. The striatal tissues obtained from the same animals as those used in Figure 1 were immunostained with TH antibody for dopamine fibers. CON: PBS as control; C: capsaicin; M: MPTP; MC: MPTP and capsaicin; MCA2: MPTP, capsaicin, and AM251; MCA6: MPTP, capsaicin, and AM630. (a) Photomicrographs of TH^+ fibers in the striatum. These data are representative of five to six animals per group. (b) The optical density of TH^+ fibers in the striatum. Bars represent the means ± SEM of five to six animals per group. $^*P < 0.001$, significantly different from control. $^\&P < 0.001$, significantly different from MPTP. $^\#P < 0.001$, significantly different from MPTP and CAP (one-way ANOVA with the Neuman-Keuls post hoc test). Scale bars: 500 μm.

by the one-way ANOVA Newman-Keuls analyses using the Instat 3.05 software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. CB Receptor Contributes to TRPV1-Activated Neuroprotection in the Nigrostriatal Dopamine Pathway of MPTP-Treated Mice In Vivo. To examine the functional interactions between the CB receptor and TRPV1 in PD, we chose a mouse MPTP model of PD. Consistent with our previous reports [11, 18, 21], TH immunohistochemical analysis reveals the significant loss of TH^+ cells in the SN (Figure 1(b)) and TH^+ fibers in the STR (Figure 3(a)) in MPTP-treated mice compared to PBS-treated control mice (Figures 1(b) and 3(a)). The number of TH^+ cells as assessed by stereology in the SN and the density of TH^+ fibers in the STR are significantly reduced by 61% (Figure 1(c); $P < 0.001$) and 63% (Figure 3(b); $P < 0.001$), respectively, in MPTP-lesioned mice compared to PBS-treated control mice.

To determine the effects of TRPV1 activation on dopamine neurons, mice received intraperitoneally a single injection of the TRPV1 agonist capsaicin (CAP; 0.5 mg/kg) or vehicle at 30 min before MPTP treatment (Figure 1(a)). Similar to our previous reports [11, 23], pretreatment with CAP significantly increased the number of TH^+ cells by 35% in the SN (Figure 1(c); $P < 0.001$) and increased the density of TH^+ fibers by 40% in the striatum (Figure 3(b); $P < 0.001$) in MPTP-lesioned mice compared to MPTP-lesioned vehicle-treated mice. Pretreatment with CAP only as a control had no effects on TH^+ cells (Figures 1(b) and 1(c)) and TH^+ fibers (Figures 3(a) and 3(b)).

As a cannabinoid (CB) receptor can functionally interact with TRPV1 [15, 16], we wondered if the CB receptor could be associated with TRPV1-activated neuroprotection on nigrostriatal dopamine neurons *in vivo*. To test this, we inhibited the activation of the CB receptor using the CB1 antagonist AM251 and the CB2 receptor antagonist AM630. Mice intraperitoneally received the CB antagonists (AM251 or AM630; 0.1 mg/kg) or PBS each day for 7 days starting at 30 min before CAP treatment and 1 hour before MPTP treatment (Figure 1(a)). Pretreatment with AM251 and AM630 inhibited CAP neuroprotection against MPTP toxicity, resulting in a significant reduction of the number of TH^+ cells in the SN (Figure 1(c); $P < 0.001$) and a reduction of the density of TH^+ fibers in the striatum (Figure 3(b); $P < 0.001$) compared to the MPTP- and CAP-treated group. By contrast, pretreatment with CB antagonists did not prevent MPTP-induced degeneration of dopamine neurons in the absence of CAP treatment (Figures 1(c) and 3(b)). These results indicate that the CB receptor might regulate TRPV1-activated neuroprotection in MPTP-lesioned mice.

3.2. Interaction between CB Receptor and TRPV1 Regulates Proinflammatory Responses and Glial Activation In Vivo. As TRPV1 activation by CAP prevents the expression of proinflammatory molecules such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and iNOS, which can impose neurotoxicity on dopamine neurons in MPTP-lesioned mice [10, 11], we wondered if its anti-inflammatory effect could be associated with the CB1 and CB2 receptors in the MPTP model. Consistent with our previous work [11], analysis by real-time PCR showed that CAP efficiently

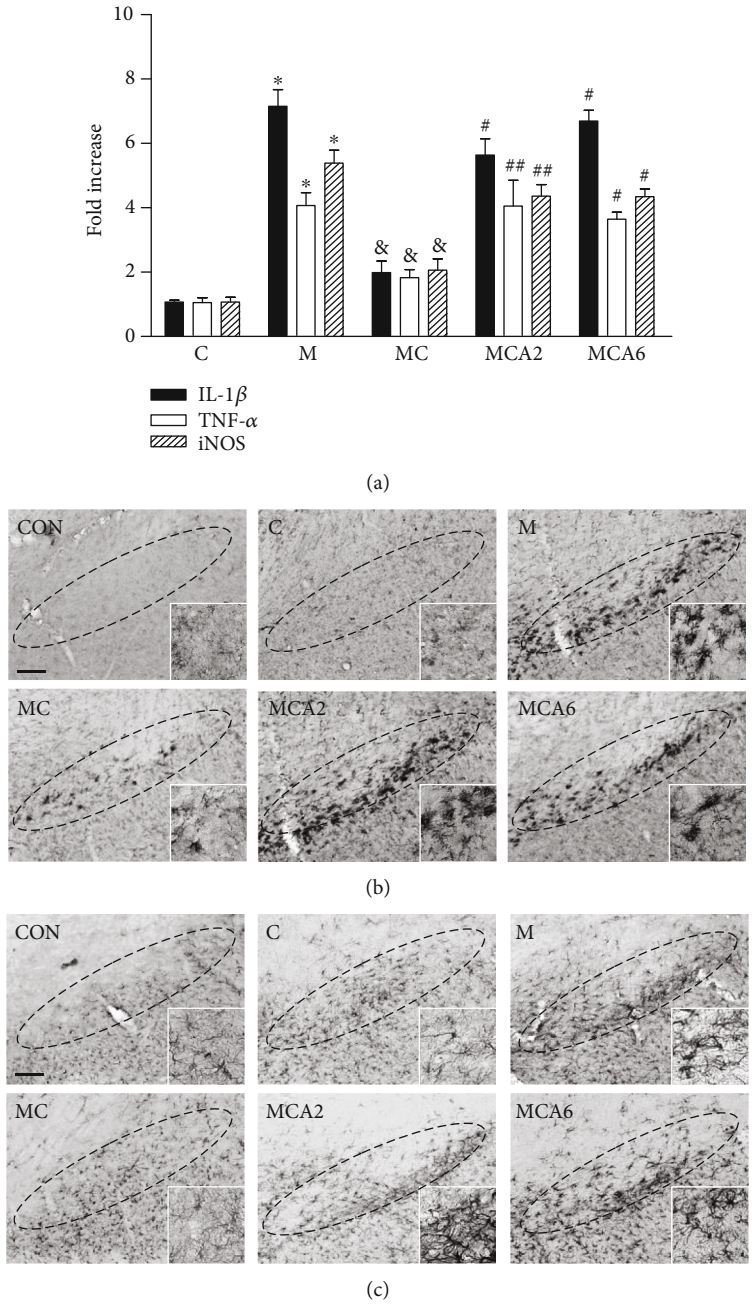


Figure 4: Crosstalk between CB and TRPV1 inhibits glial activation and expression of proinflammatory cytokines in the SN of MPTP-treated mice in vivo. Mice were intraperitoneally given an injection of PBS or MPTP. All mice intraperitoneally received vehicle as controls or cannabinoid (CB) antagonist (AM251 or AM630; 0.1 mg/kg/day) for 1 day or 3 days at 30 min before capsaicin (C) and 1 hour before MPTP and a single injection of capsaicin (0.5 mg/kg) at 30 min before MPTP. (a) Real-time PCR analysis showing mRNA expression of proinflammatory mediators (IL-1β, TNF-α, and iNOS) in the SN. Mice were sacrificed, and the total RNA was isolated from SN one day after the last injection of MPTP or vehicle in the absence or presence of CAP and CB1/2 antagonists (AM251 or AM630) (refer to Figure 1(a)). Bars represent the means ± SEM of four samples. C: control; M: MPTP; MC: MPTP and capsaicin; MCA2: MPTP, capsaicin, and AM251; MCA6: MPTP, capsaicin, and AM630. $^{*}P < 0.01$, significantly different from control. $^{\&}P < 0.01$: significantly different from MPTP. $^{\#}P < 0.01$ and $^{\#\#}P < 0.05$, significantly different from MPTP and capsaicin (one-way ANOVA with the Neuman-Keuls post hoc test). (b, c) Photomicrographs of CD11b^{+} microglia and GFAP^{+} astrocytes in the SN of MPTP-treated mice in vivo. Mice that received PBS as a control (CON); capsaicin (C); MPTP (M); MPTP and capsaicin (MC); MPTP, capsaicin, and AM251 (MCA2); or MPTP, capsaicin, and AM630 (MCA6) were sacrificed 3 days after the last MPTP injection (refer to Figure 1(a)). Brains were removed and coronal sections (30 μm) were cut using a sliding microtome. Every sixth serial section was selected and immunostained with CD11b antibody for microglia (b) or GFAP antibody for astrocytes (c). Insets show higher magnifications of (b) and (c), respectively. These data are representative of five to six animals per group. Dotted lines indicate SNpc. Scale bars: (a) 300-500 μm; (b) 250-420 μm.

decreased MPTP-induced proinflammatory responses in the SN (Figure 4(a)). These anti-inflammatory effects of CAP were significantly reversed by the treatment of AM251 and AM630, indicating interactions between TRPV1 and the CB1/2 receptors.

As the activation of glial cells such as microglia and astrocytes is involved in neurodegeneration in MPTP mice [3, 11, 18, 21], we next investigated whether interactions between the two receptors, TRPV1 and the CB receptor, could regulate glial activation in the SN *in vivo*. To test this, we performed immunostaining with the CD11b antibody for microglia and the GFAP antibody for astrocytes. In the MPTP-treated group, numerous $CD11b^+$-activated microglia (Figure 4(b)) and $GFAP^+$-reactive astrocytes (Figure 4(c)) were observed in the SN *in vivo* compared to PBS control (Figures 4(b) and 4(c)). In line with our recent reports [11, 18], TRPV1 activation by CAP suppressed microglial activation (Figure 4(b)) and astroglial activation (Figure 4(c)). These inhibitory effects of CAP on glial activation was reversed by treatment with AM251 and AM630 *in vivo* (Figures 4(b) and 4(c)). CB1 and CB2 antagonists alone had no effect on glial activation (data not shown). Moreover, analysis for microglia morphology showed that CAP significantly restored the MPTP-induced decrease in length of the microglia branch (increase in microglial activation) in the SN. This CAP effect on microglia morphology was reversed by treatment with AM251 and AM630 in vivo (Supplementary Figures 1(a) and 1(b)).

3.3. Anandamide Protects Nigrostriatal Dopamine Neurons from MPTP Neurotoxicity by Preventing Brain Inflammation and Gliosis In Vivo. Several lines of evidence have shown that TRPV1 activation by CAP produces anandamide (AEA), which activates TRPV1 and/or the CB receptor [15, 24, 25] and prevents neurodegeneration *in vivo* and *in vitro* [16, 26–28]. Accordingly, we determined whether AEA, when exogenously administered, could rescue nigrostriatal dopamine neurons. Mice intraperitoneally received AEA (0.5 mg/kg) for 1 day, starting at 12 hours after the last MPTP injection or PBS as a control (Figure 2(a)). The results of TH immunohistochemistry showed that MPTP caused the degeneration of nigrostriatal dopamine neurons, compared to PBS (Figures 2(b) and 2(c)). In MPTP-lesioned mice, posttreatment with AEA rescued TH^+ dopamine neurons in the SN *in vivo* (Figures 2(b) and 2(d)) and TH^+ fibers in the STR (Figures 2(c) and 2(d)) compared to MPTP-lesioned vehicle-treated mice. When quantified and expressed as a percentage of TH^+ neurons in the SN or TH^+ fibers in the STR of MPTP-lesioned mice, AEA was found to increase the number of TH^+ neurons by 41% (Figure 2(d); $P < 0.001$) and the optical density of TH^+ fibers by 36% (Figure 2(d); $P < 0.001$). AEA alone had no effects on the number of TH^+ neurons in the SN or TH^+ fibers in the STR (Figure 2(d)).

We next determined whether AEA neuroprotection on dopamine neurons could be associated with MPTP-induced expression of inflammatory cytokines in vivo. The expression of the mRNA levels of proinflammatory molecules was evaluated in animals receiving PBS, MPTP, and MPTP + AEA 1 day after the last MPTP injection. The results of RT-PCR showed that the mRNA levels of IL-1β, TNF-α, and iNOS were significantly increased in the SN of MPTP-lesioned mice compared with PBS-treated SN ($P < 0.01$; Figure 5(a)). Treatment with AEA reduced MPTP-induced increases in expression of IL-1β by 83% ($P < 0.01$; Figure 5(a)), TNF-α by 90% ($P < 0.01$; Figure 5(a)), and iNOS by 56% ($P < 0.05$; Figure 5(a)) in the SN. AEA alone had no effects on the mRNA levels of IL-1β, TNF-α, or iNOS.

Next, we analyzed the effects of AEA on glial activation by immunostaining with CD11b and GFAP in the SN 3 days after the last MPTP injection (Figures 2(a) and 5(b)). The majority of $CD11b^+$ microglia displayed an activated morphology, including larger cell bodies with short processes in the SN of MPTP-lesioned mice (Figure 5(b), left panel) compared to resting morphology in PBS-treated control (Figure 5(b)). Treatment with AEA dramatically attenuated the number of $CD11b^+$-activated microglia in the MPTP-lesioned SN (Figure 5(b)). This inhibitory effect of AEA on microglial activation was also confirmed by analysis of microglia morphology showing that AEA significantly restored the MPTP-induced decrease in the length of the microglia branch in the SN (Supplementary Figures 2(a)and 2(b)). Astrocytes also exhibited reactive morphology with thick processes (Figure 4(c)) in the MPTP-lesioned SN as determined by GFAP immunohistochemical staining. Treatment with AEA profoundly mitigated $GFAP^+$-reactive astrocytes in the MPTP-lesioned SN (Figure 5(b), right panel). A few of the $GFAP^+$ astrocytes were observed in the SN of PBS-treated mice (Figure 5(b)).

4. Discussion

The main findings of the present study are the existence and effects of the *in vivo* functional crosstalk between TRPV1 and the CB receptors, rescuing nigrostriatal dopamine neurons in the MPTP mouse model of PD. TRPV1-activated neuroprotection by capsaicin in the MPTP mouse is inhibited by antagonizing the CB1 receptor or the CB2 receptor. Capsaicin-induced inhibition of both glial activation and production of inflammatory mediators was also abolished by the treatment of CB antagonists. In addition, activation of two receptors (TRPV1 and CB receptor) by AEA showed the neuroprotective effects on the nigrostriatal dopamine neurons by inhibiting glial activation and production of inflammatory mediators in the MPTP mouse model of PD.

Neuroinflammation is considered the major neuropathological feature in neurodegenerative disorders including PD, Alzheimer's disease, frontal temporal dementia, and amyotrophic lateral sclerosis. Under neurodegenerative conditions, both microglia and astrocytes transformed to reactive phenotypes and participated in the production of inflammatory mediators in the central nervous system [29]. In the animal model of PD and in PD patients, reactive microglia/astrocytes (intense CD11b/GFAP immunoreactivity and hypertrophy) and increased level of proinflammatory mediators exist in the SNpc and STR, indicating the possible involvement of gliosis-derived inflammatory processes in PD [3, 5, 7, 30]. Many experimental studies have shown that inhibition of glial activation-derived inflammatory response contributes to a

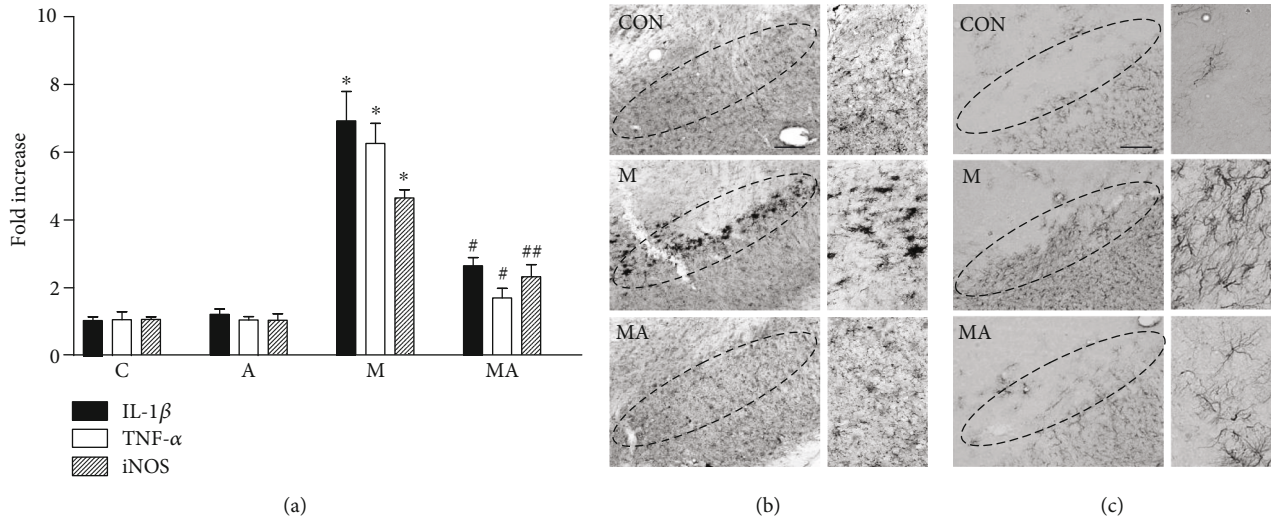


Figure 5: Anandamide inhibits glial activation and production of proinflammatory molecules in the SN of MPTP-treated mice in vivo. Mice were intraperitoneally given an injection of PBS as a control (CON) or MPTP only (M) or MPTP + anandamide (0.5 mg/kg; MA) 12 hours after the last injection of MPTP. (a) Real-time PCR showing the messenger RNA expression of proinflammatory molecules in the SN. The total RNA was isolated from the SN at 1 day after the last injection of MPTP or vehicle in the absence or presence of anandamide (refer to Figure 2(a)). These results are means ± SEM of four samples. C: control; A: anandamide; M: MPTP; MA: MPTP and anandamide. $^{*}P < 0.01$, significantly different from C. $^{\#}P < 0.01$ and $^{\#\#}P < 0.05$, significantly different from M (one-way ANOVA with the Neuman-Keuls post hoc test). (b, c) Photomicrographs of CD11b^{+} microglia and GFAP^{+} astrocytes in the SN of MPTP-treated mice in vivo. Mice that received PBS as a control (CON), MPTP (M), and MPTP and anandamide (MA) were sacrificed at 3 days after the last MPTP injection (refer to Figure 2(a)). Brains were removed, and coronal sections (30 μm) were cut using a sliding microtome. Every sixth serial section was selected and immunostained with CD11b antibody for microglia (b) or GFAP antibody for astrocytes (c). These data are representative of five to six animals per group. Dotted lines indicate SNpc. Scale bars: 300 μm (b); 250-420 μm (c).

protection of dopamine neurons *in vivo* and *in vitro* [3, 5, 31, 32]. Recent reports including ours show that the activation of TRPV1 by CAP [10, 11, 13] or the CB receptor by CB1/2 agonists [18, 21, 33] can prevent glial activation, oxidative stress, and expression of proinflammatory molecules *in vivo* in animal models of PD produced by the administration of MPTP, 6-OHDA, and LPS. These results are in line with the present data that TRPV1 activation by CAP or the activation of both TRPV1 and the CB receptor by AEA inhibits glial activation and production of proinflammatory mediators in MPTP-lesioned SN *in vivo*. Taken together, the present data suggest that the neuroprotective effect of CAP and AEA is associated with the property of TRPV1 and the CB receptor to block glial activation and production of inflammatory molecules in the MPTP mouse model of PD.

Anandamide (AEA) is an endogenous ligand for both TRPV1 and the CB receptor and modulates the endocannabinoid system in the central nervous system [8, 9, 17]. Pisani et al. reported that the level of AEA is increased in the cerebrospinal fluid of PD patients [34]. Molecular imaging studies reveal that the density of the CB1 receptor is increased in the putamen of PD patients and MPTP-treated marmosets, indicating an association of the endocannabinoid system with PD progression [35, 36]. Indeed, an increase in the levels of AEA by inhibiting fatty acid amide hydrolase (a degradation enzyme for AEA) enhanced TH immunoreactivity in the SN and STR and suppressed microglial activation in MPTP-treated mice [37]. This is in line with our data showing that exogenous delivery of AEA attenuates neurotoxicity and glial activation-derived inflammatory responses in MPTP-lesioned mice. Collectively, these results suggest that the upregulation of endocannabinoids might be implicated with the compensatory response which is aimed at reducing the loss of dopamine neurons and/or neuroinflammation in the animal model of PD and possibly in PD patients.

Several lines of evidence have shown that TRPV1 and CB receptors are widely colocalized in different types of neurons of the several brain areas including dopamine neurons in the SN [15, 38, 39]. Numerous studies including ours reported functional associations between TRPV1 and CB receptor, which is either neurotoxic [15, 40, 41] or neuroprotective [8, 16, 17] *in vitro* and *in vivo*. Regarding this, the present study demonstrates that the CB1 and CB2 receptor antagonists inhibit CAP-induced reduction in MPTP toxicity against dopamine neurons, glial activation, and mRNA expression of proinflammatory molecules, indicative of functional interactions between TRPV1 and CB receptors. This beneficial effect might result from molecules, which are synthesized by CAP-activated TRPV1 and are able to activate these two receptors (TRPV1 and CB receptors). Among them, AEA, an endogenous ligand for both TRPV1 and CB receptors, is synthesized by CAP-activated TRPV1 and activates both TRPV1 and CB receptors resulting in the neuroprotection in ouabain-induced excitotoxicity [16] and inhibition of locomotor activity *in vivo* [25]. The result of the present study shows that exogenous delivery of AEA attenuates MPTP neurotoxicity and glial activation-derived inflammatory responses. Taken together, our data carefully suggest that AEA synthesized by CAP-activated TRPV1 appears to inhibit glial activation-derived neuroinflammation and the resultant

survival of dopamine neurons in the MPTP mouse model of PD, although we did not provide any direct evidence of AEA synthesis by CAP-activated TRPV1.

MPTP is metabolized by astrocytic monoamine oxidase B (MAO-B) to 1-methyl-4-phenylpyridine (MPP^+), which is uptaken into dopamine neurons and then eventually leads to dopamine neuronal death [18, 19, 42]. Regarding this, we have shown that conversion of MPTP into MPP^+ is almost completed at 12 hours after the last MPTP injection [18, 19] and TRPV1 activation by CAP and CB receptor agonists does not interrupt the conversion of MPTP into MPP^+*in vivo* [11, 18]. Thus, it can rule out the possibilities that the observed neuroprotection by delayed treatment with AEA (12 hours after the last MPTP injection) might be attributable to reducing the metabolism of MPTP to MPP^+ or preventing MPP^+ uptake into dopamine neurons.

Finally, the present study suggests a novel neuroprotective mechanism for dopamine neurons, resulting from the crosstalk between TRPV1 and CB receptors in the MPTP mouse model of PD. The activation or interaction of TRPV1 and CB receptors may be beneficial for regulating the glial activation and production of proinflammatory mediators, resulting in an increased survival of dopamine neurons in MPTP-lesioned mice.

5. Conclusion

Finally, the present study suggests a novel neuroprotective mechanism for dopamine neurons, resulting from the crosstalk between TRPV1 and CB receptors in the MPTP mouse model of PD. The activation or interaction of TRPV1 and CB receptors may be beneficial for regulating the glial activation and production of proinflammatory mediators, resulting in an increased survival of dopamine neurons in MPTP-lesioned mice. Thus, the activation of both TRPV1 and CB receptors by compounds related to the endovanilloid/endocannabinoid system might constitute a new therapeutic strategy to treat PD.

Data Availability

The data used to support the findings of this study are all provided within the article.

Conflicts of Interest

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

Acknowledgments

This research was funded by the National Research Foundation of Korea (NRF) grant by the Korean Government (NRF-2010-355-C00080, NRF-2019R1I1A1A01061729, NRF-2019R1A2C2007897, NRF-2017M3C7A1031105, and NRF-2018R1A6A1A03025124).

Supplementary Materials

Supplementary 1. Supplementary Figure 1: crosstalk between CB and TRPV1 inhibits microglial activation in the SN of MPTP-treated mice in vivo. (a) The skeletonized images are processed using ImageJ with Skeleton plugin in Figure 4(b). (b) Quantification results of microglia process length in each animal groups. Bars represent the means ± SEM of five to six animals per group. $^{*}P < 0.001$, significantly different from control. $^{\&\&}P < 0.01$, significantly different from MPTP. $^{\#}P < 0.001$ and $^{\#\#}P < 0.01$, significantly different from MPTP and CAP (one-way ANOVA with the Neuman-Keuls post hoc test).

Supplementary 2. Supplementary Figure 2: anandamide suppresses microglial activation in the SN of MPTP-treated mice in vivo. (a) The skeletonized images are processed using ImageJ with Skeleton plugin in Figure 5(b). (b) Quantification results of microglia process length in each animal groups. Bars represent the means ± SEM of five to six animals per group. $^{*}P < 0.001$, significantly different from control. $^{\&}P < 0.001$, significantly different from MPTP (one-way ANOVA with the Neuman-Keuls post hoc test).

References

[1] J. M. Beitz, "Parkinson's disease: a review," *Frontiers in Bioscience (Scholar Edition)*, vol. 6, pp. 65–74, 2014.

[2] E. R. Dorsey, A. Elbaz, E. Nichols et al., "Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016," *The Lancet Neurology*, vol. 17, no. 11, pp. 939–953, 2018.

[3] Y. C. Chung, H. W. Ko, E. G. Bok et al., "The role of neuroinflammation on the pathogenesis of Parkinson's disease," *BMB Reports*, vol. 43, no. 4, pp. 225–232, 2010.

[4] P. Goswami, N. Joshi, and S. Singh, "Neurodegenerative signaling factors and mechanisms in Parkinson's pathology," *Toxicology In Vitro*, vol. 43, pp. 104–112, 2017.

[5] M. G. Tansey and M. S. Goldberg, "Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention," *Neurobiology of Disease*, vol. 37, no. 3, pp. 510–518, 2010.

[6] S. H. Huh, Y. C. Chung, Y. Piao et al., "Ethyl pyruvate rescues nigrostriatal dopaminergic neurons by regulating glial activation in a mouse model of Parkinson's disease," *Journal of Immunology*, vol. 187, no. 2, pp. 960–969, 2011.

[7] J. M. Taylor, B. S. Main, and P. J. Crack, "Neuroinflammation and oxidative stress: co-conspirators in the pathology of Parkinson's disease," *Neurochemistry International*, vol. 62, no. 5, pp. 803–819, 2013.

[8] G. Gambino, V. Rizzo, G. Giglia, G. Ferraro, and P. Sardo, "Cannabinoids, TRPV and nitric oxide: the three ring circus of neuronal excitability," *Brain Structure & Function*, vol. 225, no. 1, pp. 1–15, 2020.

[9] C. Muller, P. Morales, and P. H. Reggio, "Cannabinoid ligands targeting TRP channels," *Frontiers in Molecular Neuroscience*, vol. 11, p. 487, 2019.

[10] E. Bok, Y. C. Chung, K. S. Kim, H. H. Baik, W. H. Shin, and B. K. Jin, "Modulation of M1/M2 polarization by capsaicin contributes to the survival of dopaminergic neurons

in the lipopolysaccharide-lesioned substantia nigra in vivo," *Experimental & Molecular Medicine*, vol. 50, no. 7, pp. 1–14, 2018.

[11] Y. C. Chung, J. Y. Baek, S. R. Kim et al., "Capsaicin prevents degeneration of dopamine neurons by inhibiting glial activation and oxidative stress in the MPTP model of Parkinson's disease," *Experimental & Molecular Medicine*, vol. 49, no. 3, p. e298, 2017.

[12] J. H. Nam, E. S. Park, S. Y. Won et al., "TRPV1 on astrocytes rescues nigral dopamine neurons in Parkinson's disease via CNTF," *Brain*, vol. 138, no. 12, pp. 3610–3622, 2015.

[13] Z. Zhao, J. F. Wang, L. L. Wang et al., "Capsaicin protects against oxidative insults and alleviates behavioral deficits in rats with 6-OHDA-induced Parkinson's disease via activation of TRPV1," *Neurochemical Research*, vol. 42, no. 12, pp. 3431–3438, 2017.

[14] L. Cristino, L. de Petrocellis, G. Pryce, D. Baker, V. Guglielmotti, and V. di Marzo, "Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain," *Neuroscience*, vol. 139, no. 4, pp. 1405–1415, 2006.

[15] S. R. Kim, E. Bok, Y. C. Chung, E. S. Chung, and B. K. Jin, "Interactions between CB(1) receptors and TRPV1 channels mediated by 12-HPETE are cytotoxic to mesencephalic dopaminergic neurons," *British Journal of Pharmacology*, vol. 155, no. 2, pp. 253–264, 2008.

[16] W. B. Veldhuis, M. van der Stelt, M. W. Wadman et al., "Neuroprotection by the endogenous cannabinoid anandamide and arvanil against in vivo excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases," *The Journal of Neuroscience*, vol. 23, no. 10, pp. 4127–4133, 2003.

[17] S. S. Manna and S. N. Umathe, "Involvement of transient receptor potential vanilloid type 1 channels in the proconvulsant effect of anandamide in pentylenetetrazole-induced seizures," *Epilepsy Research*, vol. 100, no. 1-2, pp. 113–124, 2012.

[18] Y. C. Chung, E. Bok, S. H. Huh et al., "Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation," *Journal of Immunology*, vol. 187, no. 12, pp. 6508–6517, 2011.

[19] Y. C. Chung, S. R. Kim, and B. K. Jin, "Paroxetine prevents loss of nigrostriatal dopaminergic neurons by inhibiting brain inflammation and oxidative stress in an experimental model of Parkinson's disease," *Journal of Immunology*, vol. 185, no. 2, pp. 1230–1237, 2010.

[20] Y. C. Chung, S. R. Kim, J. Y. Park et al., "Fluoxetine prevents MPTP-induced loss of dopaminergic neurons by inhibiting microglial activation," *Neuropharmacology*, vol. 60, no. 6, pp. 963–974, 2011.

[21] Y. C. Chung, W.-H. Shin, J. Y. Baek et al., "CB2 receptor activation prevents glial-derived neurotoxic mediator production, BBB leakage and peripheral immune cell infiltration and rescues dopamine neurons in the MPTP model of Parkinson's disease," *Experimental & Molecular Medicine*, vol. 48, no. 1, article e205, 2016.

[22] K. Young and H. Morrison, "Quantifying microglia morphology from photomicrographs of immunohistochemistry prepared tissue using ImageJ," *JoVE (Journal of Visualized Experiments)*, vol. 136, no. 136, 2018.

[23] J. Y. Baek, J. Jeong, K. Kim et al., "Inhibition of microglia-derived oxidative stress by ciliary neurotrophic factor protects dopamine neurons in vivo from MPP(+) neurotoxicity," *International Journal of Molecular Sciences*, vol. 19, no. 11, p. 3543, 2018.

[24] V. Di Marzo, A. Fontana, H. Cadas et al., "Formation and inactivation of endogenous cannabinoid anandamide in central neurons," *Nature*, vol. 372, no. 6507, pp. 686–691, 1994.

[25] V. Di Marzo, I. Lastres-Becker, T. Bisogno et al., "Hypolocomotor effects in rats of capsaicin and two long chain capsaicin homologues," *European Journal of Pharmacology*, vol. 420, no. 2-3, pp. 123–131, 2001.

[26] I. Kotlar, E. Rangel-López, A. Colonnello et al., "Anandamide reduces the toxic synergism exerted by quinolinic acid and glutaric acid in rat brain neuronal cells," *Neuroscience*, vol. 401, pp. 84–95, 2019.

[27] L. D. Schurman and A. H. Lichtman, "Endocannabinoids: a promising impact for traumatic brain injury," *Frontiers in Pharmacology*, vol. 8, p. 69, 2017.

[28] C. Xu, D. J. Hermes, B. Nwanguma et al., "Endocannabinoids exert CB1 receptor-mediated neuroprotective effects in models of neuronal damage induced by HIV-1 Tat protein," *Molecular and Cellular Neurosciences*, vol. 83, pp. 92–102, 2017.

[29] S. Voet, S. Srinivasan, M. Lamkanfi, and G. Loo, "Inflammasomes in neuroinflammatory and neurodegenerative diseases," *EMBO Molecular Medicine*, vol. 11, no. 6, 2019.

[30] G. M. Halliday and C. H. Stevens, "Glia: initiators and progressors of pathology in Parkinson's disease," *Movement Disorders*, vol. 26, no. 1, pp. 6–17, 2011.

[31] H. M. Gao, B. Liu, W. Zhang, and J. S. Hong, "Critical role of microglial NADPH oxidase-derived free radicals in the in vitro MPTP model of Parkinson's disease," *The FASEB Journal*, vol. 17, no. 13, pp. 1954–1956, 2003.

[32] D. C. Wu, P. Teismann, K. Tieu et al., "NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6145–6150, 2003.

[33] E. S. Chung, E. Bok, Y. C. Chung, H. H. Baik, and B. K. Jin, "Cannabinoids prevent lipopolysaccharide-induced neurodegeneration in the rat substantia nigra in vivo through inhibition of microglial activation and NADPH oxidase," *Brain Research*, vol. 1451, pp. 110–116, 2012.

[34] V. Pisani, V. Moschella, M. Bari et al., "Dynamic changes of anandamide in the cerebrospinal fluid of Parkinson's disease patients," *Movement Disorders*, vol. 25, no. 7, pp. 920–924, 2010.

[35] R. Cilia, "Molecular imaging of the cannabinoid system in idiopathic Parkinson's disease," *International Review of Neurobiology*, vol. 141, pp. 305–345, 2018.

[36] I. Lastres-Becker, M. Cebeira, M. L. de Ceballos et al., "Increased cannabinoid CB1 receptor binding and activation of GTP-binding proteins in the basal ganglia of patients with Parkinson's syndrome and of MPTP-treated marmosets," *The European Journal of Neuroscience*, vol. 14, no. 11, pp. 1827–1832, 2001.

[37] J. M. Viveros-Paredes, R. E. Gonzalez-Castañeda, A. Escalante-Castañeda, A. R. Tejeda-Martínez, F. Castañeda-Achutiguí, and M. E. Flores-Soto, "Efecto del inhibidor de amida hidrolasa de ácidos grasos en el daño neuronal dopaminérgico inducido por MPTP," *Neurología*, vol. 34, no. 3, pp. 143–152, 2019.

[38] D. J. Cavanaugh, A. T. Chesler, A. C. Jackson et al., "Trpv1 reporter mice reveal highly restricted brain distribution and functional expression in arteriolar smooth muscle cells," *The Journal of Neuroscience*, vol. 31, no. 13, pp. 5067–5077, 2011.

[39] E. Mezey, Z. E. Toth, D. N. Cortright et al., "Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 7, pp. 3655–3660, 2000.

[40] H. Hermann, L. de Petrocellis, T. Bisogno, A. Schiano Moriello, B. Lutz, and V. di Marzo, "Dual effect of cannabinoid CB1 receptor stimulation on a vanilloid VR1 receptor-mediated response," *Cellular and Molecular Life Sciences*, vol. 60, no. 3, pp. 607–616, 2003.

[41] S. R. Kim, Y. C. Chung, E. S. Chung et al., "Roles of transient receptor potential vanilloid subtype 1 and cannabinoid type 1 receptors in the brain: neuroprotection versus neurotoxicity," *Molecular Neurobiology*, vol. 35, no. 3, pp. 245–254, 2007.

[42] V. Jackson-Lewis and S. Przedborski, "Protocol for the MPTP mouse model of Parkinson's disease," *Nature Protocols*, vol. 2, no. 1, pp. 141–151, 2007.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 4896727, 13 pages
https://doi.org/10.1155/2020/4896727

Research Article

B7-H4 Inhibits the Development of Primary Sjögren's Syndrome by Regulating Treg Differentiation in NOD/Ltj Mice

Xu Zheng,[1] Qikai Wang,[1,2] Xiang Yuan,[1] Yingbo Zhou,[1] Hui Chu,[1] Guosheng Wang,[1] Xiangpei Li,[1] Yiping Wang,[3] Li Wei,[4] Li Wang,[1] and Xiaomei Li[1]

[1]*Department of Rheumatology and Immunology, The First Affiliated Hospital of USTC, University of Science and Technology of China, Hefei, Anhui, China*
[2]*Department of Rheumatology and Immunology, The People's Hospital of Bozhou, Bozhou, Anhui, China*
[3]*Westmead Institute for Medical Research, University of Sydney, Westmead 2145 NSW, Australia*
[4]*Pharmacoepidemiology and Medication Safety Research Cluster, UCL School of Pharmacy, London WC1N 1AX, UK*

Correspondence should be addressed to Li Wang; wang_liy@aliyun.com and Xiaomei Li; lixiaomei@ustc.edu.cn

Received 21 June 2020; Revised 30 August 2020; Accepted 10 September 2020; Published 27 September 2020

Academic Editor: Lihua Duan

Background. This study is aimed at exploring the role of B7-H4 in the pathogenesis of primary Sjögren's syndrome (pSS) in NOD/Ltj mouse. *Methods*. B7-H4 expression in salivary glands was examined by IHC, and lymphocyte infiltration was showed by H&E. Next, anti-B7-H4 mAb or immunoglobulin isotype was injected into NOD/Ltj mice. Cytokine levels were measured by quantitative RT-PCR, and immunoglobulins were measured by ELISA. T cell subsets were analyzed by flow cytometry. Last, we treated NOD/Ltj mice with B7-H4Ig and control Ig. CD4+Foxp3+ T cells were assessed by immunohistochemistry. Two-tailed Student's *t*-tests were used to detect the statistical difference in various measures between the two groups. *Results*. B7-H4 expression was remarkably reduced in salivary glands of NOD/Ltj mice at 15 weeks compared with the NOD/Ltj mice at 8 weeks. Anti-B7-H4 mAb treatment increased lymphocyte infiltration in salivary glands. Inflammatory cytokines including IL-12, IL-18, IL-1α, TNF-α, IFN-α, and BAFF were upregulated markedly in anti-B7-H4 mAb-treated mice compared to IgG isotype-treated mice. Flow cytometry analysis showed that anti-B7-H4 mAb-treated mice had lower levels of CD4+Foxp3+/CD4+ T cells in spleen. Moreover, Foxp3 mRNA levels of salivary glands were diminished in anti-B7-H4 mAb-treated mice. Flow cytometry analysis showed that anti-B7-H4 mAb inhibited CD4+Foxp3+/CD4+ T cell production, while B7-H4Ig would promote naïve CD4+ T into Treg differentiation. Administration with B7-H4Ig displayed significantly decreased lymphocyte infiltration in salivary glands and low levels of total IgM and IgG in serum. Analysis of inflammatory cytokines in salivary glands after B7-H4Ig treatment revealed that the mRNA levels of IL-12, IL-6, IL-18, IL-1α, TNF-α, and IFN-α were significantly downregulated in B7-H4Ig-treated mice compared to control Ig treatment. B7-H4Ig-treated mice had significantly higher levels of CD4+Foxp3+/CD4+ T cells in spleen. IHC in salivary gland revealed that CD4+Foxp3+ T cells of B7-H4Ig treatment mouse were more than control Ig treatment. *Conclusions*. Our findings implicate that B7-H4 has a protective role for salivary gland epithelial cells (SGECs) and therapeutic potential in the treatment of pSS.

1. Introduction

Primary Sjögren's syndrome is a chronic, inflammatory autoimmune disease characterized by lymphocytic infiltration in the exocrine glands, especially the salivary and lacrimal glands, leading to a destruction of their functional components [1]. The disease may occur as primary Sjögren's syndrome (pSS) alone or in conjunction with another autoimmune disorder as secondary Sjögren's syndrome [2]. Although dry mouth and eyes are the hallmark symptoms of pSS, the disease affects other organs of the body and causes substantial morbidity [3]. Up to now, the underlying pathophysiologic mechanisms of Sjögren's syndrome remain obscure. NOD/Ltj is the most widely used pSS model animal exhibiting CD4+ lymphocyte infiltration, autoantibodies, and xerostomia. Analysis of lesion tissue of the salivary

glands shows a predominance of T lymphocytes surrounding ductal epithelial cells. The 70%~80% of these T cells are CD4+ T cells, 10% are CD8+ T cells, and the remaining infiltrating cells are B cells [4, 5]. In recent years, evidences have indicated that salivary gland epithelial cells (SGECs) in SS lesions were activated and played an important role in the induction and perpetuation of the inflammatory processes [6, 7]. Presence of costimulatory factors CD80, CD86, and CD40 on SGEC is capable to activate immune cells to secret Th1 cytokines. This leads a feed forward loop, resulting in upregulating expression of costimulatory molecules and adhesion molecules on SGEC [7].

B7-H4 is a member of the B7/CD28 costimulatory/coinhibitory superfamily. The role of B7-H4 in the inhibition of immune responses has shown in various in vitro [8–11] and in vivo studies [12–15]. Studies in experimental autoimmune encephalomyelitis (EAE) model have shown that blockage of endogenous B7-H4 by specific monoclonal antibody can promote T cell responses and accelerate the disease. These results indicate that B7-H4 has a capacity to regulate autoreactive T cell responses [9]. Moreover, deficiency in expression of B7-H4 in SGECs from pSS patients causes the lack of suppression of infiltrating CD4+ T cells [16].

In this study, we explored the regulatory mechanism of B7-H4 in the pathogenesis of pSS through B7-H4 monoclonal antibody or soluble B7-H4Ig fusion protein (intervening an Fc fusion of the extracellular domain of B7-H4 to mimic the natural ability of B7-H4). Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the decreased numbers and functions of Tregs in vivo and in vitro. The present results show that B7-H4 is expressed in glandular epithelial cells [16], and blockade of B7-H4 increases proinflammatory cytokines such as IL-12, IL-18, IL-1α, TNF-α, IFN-α, and BAFF, while reduces numbers of Tregs in NOD/Ltj mouse. Moreover, in salivary gland, we find that both TGF-β and IL-2 were reduced by anti-B7-H4 mAb [17]. In this study, we also found that B7-H4Ig treatment in NOD/Ltj mouse reduces proinflammatory cytokines, ameliorates lymphocyte infiltration, and increases the number and function of Tregs.

2. Methods

2.1. Mice Strains. Female nonobese diabetic (NOD/Ltj) mice or C57BL/6 (B6) mice were used in aged 8 weeks and were housed in a specific pathogen-free (SPF) room within the mouse facility of the Central Laboratory at Anhui Provincial Hospital. Mouse housing procedures were conducted according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998), and the breeding and use of these animals for the present studies were approved by the Institutional Animal Care and Use Committee of Anhui Provincial Hospital.

2.2. In Vivo Treatments. 8 weeks of NOD/Ltj mice were given i.p. injections with different doses of recombinant anti-B7-H4 mAb (AMP-110, Amplimmune) or IgG isotype (BD Bioscience). NOD/Ltj mice received 12.5 mg/kg of anti-B7-H4 mAb or the same dosage of IgG isotype for the first time, then were administered with anti-B7-H4 mAb 7.5 mg/kg or IgG isotype 7.5 mg/kg every 3 days for 2 weeks. To determine the therapeutic effect of B7-H4Ig (Chimerigen laboratories), female NOD/Ltj mice were administered with B7-H4Ig 7.5 mg/kg or control Ig (Chimerigen laboratories) 7.5 mg/kg every 3 days for 2 weeks [15] starting at the age of 8 weeks. All mice were sacrificed at 15 weeks of age.

2.3. Flow Cytometry Analysis and Antibodies. Percp-5.5 anti-mouse CD4, FITC anti-mouse CD3, APC anti-mouse IFN-γ, APC anti-mouse Foxp3 and PE anti-mouse IL17α, and isotype-matched mAbs were purchased from BD Biosciences; Fixation/Permeabilization Kit was purchased from eBioscience. Single-cell suspensions were generated from spleens and treated with RBC lysis buffer containing NH4Cl. Cells from spleen were fluorescently labeled by incubation with the indicated Abs in FACS buffer (PBS containing 0.3% BSA) for 30 min on ice. Subsequently, samples were washed and suspended in PBS containing 1% FCS. For intracellular cytokine analysis of IL-17A and IFN-γ, the splenocytes were stimulated for 4 h at 37°C with PMA (50 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma). Then, cells were permeabilized and fixed after surface staining and stained with fluorescence-conjugated antibodies or IgG controls. Data were acquired on a BD FACS CantoII.

2.4. Treg Induction Experiment. Naïve CD4+ T cells were isolated from the splenocytes using naive CD4+ T Cell Isolation Kit (Miltenyi), 5 × 10^5 cells/well were cultured with SGECs (5 × 10^5 cells/well) and stimulated with 1 μg/ml anti-CD3 (eBioscience) and 2 μg/ml anti-CD28 (eBioscience) plus anti-B7-H4 mAb (10 μg/ml) or IgG isotype (10 μg/ml) or B7-H4Ig (10 μg/ml) or control Ig (10 μg/ml). Cells were cultured in Treg-promoting (IL-2 30-50 U/ml, PeproTech, TGF-β 2 ng/ml, R&D Systems) conditions. After 3 days of culture, the percentage of Treg cells was detected by flow cytometry.

2.5. Pathological Assessment. NOD/Ltj mice were killed at 15 weeks of age, and blood serum had been collected. Submandibular salivary glands were snap frozen in OCT compound and cut into 6-μm serial sections (Zeiss); for histochemistry, salivary glands were embedded in paraffin, then sectioned (5 μm each) and stained with H&E. The focus score (FS) was defined as an aggregate of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue and stained with H&E for histological observation of mononuclear cell infiltration. Histological observations and photomicrography were performed using an Olympus microscope. For immunohistochemistry analysis, immunohistochemistry for mouse Foxp3 was performed as previously described, with minor modifications [18]. The antibodies used in this study were as follows: PE anti-mouse CD4 (Abcam), FITC anti-mouse Foxp3 (Abcam), and diaminidophenylindol (DAPI, Invitrogen). The number of CD4+ and CD4+Foxp3+ was counted in 5 consecutive high-power fields (40x) by means of 0.0314 mm^2 graticule fitted in the eyepiece of the microscope and expressed as ratio of CD4+Fopx3+/CD4+ T cells.

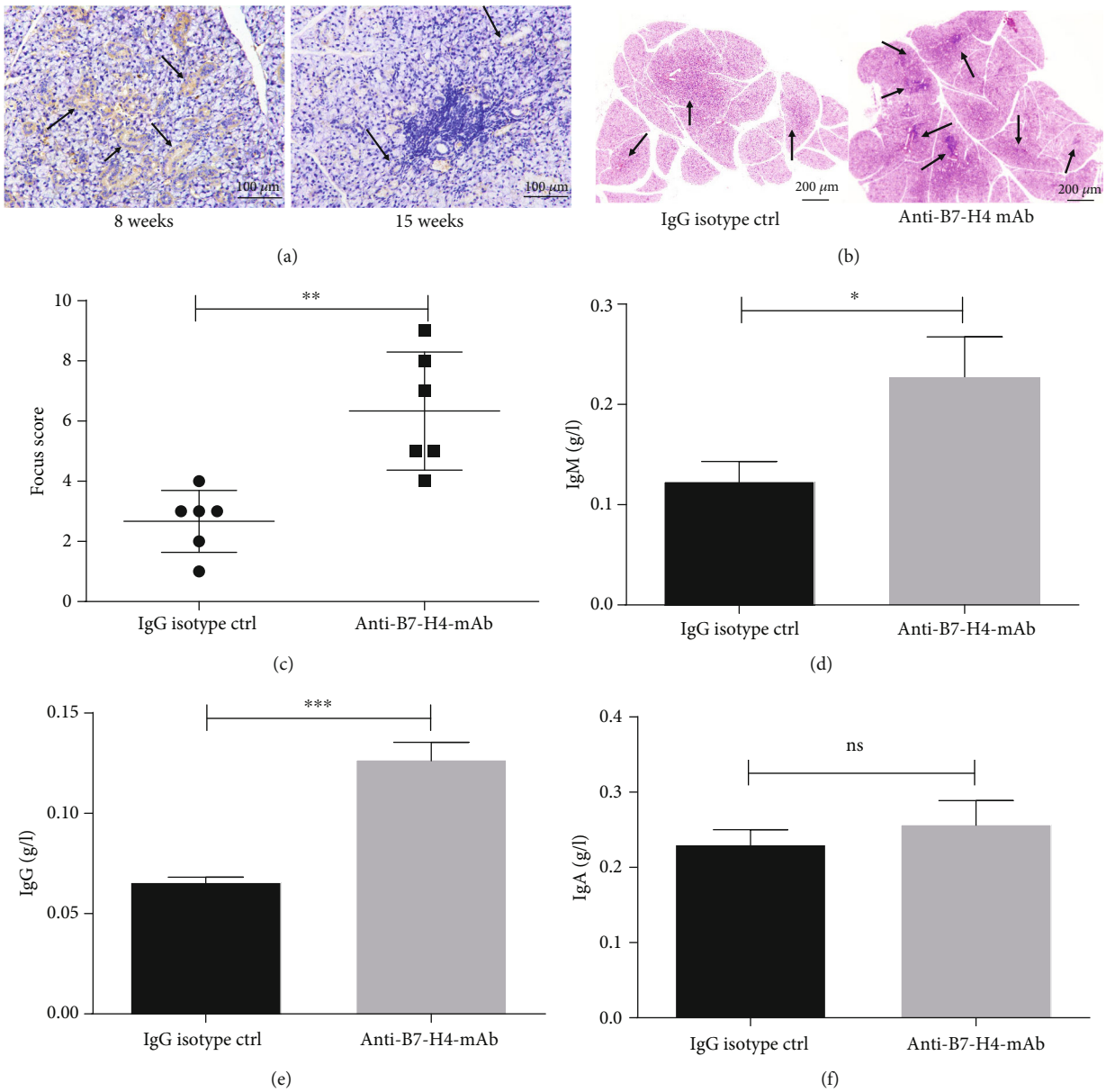


Figure 1: B7-H4 suppresses the progression of pSS in NOD/Ltj mice. (a) B7-H4 expression in salivary glands from 8 weeks or 15 weeks of NOD/Ltj mouse was examined by IHC (100x). Arrows indicate B7-H4 expression. (b, c) Female NOD/Ltj mice were injected with IgG isotype ctrl or anti-B7-H4 mAb from 8 weeks of age. The mice were sacrificed at 15 weeks of age. H&E stains of the salivary gland sections from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment mice. Arrows indicate infiltrates within the salivary gland (b). Histopathological assessment data are presented as FS (c). (d–f) Detection of pSS-associated autoantibodies in serum. Serum samples were collected from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment NOD/Ltj mice, and the levels of immunoglobulins were measured by ELISA. Immunoglobulin levels for (d) IgM, (e) IgG, and (f) IgA are shown. Two-tailed Student's *t*-test. All the data presented were from three independent experiments.

2.6. Quantitative Real-Time PCR (qRT-PCR) Assay. Total RNA was extracted using RNeasy (Qiagen) and was reverse transcribed to cDNA using Taqman reverse transcription reagents (Applied Biosystems) according to the manufacturer's instructions. Primer and probe sets were obtained from Applied Biosystems. qRT-PCR was performed using the Taqman Universal PCR Master Mix. Primers used to amplify specific gene fragments as follows:

IL-1α: 5′AAGACAAGCCTGTGTTGCTGAAGG (forward) and 5′TCCCAGAAGAAAATGAGGTCGGTC (reverse); TNF-α: 5′AGAAGTTCCCAAATGGCCT (forward) and 5′CCACTTGGTGGTTTGCTACG (reverse); IL-10: 5′CCAAGCCTTATCGGAAATGA (forward) and 5′TTTTCACAGGGGAGAAATCG (reverse); IL-2: 5′TGAGCAGGATGGAGAATTACAGG (forward) and 5′GTCCAA

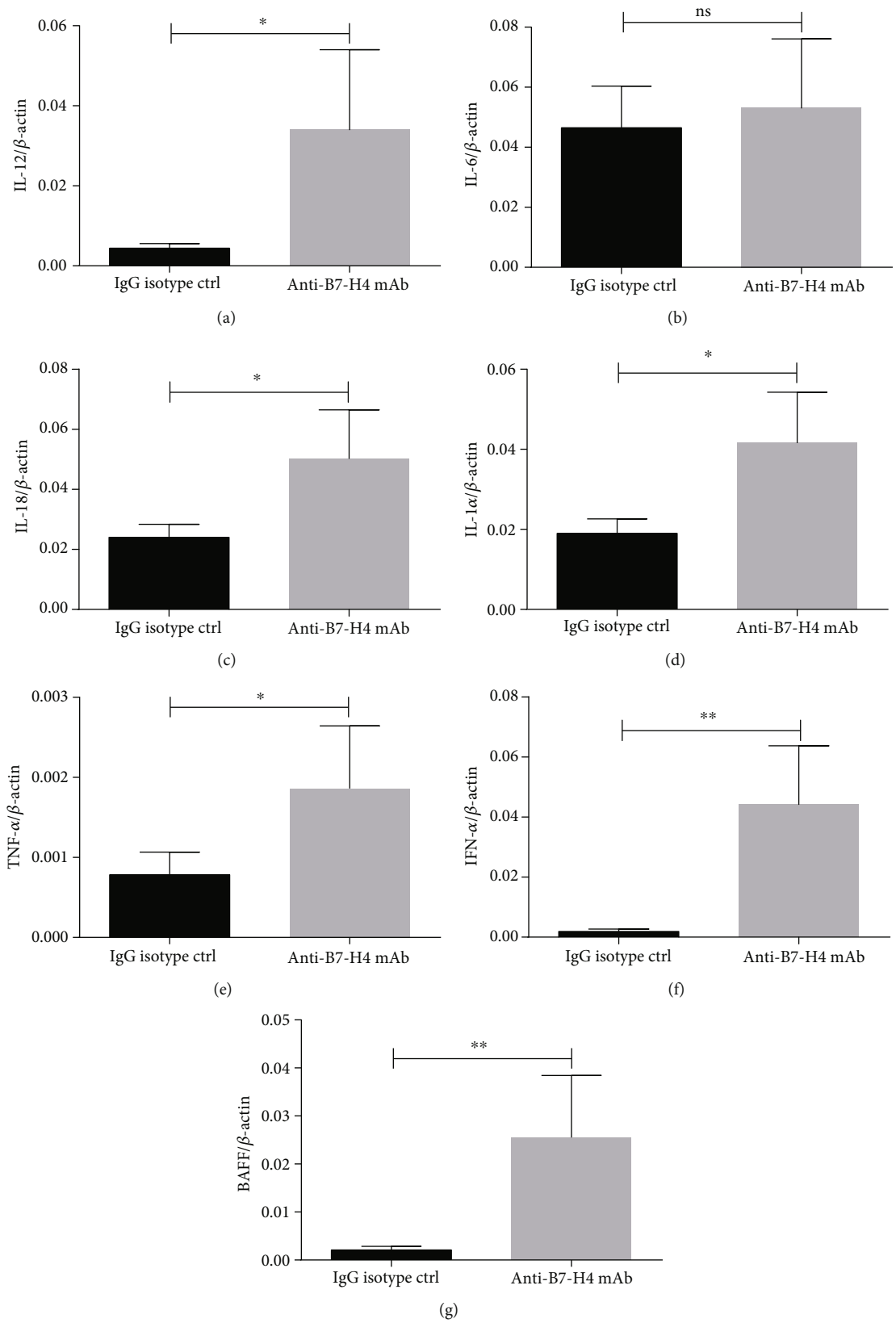


Figure 2: Blockade of B7-H4 increases proinflammatory cytokines in NOD/Ltj mice. (a–g) The salivary glands were collected from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment NOD/Ltj mice. Relative levels of (a) IL-12 mRNA, (b) IL-6 mRNA, (c) IL-18 mRNA, (d) IL-1α mRNA, (e) TNF-α mRNA, (f) IFN-α mRNA, and (g) BAFF mRNA were determined by quantitative RT-PCR. Two-tailed Student's t-test. All the data presented were from three independent experiments.

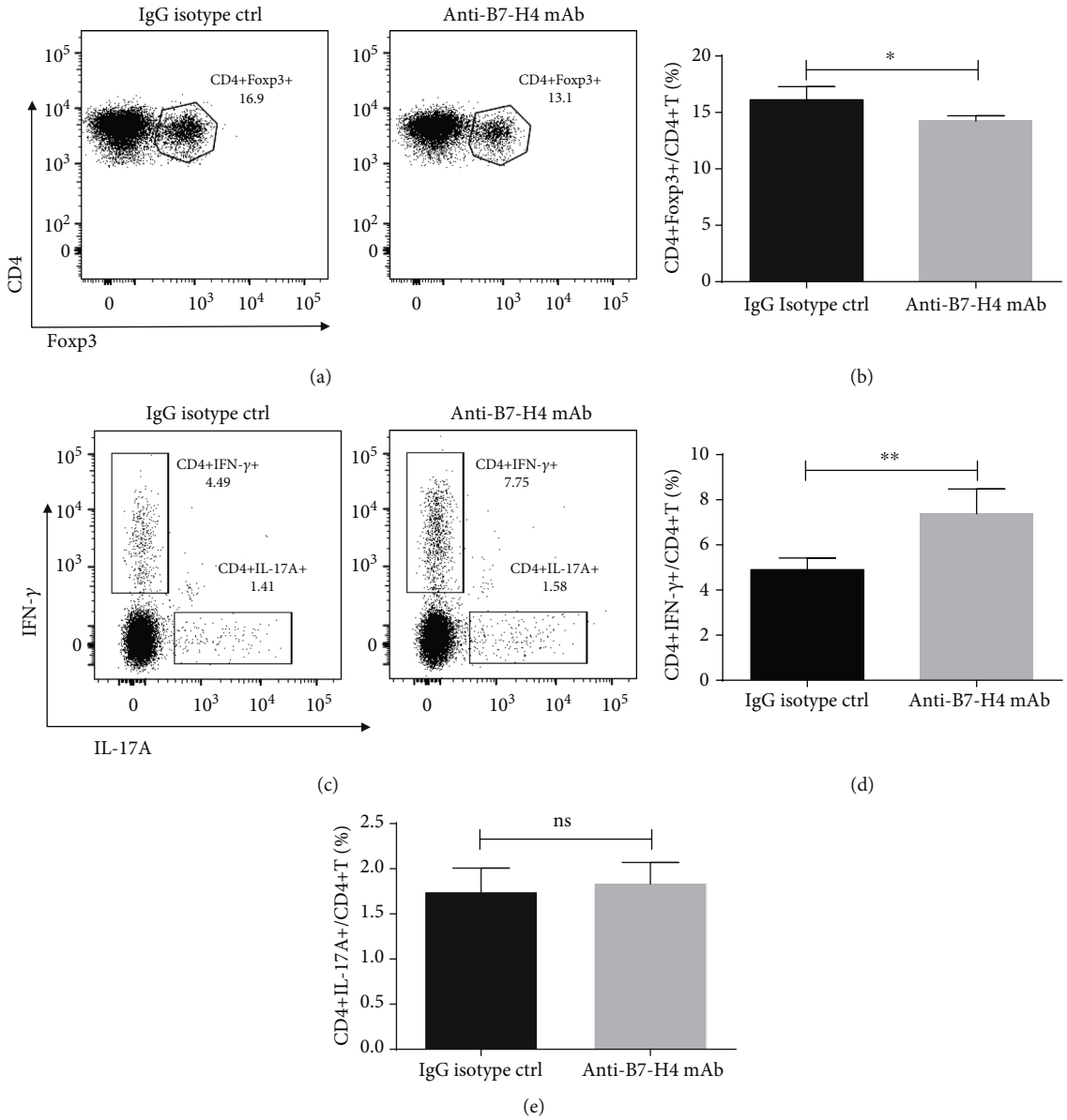


Figure 3: B7-H4 regulates CD4+ T cell response in vivo. (a, b) Splenocytes from 15-week-old NOD/Ltj mice treated with IgG isotype ctrl and anti-B7-H4 mAb, respectively, were stained with anti-CD4/anti-intracellular Foxp3 antibodies and analyzed by flow cytometry (a), and accumulated data of CD4+Foxp3+/CD4+ T cells are shown (b). (c–e) Splenocytes from 15-week-old IgG isotype ctrl or anti-B7-H4 mAb treatment NOD/Ltj mice were stimulated with PMA and ionomycin for 4 h and were stained with anti-CD4/intracellular IFN-γ or anti-CD4/intracellular IL-17A antibodies and analyzed by flow cytometry (c), accumulated data of CD4+IFN-γ+/CD4+ T cells (d), and CD4+IL-17A+/CD4+ T cells (e). Two-tailed Student's t-test. All the data presented were from three independent experiments.

GTTCATCTTCTAGGCAC (reverse); TGF-β: 5′GGAAAT CAACGGGATCAGCC (forward) and 5′GTGCCGTGAGC TGTGCAGGT (reverse); Foxp3: 5′CGAAAGTGGCAGAG AGGTATT (forward) and 5′GCATGGGTCTGTCTTC TCTAAG (reverse); IL-18: 5′CTCTGTGGTTCCATGC TTTCT (forward) and 5′GTTTGAGGCGGCTTTCTTTG (reverse); IL-6: 5′TGAACTCCTTCTCCACAAGCG (forward) and 5′TCTGAAGAGGTGAGTGGCTGTC (reverse); IL-12: 5′TCAAACCAGACCCACCGAA (forward) and 5′ GCTGACCTCCACCTGCTGA (reverse); IFN-α: 5′GGCT CTGGTGCATGAGATGT (forward) and 5′GCCTTCTTC CTGAATCTGTCTTA (reverse); BAFF: 5′AAGACCTAC GCCATGGGACATC(forward) and 5′TCTTGGTATTG CAAGTTGGAGTTCA (reverse); β-actin: 5′TACAGCTTC ACCACCACAGC (forward) and 5′TCTCCAGGGAGGAA GAT (reverse).

2.7. Immunoglobulins Measurement. Serum IgM, IgG, and IgA were detected using ELISA kits. 96-well plates were coated with 50–100 μL capture antibodies for overnight at

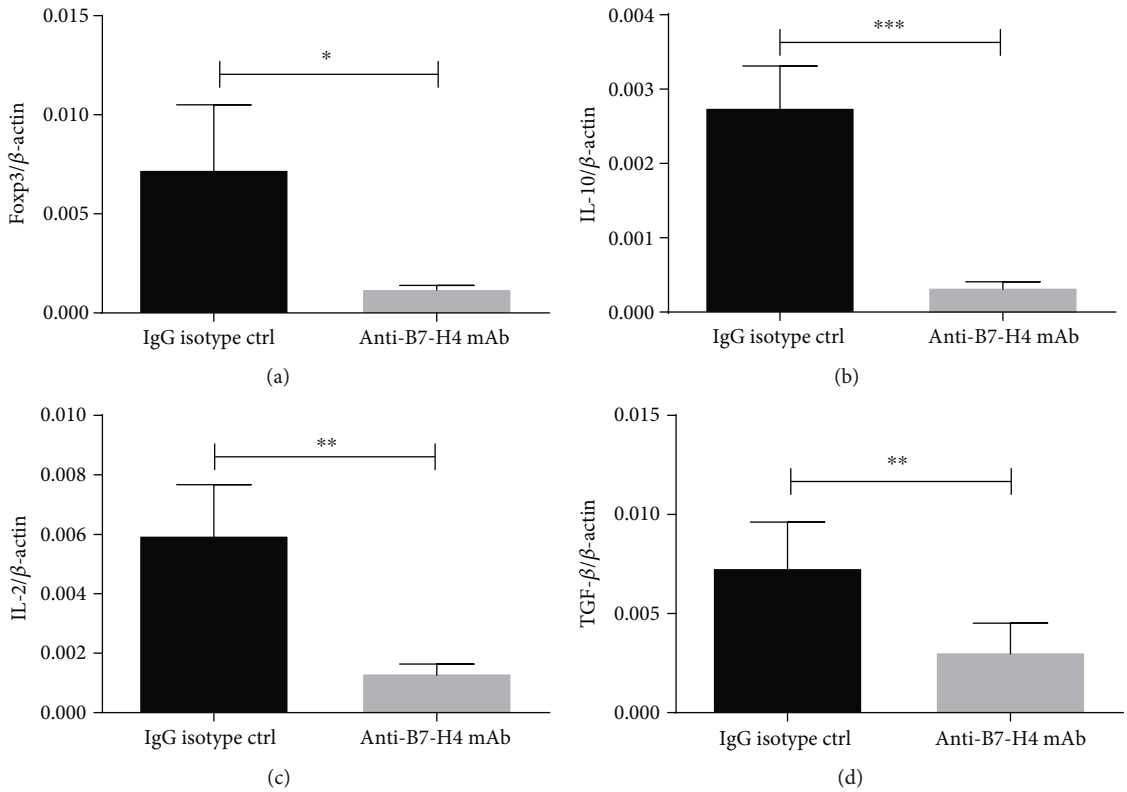


Figure 4: Blockade of B7-H4 suppresses Tregs in salivary gland. (a–d) The salivary glands were collected from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment NOD/Ltj mice. Relative levels of (a) Foxp3 mRNA, (b) IL-10 mRNA, (c) IL-2 mRNA, and (d) TGF-β mRNA were determined by quantitative RT-PCR. Two-tailed Student's t-test. All the data presented were from three independent experiments.

4°C or 2 h in room temperature following the manufacturer's instruction. Wash wells four times in using PBST and add 100 μL diluted serum or standard incubating at room temperature for 2 h. Wash wells four times and add 100 μL of diluted detection antibody to each well incubating for 1 h. After washing, add 100 μL of diluted HRP conjugate to each well for 30 min thoroughly aspirate and add 100 μL of substrate to each well and develop plate at room temperature in the dark for 30 min. Stop the reaction by adding 100 μL of stop solution. While the levels of Ig isotypes were read at 405 nm, the levels of autoantibodies were read at 450 nm. The antibody concentrations were calculated using Ig standards, provided by the manufacturer.

2.8. Statistical Analysis. The data are expressed as mean ± standard deviation (SD). Two-tailed Student's t-tests were used to detect the statistical difference in various measures between the two groups. Data were shown as a representative experiment of three independent experiments. Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. B7-H4 Suppresses the Progression of pSS in NOD/Ltj Mice. Immunohistochemical analysis of B7-H4 expression in salivary glands revealed that B7-H4 expression was remarkably reduced in salivary glands of NOD/Ltj mice at 15 weeks compared with the NOD/Ltj mice at 8 weeks (Figure 1(a)). Blocking endogenous B7-H4 by injection of anti-B7-H4 mAb, NOD/Ltj mice received 12.5 mg/kg of anti-B7-H4 mAb or the same dosage of IgG isotype for the first time, then were administered with anti-B7-H4 mAb 7.5 mg/kg or IgG isotype 7.5 mg/kg every 3 days for 2 weeks. Salivary gland H&E staining showed aggravated lymphocyte infiltration in salivary glands of anti-B7-H4 mAb treatment when compared with IgG isotype ctrl treatment in NOD/Ltj mice (Figures 1(b) and 1(c)). To evaluate the immune response following anti-B7-H4 mAb treatment, we analyzed the levels of total IgM, IgG, and IgA in the serum of anti-B7-H4 mAb-treated mice and IgG isotype-treated mice. Anti-B7-H4 mAb-treated NOD/Ltj mice displayed elevated levels of total IgG and IgM compared to IgG isotype-treated mice (Figures 1(d) and 1(e)). However, level of IgA did not show any significant differences between the groups (Figure 1(f)).

3.2. Blockade of B7-H4 Increases Proinflammatory Cytokines in NOD/Ltj Mice. Inflammatory cytokines are induced in pSS [19]. Therefore, we analyzed the levels of inflammatory cytokine mRNA in salivary glands, which were in the relevant to this model. We found that levels of IL-12 (Figure 2(a)), IL-18 (Figure 2(c)), IL-1α (Figure 2(d)), TNF-α (Figure 2(e)), IFN-α (Figure 2(f)), and BAFF (Figure 2(g)) were upregulated markedly in anti-B7-H4 mAb-treated mice compared to IgG isotype-treated mice, while the level of IL-6

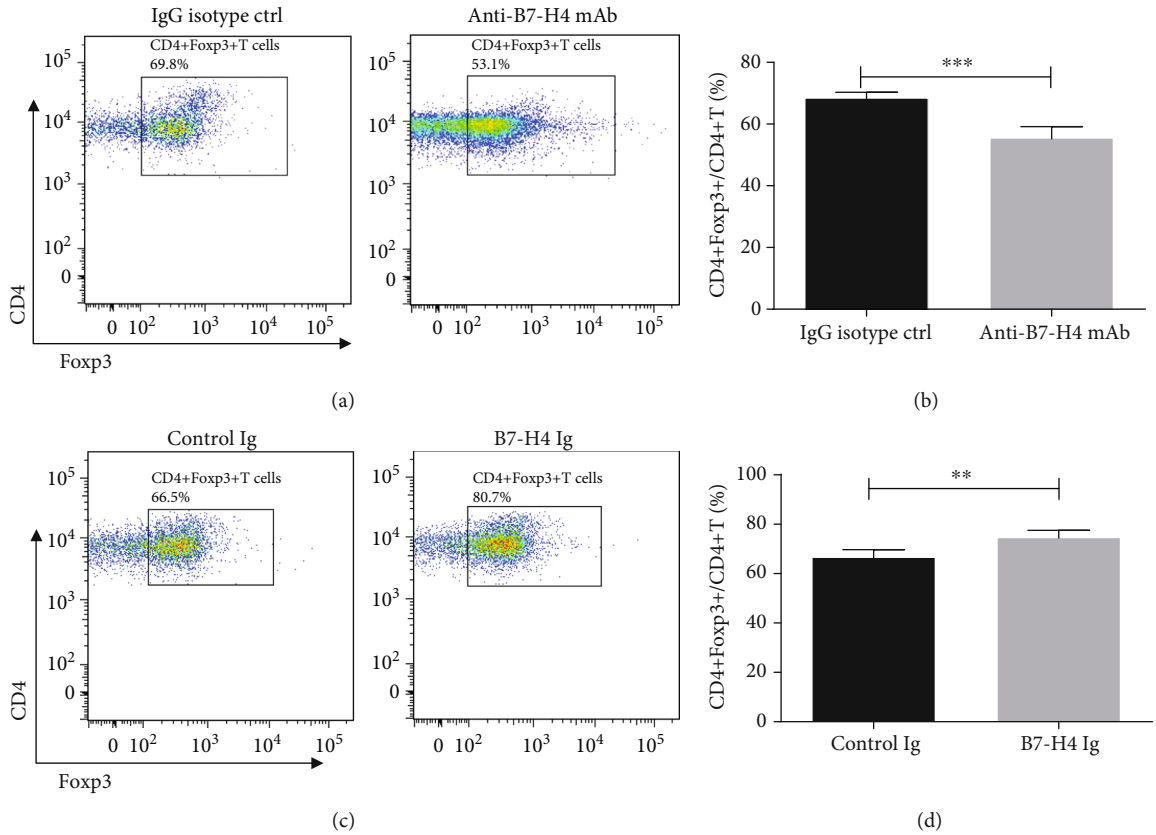


Figure 5: B7-H4 on SGEC is involved in Treg differentiation. (a, b) Naïve CD4+ T cells were isolated from NOD/Ltj mice and cultured (2.5×10^5 cells/well) with anti − CD3 (1 μg/ml) + anti − CD28 (2 μg/ml) plus an equal number of SGECs in the presence of IL-2 (30-50 U/ml), TGF-β (2 ng/ml), plus IgG isotype, or anti-B7-H4 mAb. The cells were collected on day 3 of culture, and the percentage of CD4+FoxP3+/CD4+ T cells was determined by FACS analysis (a). Accumulated data of Tregs differentiation in vitro was shown (b). (c, d) Naïve CD4+ T cells from NOD/Ltj mice were activated in Treg-promoting conditions as detailed in (a, b) with control Ig or B7-H4 Ig, and the percentage of CD4+FoxP3+/CD4+ T cells was determined by FACS analysis (c). Accumulated data of Treg differentiation in vitro was shown (d). Two-tailed Student's t-test. All the data presented were from three independent experiments.

in salivary glands showed no difference between the two treatments (Figure 2(b)).

3.3. B7-H4 Regulates CD4+ T Cell Responses In Vivo. Expression of cytokine mRNA in salivary glands suggests a role of B7-H4 in regulation of T cell immune responses. Flow cytometry analysis of CD4+ T subpopulations in the spleen showed that anti-B7-H4 mAb treatment promoted a relative expansion of the CD4+Foxp3+ T subset (Figures 3(a) and 3(b)) and CD4+IFN-γ+ T subset (Figures 3(c) and 3(d)) compared to IgG isotype. In addition, CD4+IL-17A+ T subset (Figures 3(c) and 3(e)) was found at similar levels in the two treatment groups.

3.4. Blockade of B7-H4 Suppresses Tregs in Salivary Gland. In salivary glands, Tregs in anti-B7-H4 mAb-treated mice were examined compared to control mice. Foxp3 mRNA levels of salivary glands examined with quantitative RT-PCR were diminished in anti-B7-H4 mAb-treated mice compared to that in IgG isotype-treated mice (Figure 4(a)). Moreover, mRNA level of IL-10, which Tregs functionally dependent upon [20, 21], were remarkably diminished in salivary gland of anti-B7-H4 mAb-treated mice (Figure 4(b)). TGF-β in combination with IL-2 is critical for the differentiation of Tregs [22]. IL-2 and TGF-β mRNA levels markedly decreased in NOD/Ltj mice injected with anti-B7-H4 mAb as determined by qRT-PCR analysis of salivary glands (Figures 4(c) and 4(d)).

3.5. B7-H4 on SGEC Is Involved in Treg Differentiation. We next determined whether SGEC B7-H4 is involved in the Treg cell differentiation. The ability of B7-H4 on SGEC under Treg-driving conditions to modulate the differentiation of naïve CD4+ T cells was examined in either containing anti-B7-H4 mAb or IgG isotype. Flow cytometry analysis showed that anti-B7-H4 mAb inhibited CD4+Foxp3+/CD4+ T cell production compared to the addition of IgG isotype (Figures 5(a) and 5(b)). We next asked if B7-H4Ig would promote naïve CD4+ T into Treg differentiation. Naïve CD4+ T was cocultured with SGECs in Treg-promoting conditions with or without B7-H4Ig. The data showed that B7-H4Ig treatment increased CD4+Foxp3+/CD4+ T cells compared with control Ig treatment (Figures 5(c) and 5(d)).

3.6. B7-H4Ig Ameliorates pSS in NOD/Ltj Mouse. To evaluate the potential of modulation of the B7-H4 pathway as a

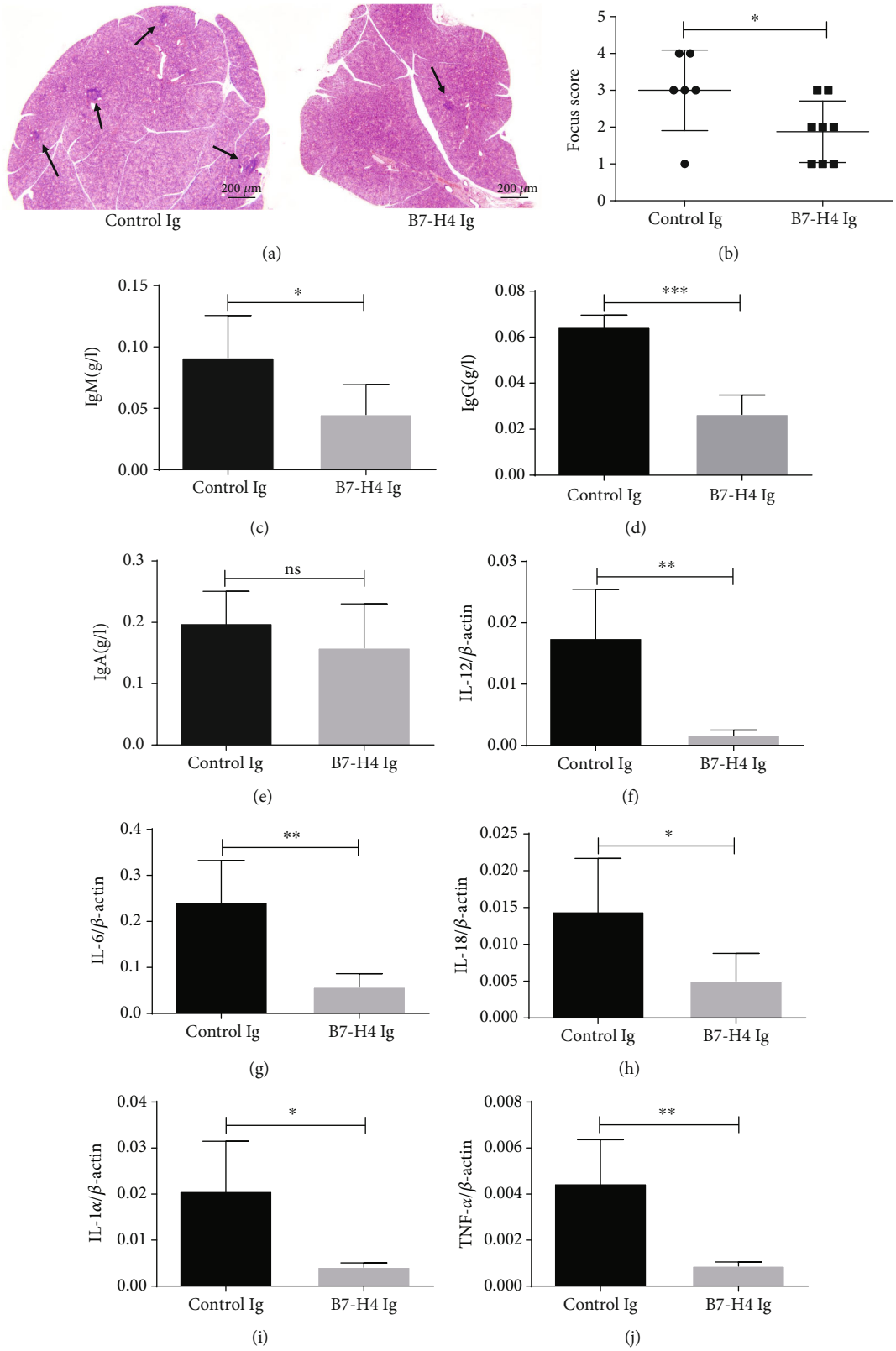


Figure 6: Continued.

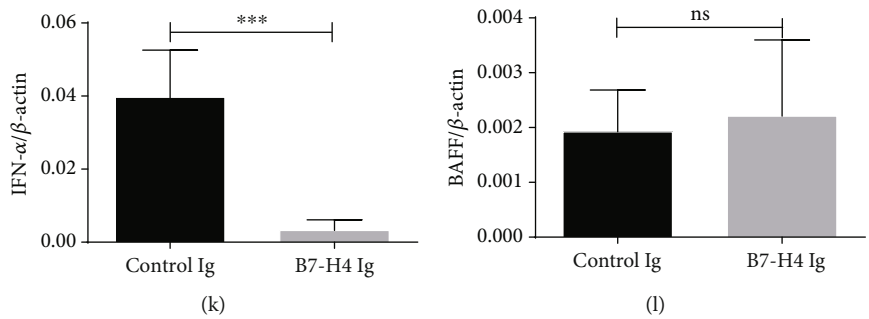


Figure 6: B7-H4Ig ameliorates pSS in NOD/Ltj mouse. (a, b) Female NOD/Ltj mice were injected with control Ig or B7-H4Ig from 8 weeks of age. The mice were sacrificed at 15 weeks of age. H&E stains of the salivary gland sections from control Ig treatment and B7-H4Ig treatment mice. Arrows indicate infiltrates within the salivary gland (a). Histopathological assessment data are presented as FS (b). (c–e) Serum samples were collected from IgG isotype ctrl treatment and B7-H4Ig treatment NOD/Ltj mice, and the levels of immunoglobulins were measured by ELISA. Immunoglobulin levels for (c) IgM, (d) IgG, and (e) IgA are shown. (f–l) The salivary glands were collected from control Ig treatment and B7-H4Ig treatment NOD/Ltj mice. Relative levels of (f) IL-12 mRNA, (g) IL-6 mRNA, (h) IL-18 mRNA, (i) IL-1α mRNA, (j) TNF-α mRNA, (k) IFN-α mRNA, and (l) BAFF mRNA were determined by quantitative RT-PCR. Two-tailed Student's t-test. All the data presented were from three independent experiments.

therapeutic option to restrict immune-mediated damage, we treated female NOD/Ltj mice with B7-H4Ig and control Ig starting from 8 weeks of age. NOD/Ltj mice were administered with B7-H4Ig (7.5 mg/kg) or control mouse IgG (7.5 mg/kg) every 2 days for 2 weeks. After 7 weeks later, pathological changes of salivary glands with H&E staining were examined. Administration with B7-H4Ig significantly decreased lymphocyte infiltration in salivary glands (Figures 6(a) and 6(b)). B7-H4Ig treatment mice displayed low levels of total IgM (Figure 6(c)) and IgG (Figure 6(d)) in serum, while IgA (Figure 6(e)) was at similar levels in both B7-H4Ig treatment and control Ig treatment. Analysis of inflammatory cytokines in salivary glands after B7-H4Ig treatment revealed that the mRNA levels of IL-12 (Figure 6(f)), IL-6(Figure 6(g)), IL-18 (Figure 6(h)), IL-1α (Figure 6(i)), TNF-α (Figure 6(j)), and IFN-α (Figure 6(k)) were significantly downregulated in B7-H4Ig-treated mice compared to control Ig treatment. The level of BAFF mRNA (Figure 6(l)) in salivary glands did not show any significant differences between the two groups.

3.7. B7-H4Ig Ameliorates pSS through Expanding Tregs. B7-H4Ig-treated mice had significantly higher levels of CD4+Foxp3+/CD4+ T cells in spleen by flow cytometry analysis than that in those treated with control Ig (Figures 7(a) and 7(b)). In salivary glands, Foxp3 mRNA (Figure 7(c)), together with IL-10 mRNA (Figure 7(d)) level increased in B7-H4Ig-treated mice compared to control Ig-treated mice. Using immunohistochemical analysis, we found that CD4+Foxp3+ T cells increased significantly in B7-H4Ig treatment mice compared to control Ig treatment (Figure 7(e)), and the ratio of CD4+Foxp3+/CD4+ T cells in salivary gland was shown (Figure 7(f)).

4. Discussion

pSS is an autoimmune disorder with infiltration of periductal lymphocytes in salivary and lacrimal glands, and it can result in downregulated secretory function, dry mouth, and dry eyes. Due to the extensive involvement of various epithelial cells, pSS has also been depicted as autoimmune epithelitis disease [23]. SGECs can present antigen and induce T cell activation in pSS immunological salivary gland lesions [24]. It was reported that abnormal activation of CD4+ T cells and B cells has close relationship with development and pathogenesis of pSS [25].

B7 costimulatory molecules play an important role in immune-regulatory networks. Previous research shows that B7-2 has been found to be expressed by human nonstimulated monocytes, B cells, and SGECs [26] and negatively regulated by activation processes to costimulate T cell proliferation [27]. B7-H4 (also known as B7S1, B7×, and Vtcn1) is another B7 family member; it is detected on most nonhematopoietic tissues but protein expression is more limited [28]. The biological activity of B7-H4 has been shown to suppress immune responses in autoimmune disease. Soluble B7-H4 can also be detected in the serum of rheumatoid arthritis patients. In mice model, soluble B7-H4 correlates with increased age and disease severity in lupus-prone BWF1 mice and mice with CIA [29]. In an EAE model, administration of B7-H4 antibodies to block B7-H4 during the T cell priming phase led to more severe of EAE [30]. Similarly, EAE was severe in B7-H4 deficient compared to that in WT mice, resulting in the expansion of Th1 and Th17 cells leading to severe disease [30]. The expression of B7-H4 is also induced rapidly in the tubular cells after stimulation with LPS in an autoimmune kidney disease mouse model [30]. In an autoimmune kidney disease mouse model, expression of B7-H4 is rapidly induced in the tubular cells after stimulation with LPS [31]. B7-H4 knockout mice have an enhanced humoral immune response and develop severe renal injury after administration of antibodies against glomerular antigens. It was also observed that B7-H4 deficiency or treatment with soluble B7-H4 could increase the disease incidence and severity in the CIA model [32].

A previous research showed a noticeable decrease in the expression of B7-H4 within the pancreatic islets at approximately 10 weeks of age but a significant loss of B7-H4 expression at 15 weeks in NOD/Ltj mouse [33]. We found the similar changes of B7-H4 expression in salivary glands of

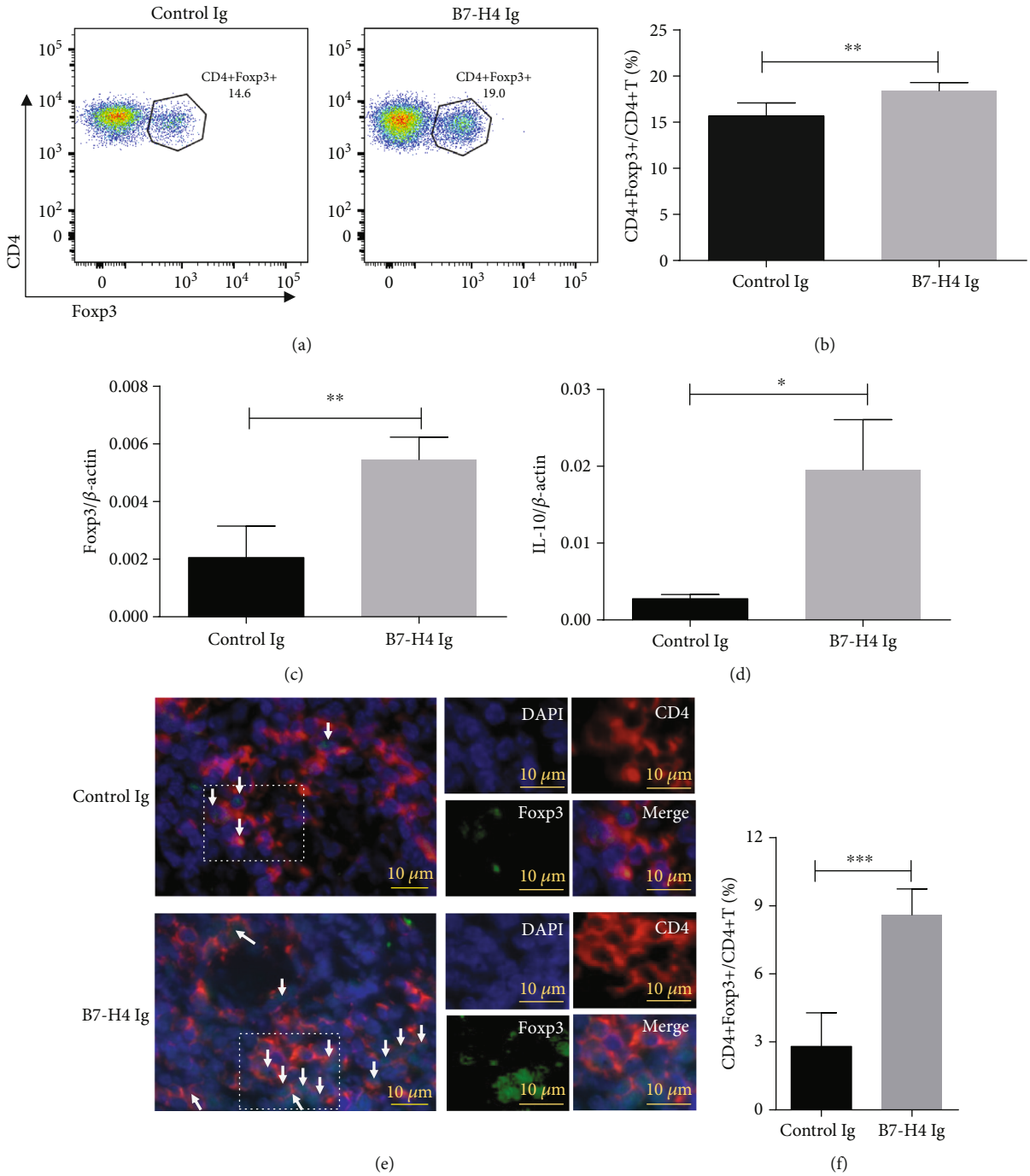


Figure 7: B7-H4Ig ameliorates pSS through expanding Tregs. (a, b) Splenocytes from 15-week-old control Ig treatment and B7-H4Ig treatment NOD/Ltj mice. Staining of (a) CD4 and intracellular staining of Foxp3 cells were performed. (b) Accumulated data of CD4+Foxp3+/CD4+ T cells. (c, d) The salivary glands were collected from control Ig treatment and B7-H4Ig treatment NOD/Ltj mice. Relative levels of (c) Foxp3 mRNA and (d) IL-10 mRNA were determined by quantitative RT-PCR. (e) Colocalization of Foxp3 with CD4 in the salivary glands from B7-H4Ig or control Ig treatment in NOD/Ltj mouse. Anti-Foxp3 (green); anti-CD4 (red); and DAPI (blue). Representative results are shown from three independent experiments (200x magnification). Examples of CD4+Fopx3+ T cells (arrow) are indicated in the left panels. The right panels represent the enlarged portion of the indicated (white hatched box) area in the left panels. (f) Ratio of CD4+Foxp3+/CD4+ T cells in the salivary glands from control Ig or B7-H4 Ig treatment in NOD/Ltj mouse. Two-tailed Student's t-test. All the data presented were from three independent experiments.

NOD/Ltj mice. B7-H4 expression was remarkably reduced in salivary glands of NOD/Ltj mice at 15 weeks compared with that in salivary glands of NOD/Ltj mice at 8 weeks. This result is consistent with the previously published study by Yu et al. [34], suggesting that B7-H4 in SGEC has a potential role in the progression of pSS. Previous studies indicate the remarkable reduction of Treg numbers in both salivary glands and peripheral blood of patients with pSS [35, 36]. It

has demonstrated that Tregs play a pivotal role in immune homeostasis by suppressing the proliferation and function of effector T lymphocytes, as well as other immunocytes [37–39]. Whether Tregs have a possible role in the pathogenesis of salivary gland destruction in pSS is not clear. In NOD/Ltj mouse model, we found that Tregs decrease in salivary gland of mice after anti-B7-H4 mAb treatment. Furthermore, blockade B7-H4 in NOD/Ltj mice aggravates the lymphocyte infiltration in salivary glands, increases the serum IgG and IgM levels, and also upregulates the major proinflammatory cytokine production, including IL-12, IL-18, IL-1α, TNF-α, IFN-α, and BAFF in salivary glands. However, there is no significant difference of IL-6 levels between anti-B7-H4 mAb and IgG isotype control treatment. To reveal the relationship between B7-H4 expression and the number/percentage of Treg cells, anti-B7-H4 mAb was found to inhibit the development of Treg cells. It has been reported that cytokines such as IL-4 and TGF-β are the factors necessary for Treg differentiation. In anti-B7-H4 mAb-treated mice, IL-4 and TGF-β secretions were reduced, suggesting that the changed cytokine expression profile might affect the differentiation of Treg cells. Our study also supports the published mechanism in which the role of B7-H4 suppresses immune responses by inhibition of CD4+ T cell activation [40]. Saliva flow rate is an important clinical index in the diagnosis of pSS. We did not detect saliva flow rate in this research for the following reasons. First, previous research showed secretory dysfunction by 16 weeks of age in NOD/Ltj mouse [41], while in our research, 15-week-old mouse was selected. Second, it has been reported that the presence or extensiveness of lymphocytic infiltration in the salivary or lacrimal glands (sialadenitis or dacryoadenitis, respectively) is not always indicative of degree of secretory dysfunction [42] which indicating that other potential factors may lead to dry mouth even in the absence of lymphocytic infiltration in the salivary glands [43, 44]. Third, this study focused on the relationship between B7-H4 and lymphocytes in NOD/Ltj mouse, and lymphatic infiltration in mouse submandibular gland suggested the preventive effect of B7-H4 in pSS. Finally, in our previous study, there was no correlation between the percentage of B7-H4 expression in the labial gland of pSS patients and clinical indicators (including saliva flow) [34]. Even so, there is no denying that saliva flow rate is an important indicator in Sjogren's syndrome research.

B7-H4Ig is a soluble fusion Fc fusion of the extracellular domain of B7-H4 which has the natural functions of B7-H4 and is able to interact with other immune cells. B7-H4Ig treatment during EAE has shown to alleviate disease through increasing the number and function of Treg cells. Treatment with B7-H4Ig can also reduce the incidence of the autoimmune diabetes in NOD/Ltj mice, as well as the incidence and severity of disease in a CIA model [14, 32]. A phase I study of AMP-110 (a B7-H4Ig fusion protein) for use in patients with RA is ongoing (NTC01878123).

5. Conclusions

Our study demonstrated that B7-H4Ig treatment could decrease lymphocyte infiltration in salivary glands of NOD/Ltj mice through expanding Tregs. In the current study, B7-H4 expression in salivary glands was significantly reduced in the model of pSS. Targeting B7-H4 by antibody inhibits Treg differentiation and accelerates pSS, suggesting an important role of B7-H4 in the pathogenesis of pSS. B7-H4Ig ameliorates pSS and promotes Tregs expansion, indicating a new therapeutic approach for clinical treatment of pSS in the future.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Authors' Contributions

Xu Zheng and Qikai Wang contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number 81871271, 81373187, and 81901615), Anhui Key Research and Development Program (grant number 1804b06020354), the National Natural Science Foundation of Anhui province (grant number 1908085QH324), and the Fundamental Research Funds for the Central Universities (grant number WK9110000052).

References

[1] P. Sandhya, B. Kurien, D. Danda, and R. Scofield, "Update on pathogenesis of Sjogren's syndrome," *Current Rheumatology Reviews*, vol. 13, no. 1, pp. 5–22, 2017.

[2] J. P. Lefaucheur, D. Sene, and A. L. Oaklander, "Primary Sjögren's syndrome," *The New England Journal of Medicine*, vol. 379, no. 1, p. 96, 2018.

[3] J. M. Meijer, P. M. Meiners, J. J. R. Huddleston Slater et al., "Health-related quality of life, employment and disability in patients with Sjogren's syndrome," *Rheumatology (Oxford)*, vol. 48, no. 9, pp. 1077–1082, 2009.

[4] N. I. Tapinos, M. Polihronis, A. G. Tzioufas, and F. N. Skopouli, "Immunopathology of Sjögren's syndrome," *Annales de Medecine Interne*, vol. 149, no. 1, pp. 17–24, 1998.

[5] F. N. Skopouli, P. C. Fox, V. Galanopoulou, J. C. Atkinson, E. S. Jaffe, and H. M. Moutsopoulos, "T cell subpopulations in the labial minor salivary gland histopathologic lesion of Sjögren's syndrome," *The Journal of Rheumatology*, vol. 18, no. 2, pp. 210–214, 1991.

[6] M. N. Manoussakis and E. K. Kapsogeorgou, "The role of intrinsic epithelial activation in the pathogenesis of Sjögren's syndrome," *Journal of Autoimmunity*, vol. 35, no. 3, pp. 219–224, 2010.

[7] S. Tsunawaki, S. Nakamura, Y. Ohyama et al., "Possible function of salivary gland epithelial cells as nonprofessional antigen-presenting cells in the development of Sjögren's syndrome," *The Journal of Rheumatology*, vol. 29, no. 9, pp. 1884–1896, 2002.

[8] G. L. Sica, I. H. Choi, G. Zhu et al., "B7-H4, a molecule of the B7 family, negatively regulates T cell immunity," *Immunity*, vol. 18, no. 6, pp. 849–861, 2003.

[9] D. V. Prasad, S. Richards, X. M. Mai, and C. Dong, "B7S1, a novel B7 family member that negatively regulates T cell activation," *Immunity*, vol. 18, no. 6, pp. 863–873, 2003.

[10] X. Zang, P. Loke, J. Kim, K. Murphy, R. Waitz, and J. P. Allison, "B7x: a widely expressed B7 family member that inhibits T cell activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10388–10392, 2011.

[11] R. Sugamata, H. Suetake, K. Kikuchi, and Y. Suzuki, "Teleost B7 expressed on monocytes regulates T cell responses," *Journal of Immunology*, vol. 182, no. 11, pp. 6799–6806, 2009.

[12] I. Kryczek, L. Zou, P. Rodriguez et al., "B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma," *The Journal of Experimental Medicine*, vol. 203, no. 4, pp. 871–881, 2006.

[13] X. Wang, J. Hao, D. L. Metzger et al., "B7-H4 pathway in islet transplantation and beta-cell replacement therapies," *Journal of Transplantation*, vol. 2011, Article ID 418902, 2011.

[14] X. Wang, J. Hao, D. L. Metzger et al., "Early treatment of NOD mice with B7-H4 reduces the incidence of autoimmune diabetes," *Diabetes*, vol. 60, no. 12, pp. 3246–3255, 2011.

[15] I. F. Lee, X. Wang, J. Hao et al., "B7-H4.Ig inhibits the development of type 1 diabetes by regulating Th17 cells in NOD mice," *Cellular Immunology*, vol. 282, no. 1, pp. 1–8, 2013.

[16] X. Li, D. Yu, N. Yu et al., "B7-H4 deficiency in salivary gland of patients with primary Sjögren's syndrome impairs the regulatory effect on T cells," *International Journal of Rheumatic Diseases*, vol. 20, no. 4, pp. 474–480, 2017.

[17] S. G. Zheng, J. Wang, P. Wang, J. D. Gray, and D. A. Horwitz, "IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells," *Journal of Immunology*, vol. 178, no. 4, pp. 2018–2027, 2007.

[18] N. R. West, S. E. Kost, S. D. Martin et al., "Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer," *British Journal of Cancer*, vol. 108, no. 1, pp. 155–162, 2013.

[19] N. Roescher, P. P. Tak, and G. G. Illei, "Cytokines in Sjogren's syndrome: potential therapeutic targets," *Annals of the Rheumatic Diseases*, vol. 69, no. 6, pp. 945–948, 2010.

[20] S. M. Pop, C. P. Wong, D. A. Culton, S. H. Clarke, and R. Tisch, "Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes," *The Journal of Experimental Medicine*, vol. 201, no. 8, pp. 1333–1346, 2005.

[21] M. Grazia Roncarolo, S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings, "Interleukin-10-secreting type 1 regulatory T cells in rodents and humans," *Immunological Reviews*, vol. 212, no. 1, pp. 28–50, 2006.

[22] J. R. Podojil, L. N. Liu, S. A. Marshall et al., "B7-H4Ig inhibits mouse and human T-cell function and treats EAE via IL-10/Treg-dependent mechanisms," *Journal of Autoimmunity*, vol. 44, pp. 71–81, 2013.

[23] M. Ramos-Casals, A. G. Tzioufas, and J. Font, "Primary Sjögren's syndrome: new clinical and therapeutic concepts," *Annals of the Rheumatic Diseases*, vol. 64, no. 3, pp. 347–354, 2005.

[24] T. Kawanami, T. Sawaki, T. Sakai et al., "Skewed production of IL-6 and TGFβ by cultured salivary gland epithelial cells from patients with Sjögren's syndrome," *PLoS One*, vol. 7, no. 10, article e45689, 2012.

[25] C. P. Mavragani, "Mechanisms and new strategies for primary Sjögren's syndrome," *Annual Review of Medicine*, vol. 68, no. 1, pp. 331–343, 2017.

[26] E. K. Kapsogeorgou, H. M. Moutsopoulos, and M. N. Manoussakis, "Functional expression of a costimulatory B7.2 (CD86) protein on human salivary gland epithelial cells that interacts with the CD28 receptor, but has reduced binding to CTLA4," *Journal of Immunology*, vol. 166, no. 5, pp. 3107–3113, 2001.

[27] P. Jeannin, G. Magistrelli, J. P. Aubry et al., "Soluble CD86 is a costimulatory molecule for human T lymphocytes," *Immunity*, vol. 13, no. 3, pp. 303–312, 2000.

[28] I. H. Choi, G. Zhu, G. L. Sica et al., "Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family," *Journal of Immunology*, vol. 171, no. 9, pp. 4650–4654, 2003.

[29] Y. Kamimura, H. Kobori, J. Piao et al., "Possible involvement of soluble B7-H4 in T cell-mediated inflammatory immune responses," *Biochemical and Biophysical Research Communications*, vol. 389, no. 2, pp. 349–353, 2009.

[30] J. Wei, P.'. Loke, X. Zang, and J. P. Allison, "Tissue-specific expression of B7x protects from CD4 T cell-mediated autoimmunity," *The Journal of Experimental Medicine*, vol. 208, no. 8, pp. 1683–1694, 2011.

[31] R. D. Pawar, B. Goilav, Y. Xia et al., "B7x/B7-H4 modulates the adaptive immune response and ameliorates renal injury in antibody-mediated nephritis," *Clinical and Experimental Immunology*, vol. 179, no. 2, pp. 329–343, 2015.

[32] T. Azuma, G. Zhu, H. Xu et al., "Potential role of decoy B7-H4 in the pathogenesis of rheumatoid arthritis: a mouse model informed by clinical data," *PLoS Medicine*, vol. 6, no. 10, article e1000166, 2009.

[33] I. A. Radichev, L. V. Maneva-Radicheva, C. Amatya et al., "Nardilysin-dependent proteolysis of cell-associated VTCN1 (B7-H4) marks type 1 diabetes development," *Diabetes*, vol. 63, no. 10, pp. 3470–3482, 2014.

[34] D. L. Yu, X. M. Li, X. M. Wang, X. Q. Wang, and X. P. Li, "B7-H4 expression of salivary gland and sera in patients with primary Sjogren's syndrome," *Zhonghua Yi Xue Za Zhi*, vol. 92, no. 39, pp. 2775–2777, 2012.

[35] X. Li, X. Li, L. Qian et al., "T regulatory cells are markedly diminished in diseased salivary glands of patients with primary Sjögren's syndrome," *The Journal of Rheumatology*, vol. 34, no. 12, pp. 2438–2445, 2007.

[36] M. F. Liu, L. H. Lin, C. T. Weng, and M. Y. Weng, "Decreased CD4+CD25+bright T cells in peripheral blood of patients with primary Sjögren's syndrome," *Lupus*, vol. 17, no. 1, pp. 34–39, 2008.

[37] R. H. Schwartz, "Natural regulatory T cells and self-tolerance," *Nature Immunology*, vol. 6, no. 4, pp. 327–330, 2005.

[38] L. S. Taams, J. M. R. van Amelsfort, M. M. Tiemessen et al., "Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells," *Human Immunology*, vol. 66, no. 3, pp. 222–230, 2005.

[39] H. von Boehmer, "Mechanisms of suppression by suppressor T cells," *Nature Immunology*, vol. 6, no. 4, pp. 338–344, 2005.

[40] S. Paust, L. Lu, N. McCarty, and H. Cantor, "Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune

disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 28, pp. 10398–10403, 2004.

[41] Y. S. Park, A. E. Gauna, and S. Cha, "Mouse models of primary Sjogren's syndrome," *Current Pharmaceutical Design*, vol. 21, no. 18, pp. 2350–2364, 2015.

[42] M. G. Humphreys-Beher, J. Brayer, S. Yamachika, A. B. Peck, and R. Jonsson, "An alternative perspective to the immune response in autoimmune exocrinopathy: induction of functional quiescence rather than destructive autoaggression," *Scandinavian Journal of Immunology*, vol. 49, no. 1, pp. 7–10, 1999.

[43] D. Cavill, S. A. Waterman, and T. P. Gordon, "Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjogren's syndrome," *Scandinavian Journal of Immunology*, vol. 59, no. 3, pp. 261–266, 2004.

[44] F. Wang, M. W. Jackson, V. Maughan et al., "Passive transfer of Sjogren's syndrome IgG produces the pathophysiology of overactive bladder," *Arthritis and Rheumatism*, vol. 50, no. 11, pp. 3637–3645, 2004.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 9690832, 10 pages
https://doi.org/10.1155/2020/9690832

Research Article

Selective Increment of Synovial Soluble TYRO3 Correlates with Disease Severity and Joint Inflammation in Patients with Rheumatoid Arthritis

Julia Vullings,[1] Juliana P. Vago,[1] Claire E. J. Waterborg,[1] Rogier M. Thurlings,[1] Marije I. Koenders,[1] Peter L. E. M. van Lent,[1] Peter M. van der Kraan,[1] Flavio A. Amaral,[1,2] and Fons A. J. van de Loo[1]

[1]*Experimental Rheumatology, Department of Rheumatology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, Netherlands*
[2]*Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*

Correspondence should be addressed to Fons A. J. van de Loo; fons.vandeloo@radboudumc.nl

Received 24 April 2020; Revised 8 July 2020; Accepted 22 July 2020; Published 11 September 2020

Academic Editor: Lihua Duan

Objective. To investigate the role of TAM receptors in rheumatoid arthritis (RA) by determining synovial tissue TAM receptor expression, synovial fluid levels of soluble TAM receptors, and the relationship between soluble TAM receptors, joint inflammation and disease activity. *Methods*. TAM receptor expression was determined by immunohistochemistry on the synovium from RA and osteoarthritis (OA) patients. Soluble (s) Tyro3, sAxl, sMer, and their ligand Gas6 were measured by ELISA in the synovial fluid of RA ($n = 28$) and OA ($n = 12$) patients and cytokine levels by multiplex immunoassay in RA samples. Correlation analyses were performed among sTAM receptors with local cytokine levels; systemic disease parameters like erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), and anticyclic citrullinated peptide antibodies (ACPA); and disease activity scores (DAS28-ESR) in RA patients. *Results*. TAM receptors were expressed on different locations in the synovial tissue (lining, sublining, and blood vessels), and a similar expression pattern was observed in RA and OA patients. Synovial fluid sTyro3 and sMer were significantly enhanced in RA compared to OA patients, whereas no significant differences in sAxl and Gas6 levels were found. In RA samples, sTyro3 levels, but not sMer, correlated positively with proinflammatory local cytokines and the systemic factor erythrocyte sedimentation rate. Moreover, stratification analysis showed high sTyro3 levels positively correlated with higher DAS28-ESR and in RF and ACPA double positive RA patients. *Conclusion*. sTyro3 in the synovial fluid of RA patients correlates with local inflammatory molecules and systemic disease activity. These findings suggest that the reduced negative control of cell activation by TAM receptors due to their shedding in the synovial fluid, mainly sTyro3, favoring joint inflammation in RA patients.

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease marked by chronic and unrestrained inflammation, hyperplasia of synoviocytes, and damage of both the articular cartilage and bone. In the arthritic joint, the synovium is infiltrated by both innate and adaptive immune cells which, together with the proliferation of tissue-resident fibroblasts, leads to pannus tissue formation at the articular cartilage and bone interface. Overall, this eventually leads to damage and loss of articular cartilage matrix and bone tissue [1, 2]. Osteoarthritis (OA) is an age-related musculoskeletal disease characterized by progressive joint destruction, including breakdown of cartilage matrix [3]. At the synovial tissue level, the osteoarthritic joint shows a high degree of similarity with RA by increased presence of macrophages and lymphocytes. Although not an autoimmune disease, synovial inflammation, one of the hallmarks of RA, is often observed in OA

[4–6]. The main difference, however, is the lack of neutrophils in OA joints, a cell that undergoes apoptosis and which clearance by TAM receptors mediates the resolution of several types of inflammation [7, 8].

The TAM receptor tyrosine kinase family consists out of Tyro3, Axl, and Mer (gene name MERTK). TAM receptors are expressed on, amongst others, monocytes, macrophages, and dendritic cells and play a critical role in natural anti-inflammatory feedback mechanisms and the phagocytosis of apoptotic cells [8–11]. The two principal TAM receptor protein ligands are Growth Arrest-Specific 6 (Gas6) and Protein S (Pros1). Gas6 is a ligand for all three TAM receptors but with the highest affinity for Axl, whereas Pros1 can only activate Tyro3 and Mer [12, 13]. TAM receptor ligands act as bridging molecules between one of the TAM receptors and phosphatidylserine (PS) that is expressed as an 'eat-me signal' on the surface of apoptotic cells, thereby effectively opsonising apoptotic cells for TAM receptor-mediated efferocytosis [8, 12, 14]. In addition, activation of TAM receptors induces, among others, suppressor of cytokine signaling (SOCS) proteins 1 and 3, which reduce production of numerous cytokines [9, 14, 15]. Although we recently showed that Mer and Axl play a protective role in mouse models of RA, the exact function of TAM receptors in RA patients remains largely unknown [16–18]. TAM receptors can be cleaved from the cell surface, leading to shedding of their soluble ectodomain and consequently a soluble form of the receptor [19, 20]. These soluble TAM (sTAM) receptors may inhibit the immune regulatory and anti-inflammatory effects of TAM receptor activation by reducing membrane-bound receptors and by neutralization of TAM ligands by acting as decoy receptors [21, 22]. Soluble Axl (sAxl) mainly binds Gas6, whereas soluble Tyro3 (sTyro3) has the highest affinity for Pros1. However, Tsou et al. showed that soluble Mer (sMer) is not able to bind TAM receptor ligands and can therefore not act as a decoy receptor [13]. Elevated sMer and sAxl plasma levels are observed in patients with systemic lupus erythematosus (SLE), and these levels positively correlate with disease activity, inflammatory processes, and nephritis [23–25]. In addition, elevated plasma sMer levels correlate with disease activity in patients with Sjögren's syndrome [26].

To date, nothing has been described about locally produced sTAM receptor levels in RA patients, and the function of sTAM receptors in this disease remains largely unknown. Therefore, in the present study, we investigated the expression of TAM receptors in the synovial tissue and levels of sTAM receptors in the synovial fluid of RA patients using OA samples as the control. In addition, we analyzed the relationship between synovial fluid sTAM receptor levels and cytokine levels, systemic disease parameters, and disease activity scores in RA patients.

2. Materials and Methods

2.1. RA and OA Patients. RA ($n = 28$) (21 females, 7 males, mean age: 58.3 ± 14.9 years) and knee OA ($n = 12$) (7 females, 5 males, mean age: 56.8 ± 7.5 years) patients were recruited at the department of Rheumatology (Radboud University Medical Center, Nijmegen, the Netherlands) and the Sint Maartenskliniek (Nijmegen, the Netherlands). In addition to gender and age, the following laboratory parameters of RA patients were recorded: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). In the RA patient group, 16 out of 28 patients (57%) were double positive for rheumatoid factor (RF) and anticyclic citrullinated peptide antibody (ACPA) (>10 IU/ml), 2 were single RF positive and 3 were single ACPA positive, whereas 7 out of 28 RA patients (64%) were RF and ACPA double negative (<10 IU/ml) (25%). For 11 RA patients, disease activity scores were determined. Disease activity scores were calculated using the 28-Joint Disease Activity Score-erythrocyte sedimentation rate (DAS28-ESR) with three variables based on assessment of 28 joints and ESR, according to the recommendations from the European League against Rheumatism (EULAR) [27]. Disease activity scores below 3.2 indicated low disease activity, whereas a score between 3.2 and 4.0 and a score above 4.0 represented medium and high disease activity, respectively. Clinical and demographic characteristics of RA and OA patients are presented in Table 1.

2.2. Human Synovial Fluid. The synovial fluid from RA and OA patients was obtained during consultations at the polyclinic to alleviate pressure and pain of knee joints caused by edema or swelling (synovial inflammation). All material was considered surplus material; therefore, ethical approval was not required. Procedures were performed in accordance to the Dutch code of conduct for responsible use of human tissue in medical research (https://www.federa.org/code-goed-gebruik). Written informed consent was obtained from all patients. Upon collection, the synovial fluid was centrifuged at 1,700 x g for 10 minutes at 4°C, followed by 30 minutes at 10,000 x g at 4°C to remove cells. The supernatant was aliquoted and stored at -80°C. To reduce viscosity, synovial fluid samples were thawed and treated with 75 U/ml of hyaluronidase (H3506; Sigma-Aldrich, Saint Louis, MO, USA) for 15 minutes at 37°C and subsequently centrifuged at 1,000 x g for 10 minutes at 4°C. Samples were aliquoted and stored at -20°C until further analysis.

2.3. Detection of Synovial Fluid sAxl, sMer, sTyro3, and Gas6 Levels by Enzyme-Linked Immunosorbent Assay (ELISA). Synovial fluid sAxl (DY154), sMer (DY6488), sTyro3 (DY859), and Gas6 (DY885B) concentrations were determined using the DuoSet sandwich ELISA kits purchased from R&D Systems (Minneapolis, MN, USA). All ELISAs were performed according to the manufacturer's instructions using the DuoSet ELISA Ancillary Reagent Kit 2 (DY008; R&D Systems). In case of detection of sAxl and Gas6, synovial fluid samples were diluted 30 times, whereas for the detection of sMer and sTyro3, samples were diluted 10 and 5 times, respectively. Synovial fluid samples were diluted in Reagent Diluent (DY995; R&D Systems). Absorbance at 450 nm with a correction wavelength of 540 nm was detected using a microplate reader (CLARIOstar, BMG LABTECH).

2.4. Detection of Synovial Fluid Cytokine Levels by Multiplex ELISA. Cytokines in the synovial fluid of RA patients were

Table 1: Demographic and clinical characteristics of rheumatoid arthritis (RA) and osteoarthritis (OA) patients.

Characteristics	RA patients ($n = 28$)	OA patients ($n = 12$)
Age, years (mean, S.D.)	58.3 ± 14.9	56.8 ± 7.5
Female (n, %)	21, 75	7, 58
Male (n, %)	7, 25	5, 42
RF^+ (n, %)[a]	19, 68	—
$ACPA^+$ (n, %)[b]	18, 64	—
ESR in mm/h (mean, S.D.)	29.3 ± 24.2	—
CRP in mg/ml (mean, S.D.)	26.7 ± 23.8	—
DAS28-ESR (mean, S.D.)	3.75 ± 0.94	—

ACPA: anticyclic citrullinated peptide antibodies; CRP: C-reactive protein; DAS: disease activity score; ESR: erythrocyte sedimentation rate; OA: osteoarthritis; RA: rheumatoid arthritis; RF: rheumatoid factor. [a]RF^+: >10 IU/ml. [b]$ACPA^+$: >10 IU/ml.

measured on a Bio-Plex 200 system using a magnetic bead-based multiplex immunoassay (Bio-Rad Laboratories, Hercules, CA, USA). The synovial fluid was diluted 1 : 4 with Bio-Plex sample diluent (10014641; Bio-Rad Laboratories). The assay was performed according to protocols specified by the manufacturer and with the reagents (diluents, calibrators, blocking reagents, and detecting-antibody mixtures) included with their kits. Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

2.5. Immunohistochemistry of TAM Receptors on Human Synovial Tissue. RA and OA synovial tissues obtained from the knee joint during surgery at the Radboud University Medical Center (Nijmegen, the Netherlands) were used to determine protein expression of Axl, Mer, and Tyro3. This material was considered surgery surplus material. Procedures were performed in accordance to the Dutch code of conduct for responsible use of human tissue in medical research (https://www.federa.org/code-goed-gebruik). Paraffin-embedded synovial tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in Tris/ethylenediamine tetraacetic acid (EDTA) buffer (pH 9) heated to 60°C for Mer and citrate buffer (pH 6) heated to 60°C for Axl and Tyro3. Endogenous peroxidase was blocked by 3% hydrogen peroxide. Sections were blocked with 10% normal goat serum and 1% bovine serum albumin (BSA) in TBS for 20 minutes at RT before incubation with rabbit anti-human Axl (1 : 600; C89E7; Cell signaling, Danvers, MA, USA), rabbit anti-human Mer (1 : 2000; ab52968; Abcam, Cambridge, UK), rabbit anti-human Tyro3 (1 : 500; ab109231; Abcam), or rabbit anti-human IgG (1 : 74000; X0936; Agilent Technologies, Santa Clara, CA, USA) overnight at 4°C. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG (1 : 400; PK-6101; Vector Laboratories, Peterborough, UK) for 30 minutes at RT. A biotin-streptavidin horseradish peroxidase detection system was used according to manufacturer's protocol (PK6101; Vector Laboratories). Bound complexes were visualized with diaminobenzidine (DAB) by incubation for 10 minutes at RT. All antibodies were diluted in 1% BSA in TBS. Sections were counterstained with hematoxylin. Pictures were taken with the Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) at 20x and 40x magnification.

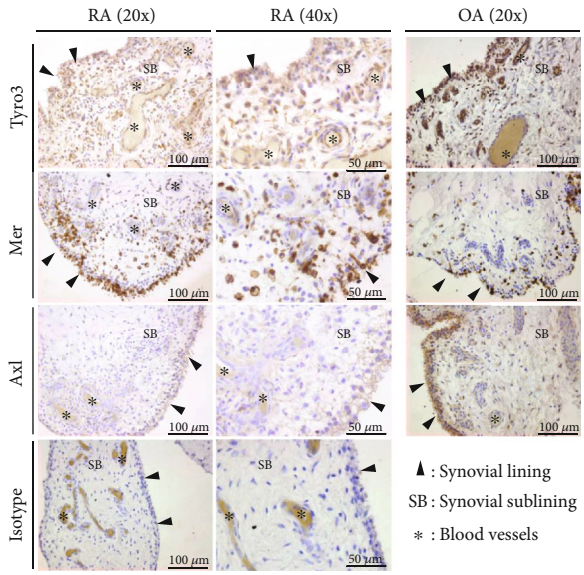


Figure 1: Immunohistochemistry of TAM receptors in the human synovium from rheumatoid arthritis and osteoarthritis patients. Synovial biopsies from rheumatoid arthritis (RA) ($n = 2$) and osteoarthritis (OA) ($n = 8$) patients were processed for immunohistochemical staining of Tyro3, Mer, Axl, and IgG isotype control. Sections were counterstained with hematoxylin. Pictures were taken at 20x and 40x magnification.

2.6. Statistical Analysis. All data were analyzed with GraphPad Prism Software (version 5.03, San Diego, CA, USA). Data were shown as dot plots with mean or as correlations. Comparisons between groups were performed using Student's unpaired t-tests or by covariance analysis (Tukey's multiple comparison test for post hoc test). Correlations between continuous data were assessed using Pearson's correlation coefficient. P values lower than 0.05 were considered statistically significant.

3. Results

3.1. Differential Synovial Expression of TAM Receptors in RA. Immunohistochemistry on the synovial tissue from RA patients revealed differences in expression of TAM receptors among different compartments in the synovium (Figure 1). Tyro3-positive cells were identified mainly in lining cells, instead sublining cells. In addition, expression of Tyro3 was also observed in association with blood vessels, in particular endothelial cells. On the other hand, vascular endothelial cells were Mer negative, whereas Mer-positive cells were observed in both the lining and sublining. Regarding the Axl expression, only cells from the synovial lining were positive, without staining in the sublining or blood vessels. The synovial tissue from OA patients was used as control, while with similar compartmentalized expression of all three TAM receptors.

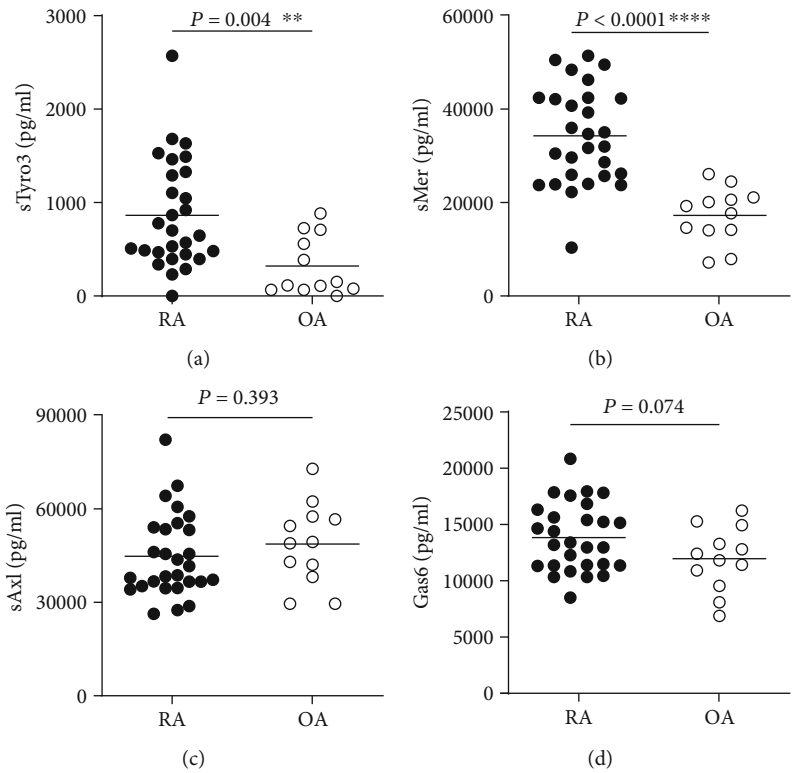


Figure 2: Soluble TAM receptors and Gas6 levels in the synovial fluid of rheumatoid arthritis and osteoarthritis patients. Soluble Tyro3 (sTyro3) (a), soluble Mer (sMer) (b), soluble Axl (sAxl) (c), and Gas6 (d) levels in the synovial fluid of rheumatoid arthritis (RA) ($n = 28$) and osteoarthritis (OA) ($n = 12$) patients were detected by ELISA. Data are presented as dot plots with mean tested by unpaired t-tests. $^{**}P < 0.01$; $^{****}P < 0.0001$.

To study if sTAM receptor levels were increased in synovial fluid of RA patients, sTyro3, sMer, sAxl, and Gas6 levels were determined by ELISA. In RA patients, both sTyro3 and sMer levels were elevated compared to OA patients ($P = 0.0042$ and $P < 0.0001$, respectively) (Figures 2(a) and 2(b)). However, no significant differences in sAxl and Gas6 levels were observed between RA and OA patients ($P = 0.3932$ and $P = 0.0740$, respectively) (Figures 2(c) and 2(d)). In fact, sAxl and Gas6 levels were comparable to levels found in plasma of both RA patients and healthy controls (data not shown). There were positive correlations between sTyro3 and sMer with the ligand Gas6 in the synovial fluid (Figures 3(a) and 3(b)). On the other hand, no significant correlations were found between sAxl with Gas6 nor among sMer, sAxl, and sTyro3 in the synovial fluid of RA patients (Figures 3(c) – 3(f)). Of note, there were no differences in sTyro3 and sMer levels in the synovial fluid between males and females (Supplementary Fig. S1) neither the observed increases in sTyro3 nor sMer in the synovial fluid were influenced by the age of the patients (Supplementary Fig. S2).

3.2. sTyro3 Positively Correlated with Proinflammatory Cytokine Levels in RA Synovial Fluid. As membrane-bound TAM receptors are involved in natural anti-inflammatory feedback mechanisms [8], the presence of high concentration of sTAM in the synovial fluid, particularly sTyro3 and sMer (as shown in Figure 2), could be associated with increased inflammation in RA joints. Thus, we next investigated if increased amounts of sTAM receptors coincide with the presence of key inflammatory molecules in RA joints. Indeed, sTyro3 levels positively correlated with synovial fluid levels of TNF-α ($r = 0.55$, $P = 0.01$), IL-1β ($r = 0.60$, $P = 0.009$), CXCL8/IL-8 ($r = 0.57$, $P = 0.009$), and IL-10 ($r = 0.69$, $P = 0.007$) (Figures 4(a) – 4(d)), but not with IL-6 ($r = 0.22$, $P = 0.36$) (Table 2) in RA patients. In addition to local inflammatory markers, sTyro3 levels also positively correlated with erythrocyte sedimentation rate (ESR) ($r = 0.60$, $P = 0.006$) (Figure 4(e)) and weakly however not significantly with CRP levels ($r = 0.38$, $P = 0.09$) (Figure 4(f)), both systemic measures of inflammation. In contrast, no significant correlations were found between sMer and local cytokine levels or ESR and CRP blood levels (Table 2). Similar to sTyro3, Gas6 in the synovial fluid also positively correlated with most of those molecules, except by IL-6, ESR, and CRP (Table 2). Thus, the presence of sTyro3 in the synovial fluid suggests a reduced control of joint inflammation in RA, impacting on the worsening of the disease.

3.3. sTyro3 Levels in RA Synovial Fluid Correlated with Systemic Disease Parameters and Disease Activity Scores. Apart from an association with inflammation, it was also investigated if sTyro3 levels in the joints correlated with systemic disease parameters and disease activity scores. Indeed, sTyro3 levels demonstrated a significant correlation to DAS28-ESR scores ($r = 0.79$, $P = 0.004$) (Figure 5(a)) and sTyro3 levels in patients with a high disease activity (>4.0) were significantly

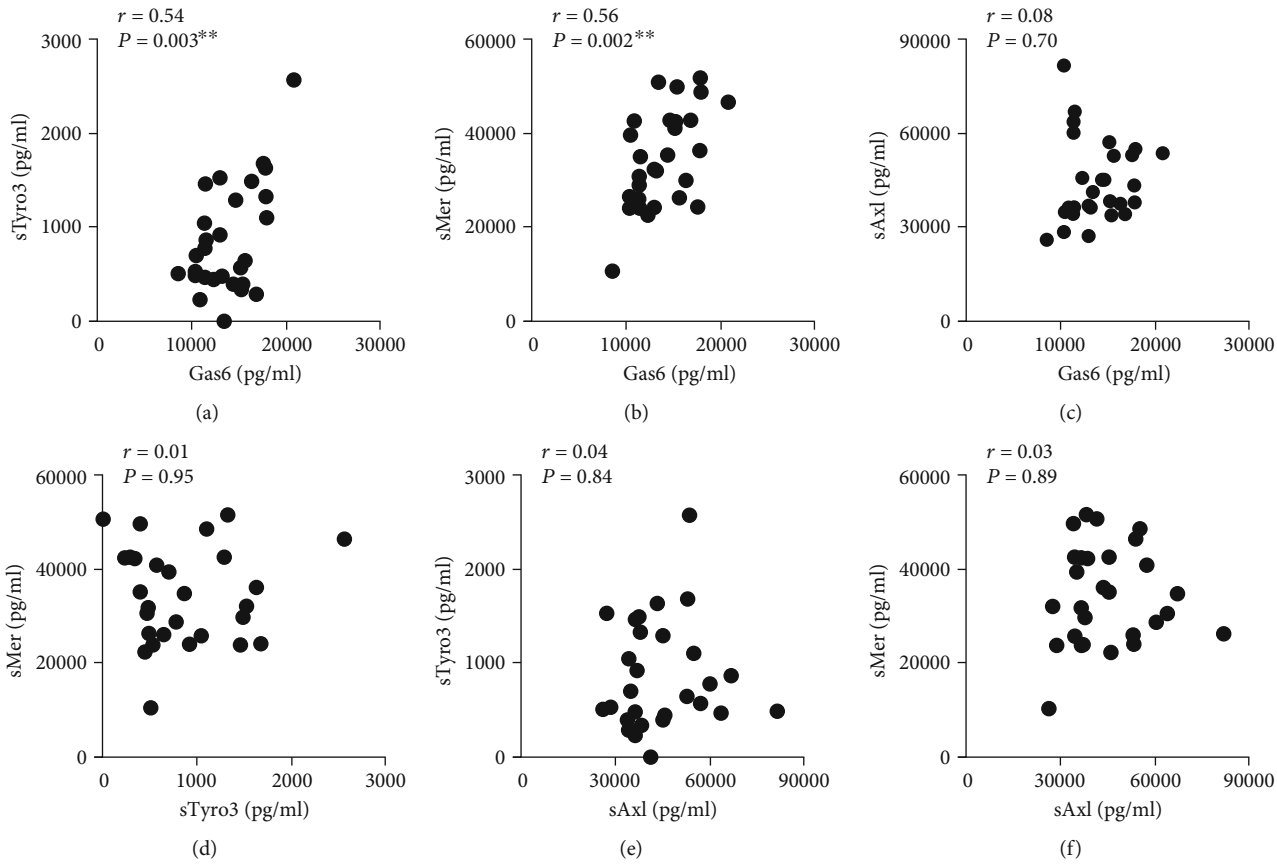


Figure 3: Relationship between soluble TAM receptor levels in the synovial fluid of rheumatoid arthritis patients. Relationship between soluble Tyro3 (sTyro3), soluble Mer (sMer), soluble Axl (sAxl), and Gas6 levels in synovial fluid of rheumatoid arthritis patients ($n = 28$). Correlations are depicted for sTyro3–Gas6 (a), sMer–Gas6 (b), sAxl–Gas6 (c), sMer–sTyro3 (d), sTyro3–sAxl (e), and sMer–sAxl (f). Data are presented as the Pearson r value (r) and P value (P) for each correlation. $^{**}P < 0.01$.

higher than those with a low (<3.2) or medium (3.2-4.0) DAS28-ESR score ($P = 0.0006$) (Figure 5(b)). Furthermore, sTyro3 levels were significantly higher in rheumatoid factor (RF) and anticitrullinated protein antibody (ACPA) double positive patients compared to double negative patients ($P = 0.005$) (Figure 5(c)). Due to the low number of single RF (3) and ACPA (2) positive RA patients enrolled in this study, the statistical analysis was impaired, although their mean sTyro3 values were comparable to double negative RA patients. No significant correlations were found between sMer and DAS28-ESR (Figure 5(d)), disease activity (Figure 5(e)) or RF, and ACPA (Figure 5(f)), as well as Gas6 (Figures 5(g)–5(i)). Together, these results suggested that sTyro3 correlates with inflammation associated mechanisms and disease activity of RA.

4. Discussion

In this study, we showed that, in RA patients, local sTyro3 and sMer levels were increased as compared to OA patients, whereas sAxl and Gas6 levels in both RA and OA patients were within the normal plasma level range as found in healthy controls. In addition, elevated synovial fluid levels of sTyro3 were associated with local inflammatory mediators, systemic disease parameters, and disease activity scores, indicating a role for sTyro3 in RA pathology.

Proper efferocytosis during homeostasis or inflammation has been shown to restrict subsequent immune responses. This effect is pivotal in the maintenance of the immune tolerance. The role of TAM receptors has been described in various autoimmune diseases, including RA, multiple sclerosis (MS), and systemic lupus erythematosus (SLE) [28, 29]. In the context of RA, the expression of Axl has already been described in joints of patients [30]. Also, a recent study using a murine model of K/BxN serum-transfer arthritis showed that Axl was expressed by a distinct subset of CX_3CR1^+ tissue-resident macrophages, which form an internal immunological barrier at the synovial lining [31]. These findings reinforce our data regarding the detection of Axl in lining cells. In addition, to the best of our knowledge, this is the first work that describes the expression of Tyro3 and Mer in the human synovial tissue of RA patients.

Although measured in plasma, Xu et al. found a comparable result of elevated sTyro3 and sMer in RA compared to OA patients and that only sTyro3 levels linked to some clinical RA features, although correlations were weak [32]. In addition, Wu et al. reported elevated sTyro3 and sMer plasma levels in RA patients compared to healthy controls, while plasma sTyro3 did not correlate with clinical features

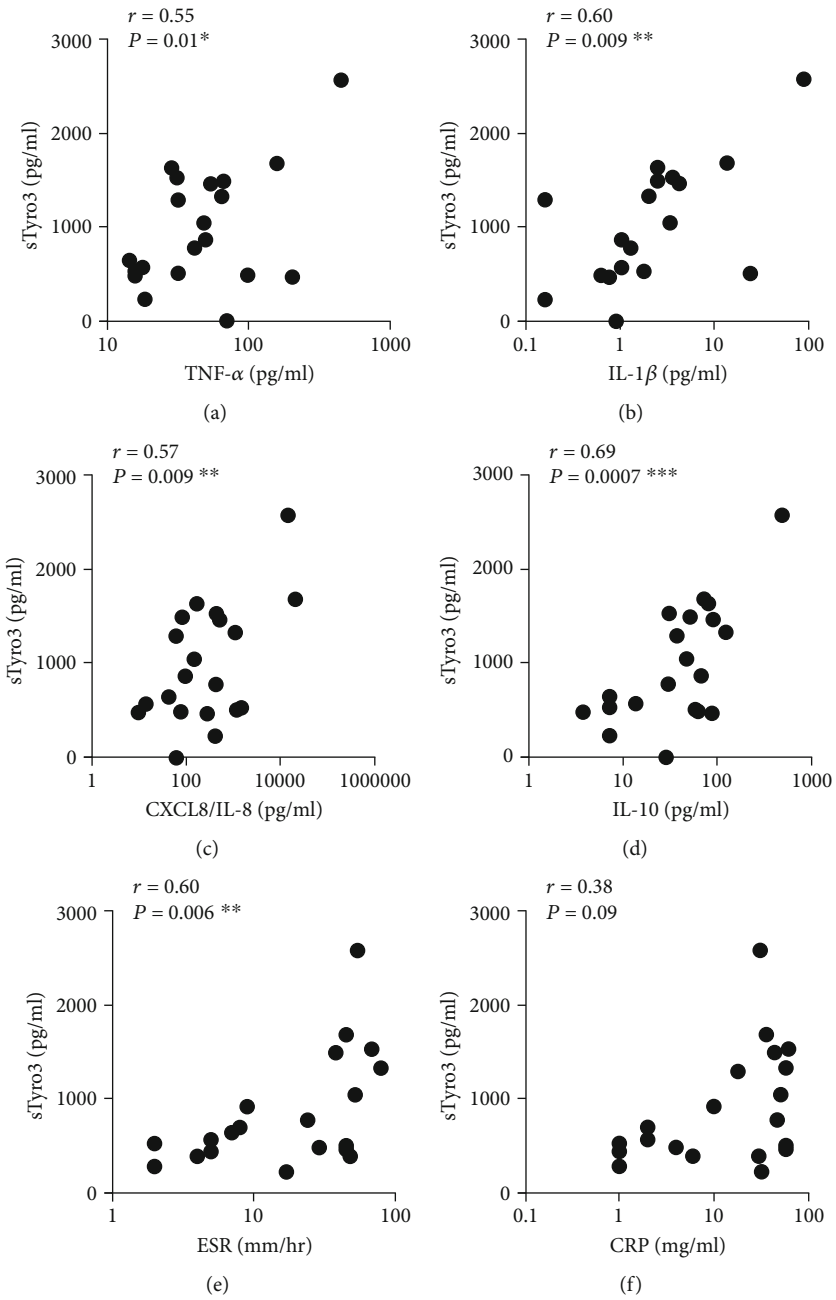


Figure 4: Relationship between soluble Tyro3 levels and inflammatory markers in the blood and synovial fluid of RA patients. Relationship between soluble Tyro3 (sTyro3) and the cytokines TNF-α, IL-1β, IL-8, and IL-10 in the synovial fluid of rheumatoid arthritis patients ($n = 20$). Correlations are depicted for sTyro3–TNF-α (a), sTyro3–IL-1β (b), sTyro3–IL-8 (c), and sTyro3–IL-10 (d). Relationship between soluble Tyro3 (sTyro3) and erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in the blood of rheumatoid arthritis patients ($n = 20$). Correlations are depicted for sTyro3–ESR (e) and sTyro3–CRP (f). Data are presented as Pearson r value (r) and P value (P) for each correlation. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

of RA [23]. In our study, using the synovial fluid rather than plasma, we found strong positive correlations between sTyro3 levels in both inflammation and disease activity scores, indicating that the synovial fluid sTyro3 is a more reliable measure of joint inflammation in RA compared to plasma sTyro3 levels. Although the focus of our study was the analysis of local TAM expression, not in plasma, we believe our data are complementary and reinforce the findings from the published studies so far [23, 32]. Interestingly, changes in sTAM levels are not consistent when comparing different arthritis related autoimmune diseases. Elevated plasma levels of both sMer and sAxl were reported in SLE patients [23–25], whereas Sjörgen's syndrome patients presented elevation of only sMer [26]. In SLE and Sjögren's syndrome, sMer rather than sTyro3 levels seem to be associated with inflammation and disease activity [23, 24, 26],

Table 2: Relationship between soluble TAM receptor levels and local and systemic inflammatory markers.

RA	TNF-α		IL-1β		IL-6		IL-8		IL-10		ESR		CRP	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
sTyro3	0.555	0.011*	0.597	0.009**	0.216	0.361	0.567	0.009**	0.695	0.001***	0.595	0.006**	0.396	0.084
sMer	0.231	0.328	0.115	0.649	-0.388	0.091	-0.011	0.963	0.230	0.199	0.057	0.810	-0.097	0.684
sAxl	0.325	0.162	-0.025	0.922	-0.397	0.083	0.129	0.589	0.168	0.479	-0.012	0.961	-0.141	0.552
Gas6	0.477	0.033*	0.481	0.043*	-0.083	0.727	0.529	0.017*	0.562	0.01**	0.223	0.345	-0.034	0.887

Relationship between soluble Mer (sMer), soluble Tyro3 (sTyro3), soluble Axl (sAxl), and Gas6 and cytokine levels in the synovial fluid and erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in the blood of rheumatoid arthritis ($n = 20$). Data are presented as Pearson r value (r) and P value for each correlation. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

indicating that sTyro3 could be more specific for RA disease pathology.

TAM receptors could be differentially regulated, expressed on different cell types, as demonstrated in this study, or exhibit different functions in distinct autoimmune diseases [14]. This could explain the difference in the levels of soluble TAM receptors observed here as well as the lack of correlation among them. Thus, additional studies regarding the modulation of TAM receptors in the context of arthritis are needed. In addition, it has been described that inflammatory autoimmune diseases are often characterized by deregulated metalloproteinase activities [33], indicating that changes in the levels of sTAM receptors observed in RA patients could indeed be caused by differential activation of shedding mechanisms. For example, shedding of TAM receptors can decrease TAM-mediated anti-inflammatory signaling and thereby increase cytokine production by both reducing the amount of membrane-bound TAM receptors and by acting as a decoy receptor, capturing the TAM receptors ligands and thereby reducing activation of membrane-bound receptors [21, 22, 34]. The metalloproteinase ADAM17 has been described as a key enzyme responsible for the proteolytic cleavage of TAM receptors [19, 20, 34]. Although most studies were performed in mice, there are some indications that ADAM17 is also responsible for cleavage of TAM receptors in humans. For instance, patients with chronic kidney disease have increased plasma levels of sMer and sAxl with increased expression of ADAM17 on monocytes [35]. In addition, different works have already shown that ADAM17 is expressed higher in the serum and synovial fluid or tissue of RA compared to OA patients [36–38]. Thus, higher levels of synovial sTAM in RA, particularly sTyro3, impair an important endogenous anti-inflammatory branch to control joint inflammation.

The decreased membrane bound Tyro3 on synovial cells could have a direct effect in reducing the control of joint inflammation, favoring the positive correlation between sTyro3 and local inflammatory parameters or disease activity in RA. However, much of the knowledge about the anti-inflammatory properties of Tyro3 came through triple TAM receptor knock out studies, although few studies explore individual anti-inflammatory properties of Tyro3. For instance, neutralizing anti-Tyro3 antibody reverted the effect of Pros1 in the reduction of proinflammatory cytokines by the human gingival epithelial cell line stimulated with *Porphyromonas gingivalis* LPS [39]. Thus, the reduced availability of joint TAM receptor ligands through the scavenger property of sTyro3 might have a stronger impact in a defective control of joint inflammation in RA. At this point, TAM receptor ligands, particularly Gas6, are involved in the resolution of inflammation [40] and present protective effects in experimental arthritis [41]. On the other hand, sMer barely binds to TAM receptor ligands [13], weakening its contribution as a decoy receptor. For that reason, the effects of reduced Mer surface expression due to its shedding to the synovial fluid were not enough to reduce anti-inflammatory feedback, explaining why no correlation was observed between sMer and cytokine levels or disease activity.

sTyro3 and sAxl act as effective ligand antagonists by blocking Pros1 and Gas6, respectively, while sMer shows weak inhibitory activities toward both ligands [13]. Here, we showed that sTyro3 levels were associated with disease phenotype, as RF and ACPA double positive patients exhibited higher sTyro3 levels. Previous studies have demonstrated that Gas6 and Pros1 limit the immune response by inhibition of DC maturation and reduction of antigen presentation to T cells [42, 43]. The pronounced disease activity associated with RF and ACPA double positive patients could be explained by the blocking of TAM ligands due to elevated sTyro3 in RA patients. Although we have not seen a significant increase of Gas6 in the synovial fluid of RA patients, there was a trend for its increase ($P = 0.074$, Figure 2(d)). In experimental arthritis, Ruiz-Heiland et al. found increased levels of circulating Gas6 after induction of a murine model of arthritis [44], which can be speculated as an attempt to control tissue inflammation. Thus, this could explain the positive correlations between Gas6 and sTyro3 and sMer. On the other hand, increased local soluble TAM receptors could scavenge TAM ligands, exemplified here only by Gas6. Thus, reduced availability of Gas6 would impair the anti-inflammatory function of this molecule due reduced membrane TAM receptor activation. In fact, the protective role of both TAM receptor ligands Gas6 and Pros1 has already been described by our group in murine models of arthritis [16, 17, 41]. In these studies, Gas6 and Pros1 overexpression decreased arthritis severity, by reducing inflammation and by inhibiting the expression of proinflammatory cytokines. Although we have not measured Pros1 in this study, we believe that high levels of sTyro3 in the synovial fluid could indeed scavenge both TAM ligands, reducing their anti-inflammatory function in tissue inflammation in RA.

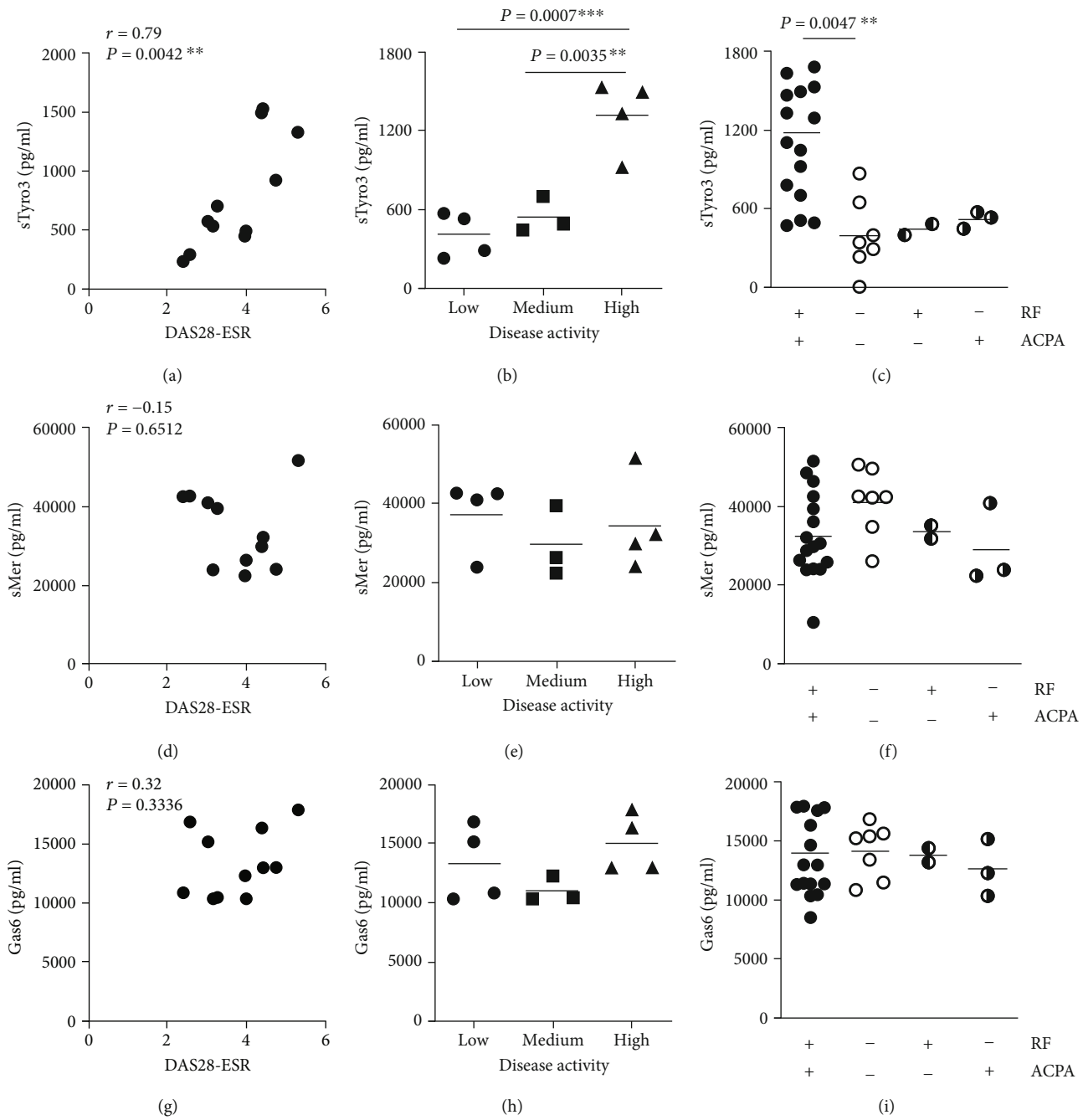


Figure 5: Relationship between soluble Tyro3 levels, disease activity scores, and systemic disease parameters. Relationship between soluble Tyro3 (sTyro3), soluble Mer (sMer), and Gas6 levels in synovial fluid of RA patients ($n = 11$) and disease activity scores based on erythrocyte sedimentation rate (DAS28-ESR) (a, d, g). sTyro3 levels in patients with low (<3.2), medium (3.2–4.0), and high (>4.0) DAS28ESR scores (b, e, h). Differences between soluble Tyro3 (sTyro3) in the synovial fluid of RA patients double positive (>10 IU/ml) for rheumatoid factor (RF) and anticyclic citrullinated peptide antibody (ACPA) (16 samples), single positive for RF (2 samples) or ACPA (3 samples), and double negative (<10 IU/ml) (7 samples) (c, f, i). Data are presented as P value for each comparison (unpaired t-tests) or as Pearson r value (r) and P value (P) for correlation. $^{**}P < 0.01$; $^{***}P < 0.001$.

In summary, in the present study, we showed that sTyro3 and sMer levels are increased in the synovial fluid of RA patients, whereas only sTyro3 correlates with inflammation and disease parameters. This indicates a role for sTyro3 in RA disease pathology, especially in RF- and ACPA-positive patients. Further functional studies are needed to elucidate the role of sTyro3 in the development of RA and to investigate whether sTyro3 is a marker that will be helpful in the evaluation of RA disease activity and local inflammation.

Data Availability

All relevant data are within the paper and its supporting information files.

Additional Points

Key message. TAM receptors are expressed on different locations in synovial tissue (lining, sublining, blood vessels). Soluble Tyro3 and soluble Mer levels are increased in synovial fluid of RA patients compared to OA patients. In RA patients, soluble Tyro3 in synovial fluid positively correlates with joint inflammation and disease activity.

Conflicts of Interest

There are no conflicts of interest.

Acknowledgments

We thank Birgitte Walgreen for helping with immunohistochemical staining, Monique Helsen for performing the Multiplex ELISA, and members of the Experimental Rheumatology department for useful dialogues. This work was supported by the Dutch Arthritis Foundation (RF15-2-403 and RN19-1-204).

Supplementary Materials

Figure S1: soluble TAM receptor levels in synovial fluid of male versus female rheumatoid arthritis. Soluble Tyro3 (sTyro3) (A), soluble Mer (sMer) (B), soluble Axl (sAxl) (C), and Gas6 (D) levels in the synovial fluid of male versus female rheumatoid arthritis patients ($n = 28$). Data are presented dot plots with mean unpaired t-tests. Figure S2: relationship between soluble TAM receptor levels and age. Relationship between soluble Tyro3 (sTyro3) (A), soluble Mer (sMer) (B), soluble Axl (sAxl) (C), and Gas6 (D) levels in the synovial fluid and the age in years of rheumatoid arthritis patients ($n = 28$). Data are presented as the Pearson r value (r) and P value (P) for each correlation. *(Supplementary Materials)*

References

[1] G. S. Firestein, "Evolving concepts of rheumatoid arthritis," *Nature*, vol. 423, no. 6937, pp. 356–361, 2003.

[2] E. Calabresi, F. Petrelli, A. F. Bonifacio, I. Puxeddu, and A. Alunno, "One year in review 2018: pathogenesis of rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 36, no. 2, pp. 175–184, 2018.

[3] D. Hamerman and M. Klagsbrun, "Osteoarthritis. Emerging evidence for cell interactions in the breakdown and remodeling of cartilage," *The American Journal of Medicine*, vol. 78, no. 3, pp. 495–499, 1985.

[4] F. Berenbaum, "Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!)," *Osteoarthritis and Cartilage*, vol. 21, no. 1, pp. 16–21, 2013.

[5] X. Houard, M. B. Goldring, and F. Berenbaum, "Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis," *Current Rheumatology Reports*, vol. 15, no. 11, p. 375, 2013.

[6] A. Mathiessen and P. G. Conaghan, "Synovitis in osteoarthritis: current understanding with therapeutic implications," *Arthritis Research & Therapy*, vol. 19, no. 1, p. 18, 2017.

[7] B. J. E. de Lange-Brokaar, A. Ioan-Facsinay, G. J. V. M. van Osch et al., "Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review," *Osteoarthritis and Cartilage*, vol. 20, no. 12, pp. 1484–1499, 2012.

[8] C. V. Rothlin, E. A. Carrera-Silva, L. Bosurgi, and S. Ghosh, "TAM receptor signaling in immune homeostasis," *Annual Review of Immunology*, vol. 33, no. 1, pp. 355–391, 2015.

[9] C. V. Rothlin, S. Ghosh, E. I. Zuniga, M. B. A. Oldstone, and G. Lemke, "TAM receptors are pleiotropic inhibitors of the innate immune response," *Cell*, vol. 131, no. 6, pp. 1124–1136, 2007.

[10] K. Q. Nguyen, W. I. Tsou, S. Kotenko, and R. B. Birge, "TAM receptors in apoptotic cell clearance, autoimmunity, and cancer," *Autoimmunity*, vol. 46, no. 5, pp. 294–297, 2013.

[11] G. Lemke and T. Burstyn-Cohen, "TAM receptors and the clearance of apoptotic cells," *Annals of the New York Academy of Sciences*, vol. 1209, no. 1, pp. 23–29, 2010.

[12] E. D. Lew, J. Oh, P. G. Burrola et al., "Differential TAM receptor-ligand-phospholipid interactions delimit differential TAM bioactivities," *eLife*, vol. 3, 2014.

[13] W. I. Tsou, K. Q. N. Nguyen, D. A. Calarese et al., "Receptor tyrosine kinases, TYRO3, AXL, and MER, demonstrate distinct patterns and complex regulation of ligand-induced activation," *The Journal of Biological Chemistry*, vol. 289, no. 37, pp. 25750–25763, 2014.

[14] A. Zagórska, P. G. Través, E. D. Lew, I. Dransfield, and G. Lemke, "Diversification of TAM receptor tyrosine kinase function," *Nature Immunology*, vol. 15, no. 10, pp. 920–928, 2014.

[15] S. Zheng, M. Hedl, and C. Abraham, "TAM receptor-dependent regulation of SOCS3 and MAPKs contributes to proinflammatory cytokine downregulation following chronic NOD2 stimulation of human macrophages," *Journal of Immunology*, vol. 194, no. 4, pp. 1928–1937, 2015.

[16] C. E. J. Waterborg, S. Beermann, M. G. A. Broeren et al., "Protective role of the MER tyrosine kinase via efferocytosis in rheumatoid arthritis models," *Frontiers in Immunology*, vol. 9, p. 742, 2018.

[17] C. E. J. Waterborg, M. G. A. Broeren, E. N. Blaney Davidson et al., "The level of synovial AXL expression determines the outcome of inflammatory arthritis, possibly depending on the upstream role of TGF-β1," *Rheumatology (Oxford)*, vol. 58, no. 3, pp. 536–546, 2019.

[18] C. E. J. Waterborg, M. I. Koenders, P. L. E. M. van Lent, P. M. van der Kraan, and F. A. J. van de Loo, "Tyro3/Axl/Mertk-deficient mice develop bone marrow edema which is an early pathological marker in rheumatoid arthritis," *PLoS One*, vol. 13, no. 10, article e0205902, 2018.

[19] L. Guo, J. R. Eisenman, R. M. Mahimkar et al., "A proteomic approach for the identification of cell-surface proteins shed by metalloproteases," *Molecular & Cellular Proteomics*, vol. 1, no. 1, pp. 30–36, 2002.

[20] J. P. O'Bryan, Y. W. Fridell, R. Koski, B. Varnum, and E. T. Liu, "The transforming receptor tyrosine kinase, Axl, is post-translationally regulated by proteolytic cleavage," *The Journal of Biological Chemistry*, vol. 270, no. 2, pp. 551–557, 1995.

[21] C. Ekman, J. Stenhoff, and B. Dahlbäck, "Gas6 is complexed to the soluble tyrosine kinase receptor Axl in human blood," *Journal of Thrombosis and Haemostasis*, vol. 8, no. 4, pp. 838–844, 2010.

[22] S. Sather, K. D. Kenyon, J. B. Lefkowitz et al., "A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation," *Blood*, vol. 109, no. 3, pp. 1026–1033, 2006.

[23] J. Wu, C. Ekman, A. Jönsen et al., "Increased plasma levels of the soluble Mer tyrosine kinase receptor in systemic lupus erythematosus relate to disease activity and nephritis," *Arthritis Research & Therapy*, vol. 13, no. 2, p. R62, 2011.

[24] G. Zizzo, J. Guerrieri, L. M. Dittman, J. T. Merrill, and P. L. Cohen, "Circulating levels of soluble MER in lupus reflect M2c activation of monocytes/macrophages, autoantibody specificities and disease activity," *Arthritis Research & Therapy*, vol. 15, no. 6, p. R212, 2013.

[25] C. Ekman, A. Jonsen, G. Sturfelt, A. A. Bengtsson, and B. Dahlback, "Plasma concentrations of Gas6 and sAxl correlate with disease activity in systemic lupus erythematosus," *Rheumatology (Oxford)*, vol. 50, no. 6, pp. 1064–1069, 2011.

[26] B. Qin, J. Wang, N. Ma et al., "The association of Tyro3/Axl/-Mer signaling with inflammatory response, disease activity in patients with primary Sjögren's syndrome," *Joint, Bone, Spine*, vol. 82, no. 4, pp. 258–263, 2015.

[27] J. Fransen and P. L. C. M. van Riel, "The disease activity score and the EULAR response criteria," *Rheumatic Diseases Clinics of North America*, vol. 35, no. 4, pp. 745–757, 2009.

[28] M. Wium, J. Paccez, and L. Zerbini, "The dual role of TAM receptors in autoimmune diseases and cancer: an overview," *Cell*, vol. 7, no. 10, p. 166, 2018.

[29] C. V. Rothlin and G. Lemke, "TAM receptor signaling and autoimmune disease," *Current Opinion in Immunology*, vol. 22, no. 6, pp. 740–746, 2010.

[30] K. O'Donnell, I. C. Harkes, L. Dougherty, and I. P. Wicks, "Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel endothelial cell survival pathway," *The American Journal of Pathology*, vol. 154, no. 4, pp. 1171–1180, 1999.

[31] S. Culemann, A. Grüneboom, J. Á. Nicolás-Ávila et al., "Locally renewing resident synovial macrophages provide a protective barrier for the joint," *Nature*, vol. 572, no. 7771, pp. 670–675, 2019.

[32] L. Xu, F. Hu, H. Zhu et al., "Soluble TAM receptor tyrosine kinases in rheumatoid arthritis: correlation with disease activity and bone destruction," *Clinical and Experimental Immunology*, vol. 192, no. 1, pp. 95–103, 2018.

[33] S. Lisi, M. D'Amore, and M. Sisto, "ADAM17 at the interface between inflammation and autoimmunity," *Immunology Letters*, vol. 162, no. 1, pp. 159–169, 2014.

[34] E. Thorp, T. Vaisar, M. Subramanian, L. Mautner, C. Blobel, and I. Tabas, "Shedding of the Mer tyrosine kinase receptor is mediated by ADAM17 protein through a pathway involving reactive oxygen species, protein kinase Cδ, and p38 mitogen-activated protein kinase (MAPK)," *The Journal of Biological Chemistry*, vol. 286, no. 38, pp. 33335–33344, 2011.

[35] I. J. Lee, B. A. Hilliard, M. Ulas et al., "Monocyte and plasma expression of TAM ligand and receptor in renal failure: links to unregulated immunity and chronic inflammation," *Clinical Immunology*, vol. 158, no. 2, pp. 231–241, 2015.

[36] S. Ohta, M. Harigai, M. Tanaka et al., "Tumor necrosis factor-alpha (TNF-alpha) converting enzyme contributes to production of TNF-alpha in synovial tissues from patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 28, no. 8, pp. 1756–1763, 2001.

[37] S. Ishii, T. Isozaki, H. Furuya et al., "ADAM-17 is expressed on rheumatoid arthritis fibroblast-like synoviocytes and regulates proinflammatory mediator expression and monocyte adhesion," *Arthritis Research & Therapy*, vol. 20, no. 1, p. 159, 2018.

[38] M. Charbonneau, K. Harper, F. Grondin, M. Pelmus, P. P. McDonald, and C. M. Dubois, "Hypoxia-inducible factor mediates hypoxic and tumor necrosis factor alpha-induced increases in tumor necrosis factor-alpha converting enzyme/ADAM17 expression by synovial cells," *The Journal of Biological Chemistry*, vol. 282, no. 46, pp. 33714–33724, 2007.

[39] L. Jiang, X. Q. Chen, M. J. Gao et al., "The Pros1/Tyro3 axis protects against periodontitis by modulating STAT/SOCS signalling," *Journal of Cellular and Molecular Medicine*, vol. 23, no. 4, pp. 2769–2781, 2019.

[40] S. Nepal, C. Tiruppathi, Y. Tsukasaki et al., "STAT6 induces expression of Gas6 in macrophages to clear apoptotic neutrophils and resolve inflammation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 116, no. 33, pp. 16513–16518, 2019.

[41] B. T. van den Brand, S. Abdollahi-Roodsaz, E. A. Vermeij et al., "Therapeutic efficacy of Tyro3, Axl, and Mer tyrosine kinase agonists in collagen-induced arthritis," *Arthritis and Rheumatism*, vol. 65, no. 3, pp. 671–680, 2013.

[42] S. Scutera, T. Fraone, T. Musso et al., "Survival and migration of human dendritic cells are regulated by an IFN-alpha-inducible Axl/Gas6 pathway," *Journal of Immunology*, vol. 183, no. 5, pp. 3004–3013, 2009.

[43] E. A. Carrera Silva, P. Y. Chan, L. Joannas et al., "T cell-derived protein S engages TAM receptor signaling in dendritic cells to control the magnitude of the immune response," *Immunity*, vol. 39, no. 1, pp. 160–170, 2013.

[44] G. Ruiz-Heiland, Y. Zhao, A. Derer et al., "Deletion of the receptor tyrosine kinase Tyro3 inhibits synovial hyperplasia and bone damage in arthritis," *Annals of the Rheumatic Diseases*, vol. 73, no. 4, pp. 771–779, 2014.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 9430763, 11 pages
https://doi.org/10.1155/2020/9430763

Research Article

Elemental Diet Enriched with Amino Acids Alleviates Mucosal Inflammatory Response and Prevents Colonic Epithelial Barrier Dysfunction in Mice with DSS-Induced Chronic Colitis

Di Guo,[1] Jun Yang,[2] Fangmei Ling,[1] Lei Tu,[1] Junrong Li,[1] Yidong Chen,[1] Kaifang Zou,[1] Liangru Zhu,[1] and Xiaohua Hou[1]

[1]*Division of Gastroenterology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, 430022 Hubei, China*
[2]*Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, 430022 Hubei, China*

Correspondence should be addressed to Liangru Zhu; zhuliangru@hust.edu.cn and Xiaohua Hou; houxh@hust.edu.cn

Received 6 May 2020; Accepted 8 July 2020; Published 14 August 2020

Academic Editor: Lihua Duan

Background. Clinical data suggest that enteral nutrition (EN) effectively decreases disease activity and maintains remission in patients with inflammatory bowel disease (IBD). However, the modulatory effects of EN on the intestinal mucosal immune system remain unclear. *Aims*. This study first aimed at comparing the therapeutic effects of three EN formulas on ameliorating dextran sulfate sodium- (DSS-) induced chronic colitis; with the most effective formula, we then examined its influence on the mucosal inflammatory response and epithelial barrier function. *Methods*. The effect of EN formulas on colitis in mice was assessed by body weight, disease activity index scores, colon length, and H&E staining for pathological examination. Colonic and circulating cytokine expression levels and the frequencies of immune cells were also analyzed. Intestinal epithelial barrier function was evaluated by detecting tight junction proteins. *Results*. We found that among the three EN formulas, an elemental diet (ED) containing enriched amino acids restored the colitis-related reduction in body weight better than the other two EN formulas. ED amino acids suppressed the release of colonic proinflammatory mediators and maintained the expression of tight junction proteins in these mice. ED amino acid treatment mitigated the colitis-induced increase in $CD103^+CD11b^+$ dendritic cells and $CD4^+$ and $CD8^+$ T cells and inhibited the predominant Th1/Th17 responses particularly in the colonic mucosal lamina propria of mice with colitis. *Conclusions*. We showed that ED amino acids can be an effective immunomodulatory agent to reduce colitis-related inflammation by inhibiting proinflammatory mediators and Th1/Th17 cell responses and by repairing the disrupted epithelial barrier.

1. Introduction

Nutritional disturbances represent a frequent manifestation in inflammatory bowel disease (IBD) patients, especially those with Crohn's disease (CD) [1]. There is increasing evidence that dietary factors might play a role in the pathogenesis of IBD, and especially CD [2]. Moreover, enteral nutrition (EN) has successfully been used as a nutritional therapy for patients with CD. EN remains of interest for patients presenting with malnutrition, those who failed with other therapeutics, patients with complications, or selected patients on long-term maintenance therapy with fewer side effects [3–5]. It has also been used as a first-line therapy for pediatric patients with CD [6] and was shown to induce clinical remission and mucosal healing and improve body composition in patients with CD [7–9].

These data suggested that EN effectively decreases disease activity and maintains remission in patients with IBD, but the potential mechanism is multifactorial, generally due to its anti-inflammatory effects and regulation of the intestinal

Table 1: A comparison of three different EN formulas.

Name	Nitrogen source	Fat (g/100 kcal)	Protein (g/100 kcal)	Carbohydrate (g/100 kcal)
Enteral nutritional powder (AA)	17 amino acids	0.17	4.7	21.14
Enteral nutritional suspension (SP)	Whey protein hydrolysates	1.7	4.0	17.6
Enteral nutritional powder (TP)	Casein-calcium, casein-sodium, soybean protein	3.53	3.53	13.5

microflora [1]. However, few studies have addressed the regulatory effects of EN on adaptive and innate immune cell profiles in the immunomicroenvironment of colitis.

It has been previously reported that EN reduces inflammation in murine IL-$10^{-/-}$ cell transfer colitis models [10]. However, the anti-inflammatory effect of different EN formulas in a chronic colitis model remains unclear. In the treatment of IBD, three different types of EN formulas are commonly used, including an elemental diet (ED) containing enriched amino acids, a semielemental formula containing short peptides, and a polymeric formula diet containing intact protein. Meanwhile, studies comparing different EN treatments have obtained conflicting results. An earlier study showed a significantly higher remission rate in acute CD patients treated with ED amino acids than in patients receiving a polymeric diet (75% vs. 36%, $p < 0.03$) [11]. But later observation found that elemental and polymeric diets were equally effective [12]. It was speculated that the nitrogen source and fat content might be relevant to the therapeutic efficacy of EN. However, recent comprehensive studies providing open comparisons of the therapeutic efficacy of these different EN formulas are still lacking.

In the present study, we aimed to examine whether these three EN formulas could ameliorate disease activity in mice with chronic colitis induced by dextran sulfate sodium (DSS) and to compare their therapeutic efficacy. Next, with the most effective EN formula, we further investigated its regulatory effects on immune cell and inflammatory cytokine profiles, as well as intestinal epithelial barrier function, to explore its potential immunological mechanism of action.

2. Materials and Methods

2.1. Mice and Disease Model. All experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (S2257). Additionally, the study was performed in accordance with institutional guidelines. Male C57BL/6 mice, 10 weeks of age, were obtained from HFK Bioscience (Beijing, China). The mice were housed in a specific pathogen-free facility at the Experimental Animal Center. They were given a standard chow diet and tap water ad libitum. Experimental chronic colitis was induced by an oral administration of water containing 2% (wt/vol) dextran sodium sulfate (DSS, MP Biomedicals, Illkirch, France) for three cycles (5 days with 2% DSS and 4 days of water). The control and colitis groups of mice were allowed free access to food and water or DSS solution. The mice in the three colitis groups received three different exclusive enteral nutrition formulas ($n = 10$ per group) daily beginning on the 24th day, and all the mice were sacrificed on day 42. All exclusive enteral nutrition diets were isocaloric and equivalent concentrations (every mouse received 20 kcal per day). The exclusive enteral nutrition formulas are as follows: Enteral Nutritional Powder (AA), an amino acid-enriched elemental diet (Wanhe Pharmaceutical Co., Ltd., Shenzhen, China); Enteral Nutritional Suspension (SP), a semielemental formula containing short peptides (Nutricia Pharmaceutical Co., Ltd., Wuxi, China); and Enteral Nutritional Powder (TP), a polymeric formula diet containing intact protein (Abbott Lab., Zwolle, Netherlands). A comparison of three different EN formulas are listed in Table 1, and the composition of the different formulas are listed in Table S1. Disease activity index (DAI) scores were assessed by combining the scores of weight loss, stool consistency, and bleeding [13]. At the end of the experiment, all mice were euthanized, and their colons were collected and photographed.

2.2. Hematoxylin-Eosin (H&E) Staining. The medial colon tissues were fixed in 10% buffered formalin overnight and embedded in paraffin. The tissue sections (5 μm) were stained with H&E and examined by light microscopy.

2.3. Isolation of Cells. Mouse splenic mononuclear cells (MNCs), mesenteric lymph nodes (MLNs), and lamina propria mononuclear cells (LPMCs) were isolated as previously reported [14]. To isolate LPMCs, in brief, the colonic tissues were flushed and cut into small pieces, followed by washing with 1 mM DTT in Hanks' balanced salt solution at 37°C for 20 min. To remove the epithelium, the pieces were incubated in 1.3 mM EDTA Hank's balanced salt solution at 37°C for 30 min. To isolate LPMCs, the remaining colonic tissue pieces were digested with 0.1 mg/mL Collagenase D (Roche Applied Science, Basel, Switzerland) in serum-free Iscove's modified Dulbecco's medium at 37°C for 1 h and filtered through a cell strainer, which was followed by centrifugation. Subsequently, the LPMCs were enriched by 40%/70% Percoll gradient centrifugation, and the cells at the interface were harvested for further experiments.

2.4. Reagents and Flow Cytometric Analysis. Mouse MNCs were stained with the following fluorescence-conjugated surface mAbs: anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE-cy7, anti-NK1.1-PE, anti-CD11b-APC-cy7, anti-CD103-BV421, anti-CD11c-BV510, and anti-CD25-APC antibodies (BD PharMingen, USA). The cells were stained according to the standard procedure of BD PharMingen. Fixation and permeabilization were performed using the Transcription Factor Buffer Set (BD PharMingen, USA), and the cells were

then stained with the anti-FoxP3-BV421 intracellular antibodies for 40 min at 4°C. To detect Th1 and Th17 cytokines, MNCs suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum were stimulated with a cell stimulation cocktail (eBioscience, USA) containing phorbol-12-myristate-13-acetate (PMA, 50 ng/mL), ionomycin (1 μg/mL), and monensin (2 μg/mL) at 37°C with 5% CO_2 for 6 h, and followed by intracellular staining with fluorescently labeled anti-IFN-γ and anti-IL-17 antibodies (BioLegend, USA) after washing, fixing, and permeabilizing according to the manufacturer's instructions. Isotype IgGs were used as a control. All samples were detected using a BD LSR Fortessa X-20 Flow Cytometry System (BD, USA) and analyzed using the FlowJo software.

2.5. Quantitative Real-Time PCR. Total RNA was extracted from mouse colonic tissue samples using TRIzol reagent (Takara, Shiga, Japan) and then transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The levels of target gene mRNA transcripts relative to the control GAPDH were determined by quantitative RT-PCR analyses on a Roche Light Cycler 480 System using SYBR Green Real-Time PCR Master Mix (Toyobo) and specific primers. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used are as follows: IL-1β, forward 5′-CTTCAGGCAGGCAGTATCACTC-3′ and reverse 5′-TGCAGTTGTCTAATGGGAACGT-3′; TNF-α, forward 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and reverse 5′-TGGGAGTAGACAAGGTACAACCC-3′; IFN-γ forward 5′-CAGGTGTGATTCAATGACGCT-3′ and reverse 5′-AACTCAAGTGGCATAGATGTGGA-3′; IL-17 forward 5′-CCTCAGACTACCTCAACCGTTC-3′ and reverse 5′-CTCTTCAGGACCAGGATCTCTT-3′; TGFβ, forward 5′-GACCGCAACAACGCCATCTAT-3′ and reverse 5′-GACAGCCACTCAGGCGTATCAG-3′; IL-10, forward 5′-GGACAACATACTGCTAACCGAC-3′ and reverse 5′-CATGGCCTTGTAGACACCTTG-3′; and GAPDH, forward 5′-CGGATTTGGTCGTATTGGG-3′ and reverse 5′-CTCGCTCCTGGAAGATGG-3′.

2.6. Immunofluorescence. The paraffin-embedded mouse colon sections (4 μm) were deparaffinized and stained with primary polyclonal antibodies including anti-rabbit claudin-1, anti-rabbit occludin, and anti-rabbit ZO-1 (all 1 : 100 dilution, Invitrogen, USA) overnight at 4°C, which was followed by secondary staining with Alexa Flour 488 donkey anti-rabbit IgG and then examination under a laser confocal microscopy.

2.7. Cytokines Analysis. Serum samples were prepared for the quantification of interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17A (IL-17A), and tumor necrosis factor-α (TNF-α) cytokines. Serum samples were analyzed by flow cytometry using the Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, USA) according to the manufacturer's instructions. Data were formatted and further analyzed using the BD CBA software.

2.8. Statistical Analysis. Statistical analyses were performed using the SPSS version 17.0 and GraphPad Prism version 5.0. The differences between the two groups were analyzed by performing unpaired Student's t-tests. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). Data are expressed as the means ± SEM. $p < 0.05$ was considered statistically significant.

3. Results

3.1. An Elemental Diet Enriched in Amino Acids Ameliorates DSS-Induced Chronic Colitis. After we established a chronic colitis mice model through three cycles of 2% DSS induction, EN amino acids, short peptides, and intact proteins were applied to study their therapeutic effects. Mice with colitis developed severe diarrhea, body weight loss (Figure 1(a)), and bloody stools. A significant reduction in colon length (Figure 1(b)) and an increase in DAI scores (Figure 1(c)) were observed in mice with colitis when compared to those in the control group. H&E staining showed architectural derangements, epithelial necrosis, and diffuse lymphocytic infiltration in the colon of colitis mice (Figure 1(d)). However, the three different ENs resulted in the obvious suppression of colitis severity, including significant reversals in body weight loss and colon length reduction, reduced DAI scores, and decreased lymphocytic infiltration in the colon (Figures 1(a)–1(d)). Between the three EN formulas used to treat colitis mice, although no significant differences were observed in the reversion of colon length reductions or in the degree of reductions in DAI scores, the restoration of body weight by ED amino acids was significantly enhanced compared to that with EN enriched in short peptides and intact proteins. The mice's body weight improvement started on day 28 for ED amino acids treatment group, but for the other two treatment groups, the mice's body weight did not improve until day 38. Therefore, with the most effective formula, we then investigated the potential anti-inflammatory mechanism of ED amino acids underlying the therapeutic effects on inducible colitis.

3.2. An Elemental Diet Enriched in Amino Acids Suppresses Proinflammatory Cytokines in the Colon. First, we found that the colonic mRNA expression levels of the proinflammatory mediators *IL-1β*, *IFN-γ*, *IL-17A*, and *TNF-α*, which had been confirmed to be involved in IBD, were upregulated notably in colitis mice compared to levels in normal controls (Figure 2(a)). Meanwhile, the expression levels of *TGFβ* mRNA, encoding an important cytokine that suppresses inflammation, were reduced dramatically in colitis mice compared to those in the control group (Figure 2(b)). Furthermore, treatment with ED amino acids was only found to downregulate these upregulated genes, namely *IL-1β*, *IFN-γ*, *IL-17A*, and *TNF-α* but had no impact on *TGFβ* and *IL-10* mRNA transcripts in the intestinal tissues

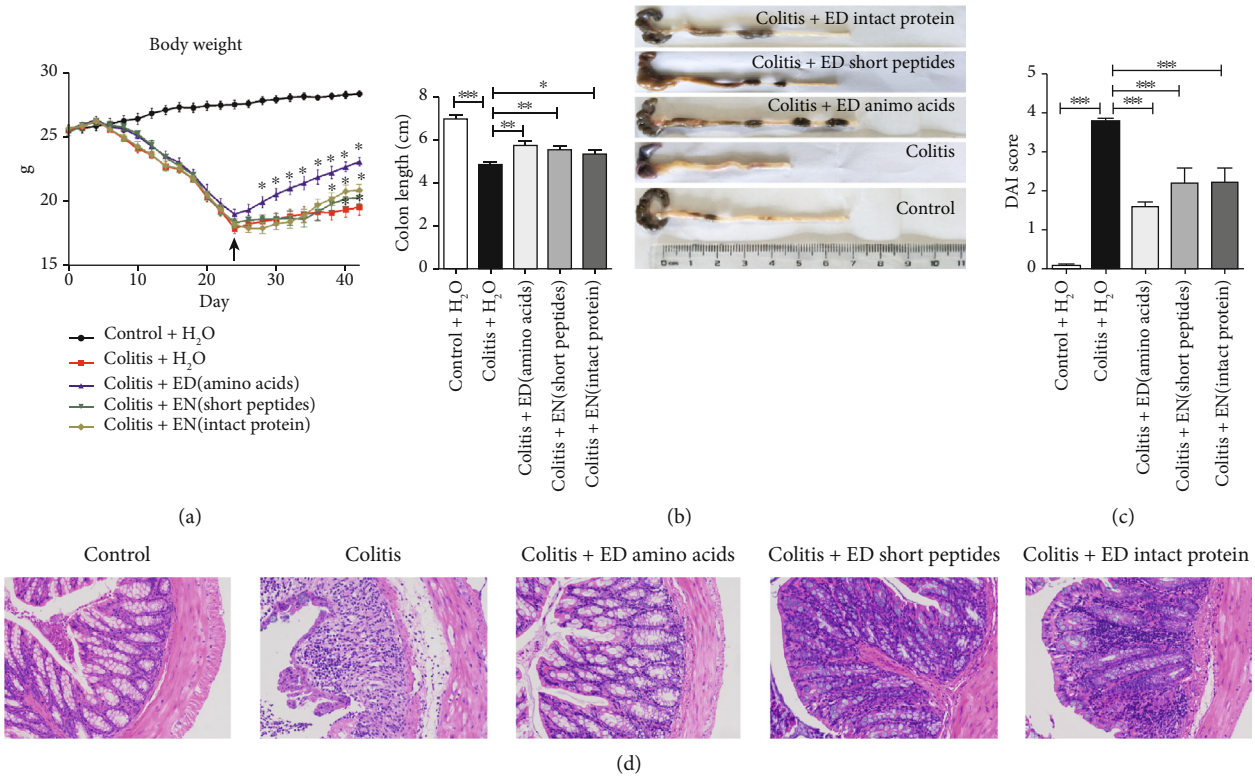


Figure 1: An elemental diet enriched in amino acids ameliorates dextran sulfate sodium- (DSS-) induced chronic colitis. (a) The body weights (g) of individual groups of mice ($n = 10$ per group). (b) Colon length (cm). (c) Disease activity index (DAI) scores. (d) Pathological changes in the colons (H&E staining, magnification, ×200). Data are representative images or expressed as the mean ± SEM of each group from three separate experiments, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

(Figures 2(a) and 2(b)). In conclusion, ED amino acids resulted in predominant resistance to the release of inflammatory cytokines in the colon.

Additionally, to determine the influence of ED amino acid treatment on inflammation in the peripheral circulation, the protein levels of several cytokines were measured in the serum from each group using a CBA Mouse Th1/Th2/Th17 Cytokine Kit. DSS treatment elevated the levels of IL-6 and reduced IL-2 and IFN-γ levels but had no effect on IL-17A and TNF-α (Figure 2(c)). DSS treatment also decreased the basal level of the anti-inflammatory meditators IL-10 and IL-4 (Figure 2(c)). However, the treatment of colitis mice with ED amino acids effectively eliminated the DSS-mediated increase in IL-6 and reduction in IL-10 protein levels in the serum. ED amino acids also resulted in an increase in IFN-γ, TNF-α, and IL-17A levels but had no effect on IL-2 and IL-4 levels in colitis mice. Overall, ED amino acids exerted a bidirectional regulatory effect on proinflammatory and anti-inflammatory cytokine release in the peripheral circulation.

3.3. An Elemental Diet Enriched in Amino Acids Affects the Accumulation of Intestinal Lamina Propria Dendritic Cells in Chronic Colitis Mice. It has been reported that IFN regulatory factor 4- (IRF4-) dependent $CD103^+CD11b^+$ dendritic cells (DCs) have a role in the generation of intestinal T helper 17 (Th17) cells, and evidence was provided that $CD103^+$-$CD11b^+$ DCs are important for Th17 cell differentiation in the intestinal draining MLN [15]. In addition, natural killer cells (NK) are the main component of innate immunity, but whether ED amino acids have a regulatory role in NK cells and DC cells in a chronic colitis model is not clearly understood. We next evaluated the effects of ED amino acids on the $CD103^+CD11b^+$ DC and $CD3^-$ $NK1.1^+$ NK cells by flow cytometry.

We found that the frequency of $CD11C^+CD103^+CD11b^+$ DC cells was significantly increased in LPMCs but was dramatically reduced in the spleen and MLN in colitis mice when compared to those in normal controls (Figures 3(a) and 3(b)). As one of our major findings, ED amino acid administration led to a significant decrease in the migration of $CD103^+CD11b^+$ DCs into the colon lamina propria (LP) of colitis mice (Figure 3(b)). Moreover, ED amino acid administration was able to affect the migration of LP $CD103^+CD11b^+$ DCs, which are responsible for Th17 cell differentiation in the intestine, further indicating that ED amino acids might have an effect on the Th17 response during intestinal mucosal immunity. However, no statistical differences in NK cells among different organs of control, colitis, and treated groups were found (Figures 3(c) and

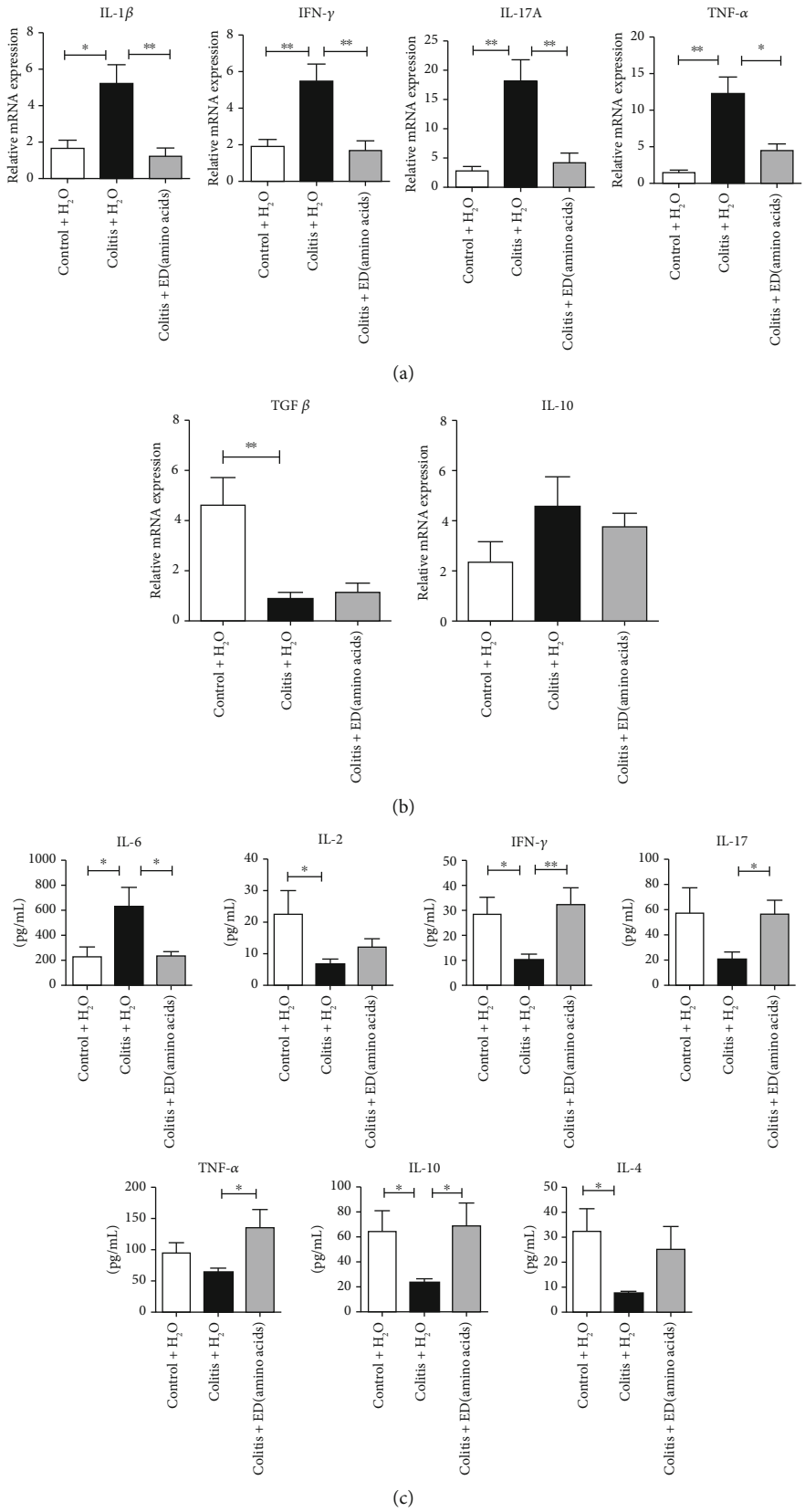


Figure 2: An elemental diet enriched in amino acids suppresses proinflammatory cytokines in the colon. The mRNA levels of proinflammatory (a) and anti-inflammatory cytokines (b) relative to the control GAPDH ($n = 5$ per group) in individual groups. (c) Levels of multiple cytokines produced in the serum samples of individual groups were analyzed by flow cytometry using a CBA mouse Th1/Th2/Th17 cytokine kit. Data are expressed as the mean ± SEM of each group from three separate experiments. $*p < 0.05$, $**p < 0.01$.

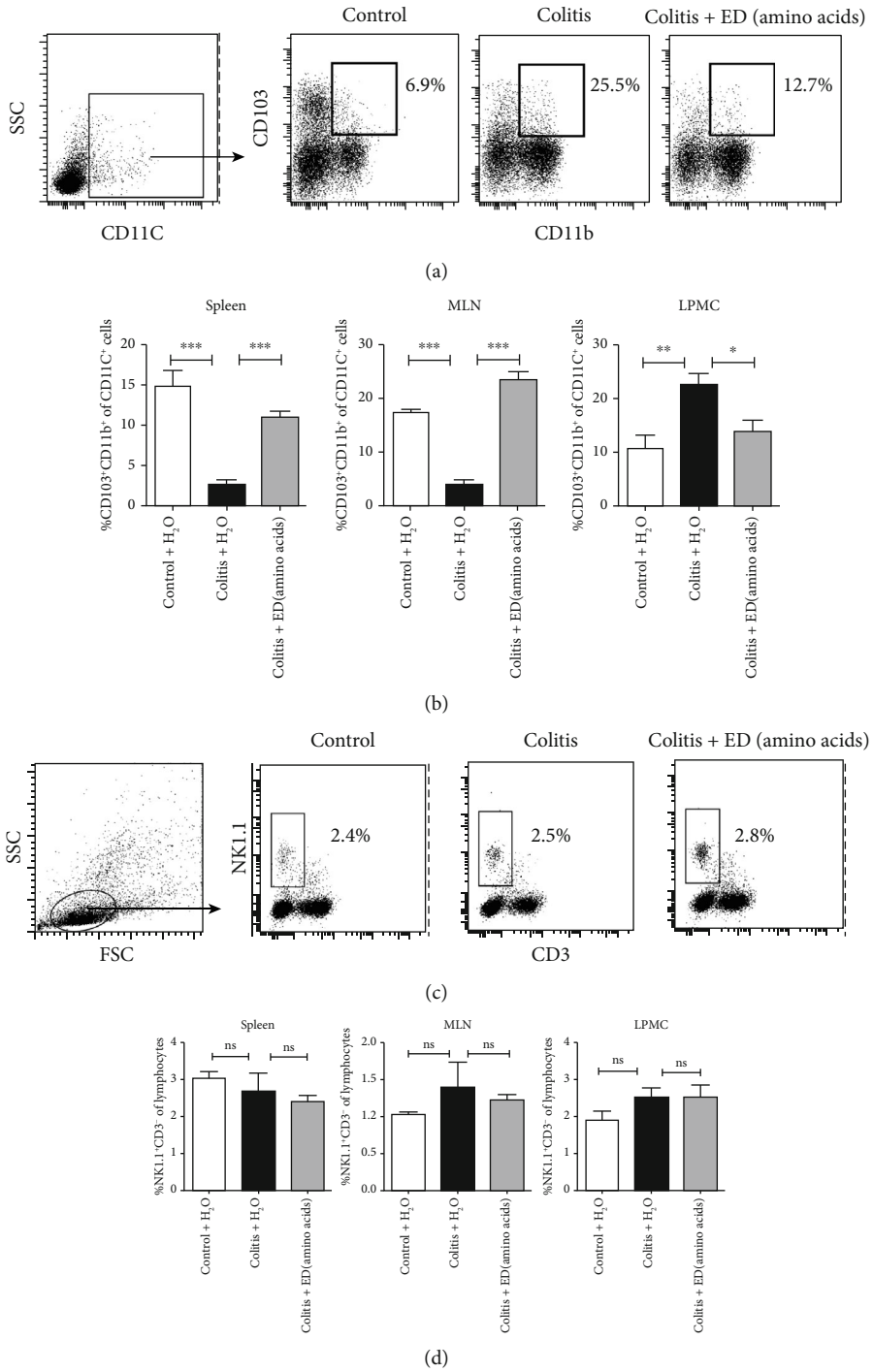
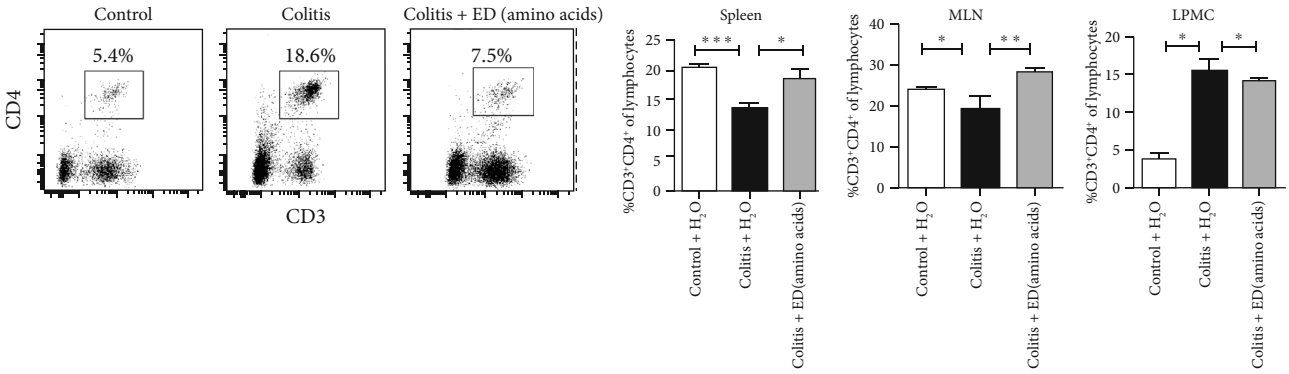


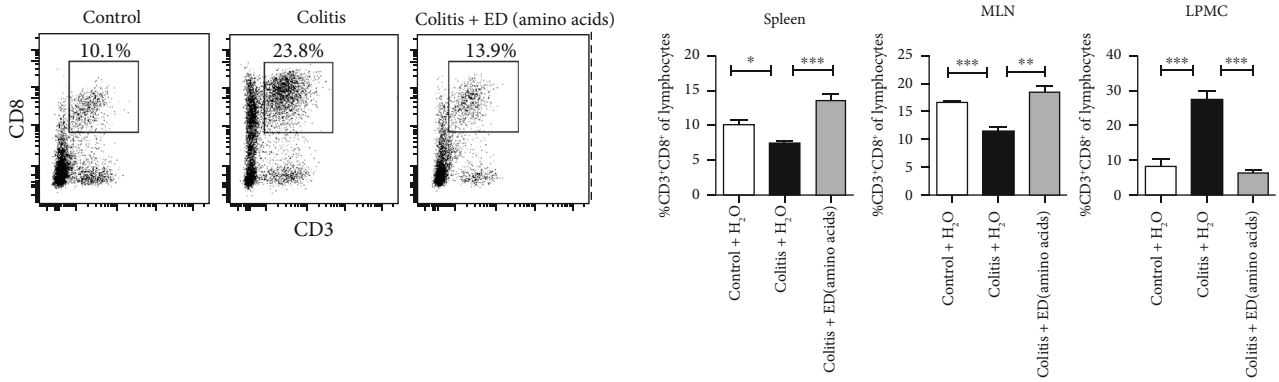
Figure 3: An elemental diet enriched in amino acids affects the accumulation of intestinal lamina propria dendritic cells (DCs) in chronic colitis mice. Mouse splenic mononuclear cells (MNCs), mesenteric lymph nodes (MLNs), and colonic lamina propria mononuclear cells (LPMCs) were isolated from individual groups of mice. Representative flow cytometric analyses of $CD11C^+CD103^+CD11b^+$ DCs (a); MNCs were gated in $CD11C^+$, and the percentages of $CD103^+CD11b^+$ DCs were analyzed in the spleen; MLNs and LPMCs in different groups of mice (b). Representative flow cytometric analyses of $CD3^-$ $NK1.1^+$ NK cells (c) and NK proportions in MNCs (d) in the spleen, MLN, and LPMCs of different groups of mice ($n = 5$ per group). Data are representative images or expressed as the mean ± SEM of each group from three separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ns: not significant.

3(d)). This suggested that NK cells might not be the predominant cell that contributes to the pathological destruction of the colon in DSS-induced chronic colitis.

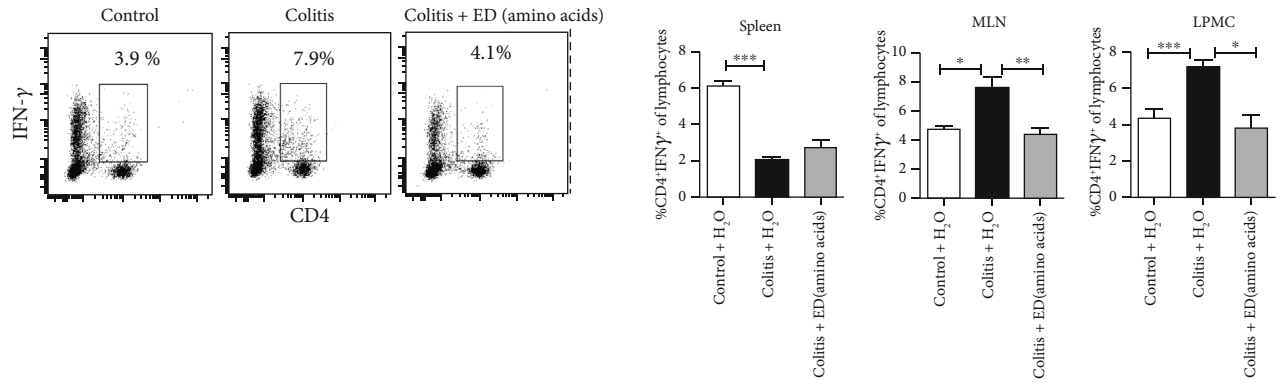
3.4. An Elemental Diet Enriched in Amino Acids Inhibits the Predominant Th1/Th17 Responses in the Colon of Colitis-Bearing Mice. To further assess the effects of ED amino acids



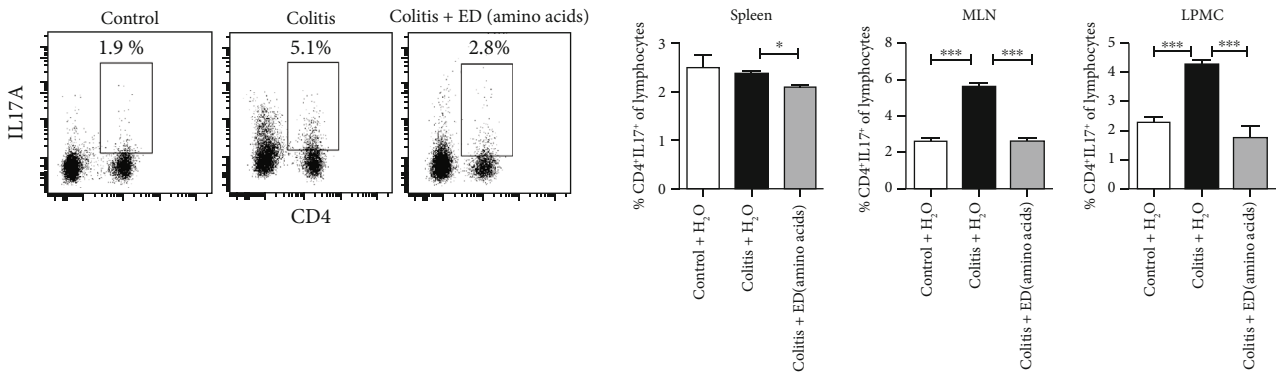
(a)



(b)



(c)



(d)

Figure 4: Continued.

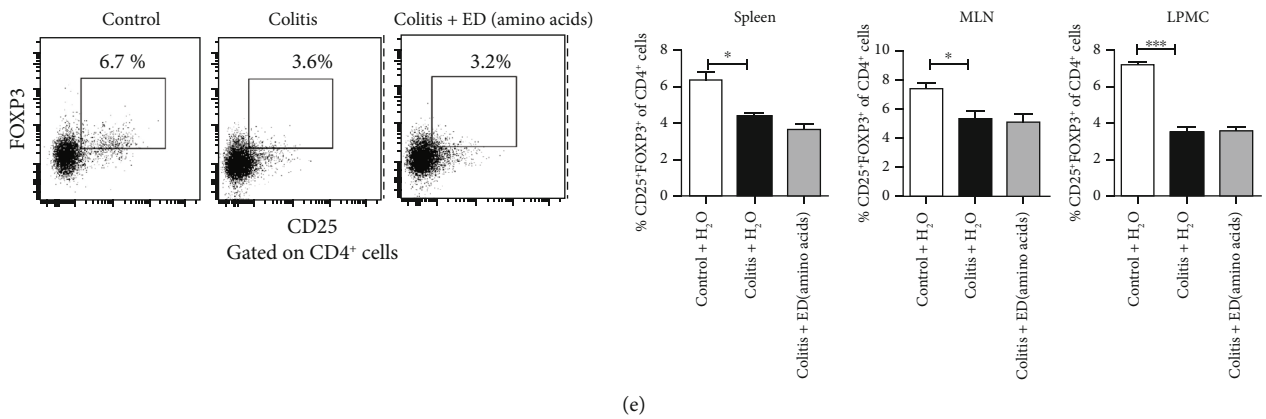


Figure 4: An elemental diet enriched in amino acids inhibits the predominant Th1/Th17 responses in the colon of colitis-bearing mice. Flow cytometric analysis of $CD3^+CD4^+$ T (a), $CD3^+CD8^+$ T (b), $CD4^+$ IFN-γ^+ Th1 (c), and $CD4^+$ $IL17A^+$ Th17 cells (d) in the spleen, mesenteric lymph node (MLNs), and lamina propria mononuclear cells (LPMCs) from individual groups of mice were calculated. $CD4^+CD25^+FoxP3^+$ Treg (e) frequency in $CD4^+$ T cells was examined. Representative flow cytometric dot plots (left) and percentages of positive cells (right) are depicted ($n = 5$ per group). Data are representative images or expressed as the mean ± SEM of each group from three separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

on the Th1/Th17/regulatory T (Treg) cell profile, we characterized the frequency of different subsets of T cells by flow cytometry. In comparison to that in the control mice, a significantly lower frequency of splenic and MLN $CD4^+$ and $CD8^+$ cells and Tregs, as well as splenic Th1 cells, was detected in the colitis group (Figures 4(a), 4(b), 4(c), and 4(e)). Further, a significantly increased frequency of $CD4^+$, $CD8^+$ T, $CD4^+$ IFN-γ^+ Th1, and $CD4^+$ $IL17^+$ Th17 cells, but decreased Tregs, were detected in the colon mucosal LP of the colitis group (Figures 4(a)–4(e)), indicating that $CD4^+$, $CD8^+$, Th1, and Th17 cells infiltrate into the LP of the colon during the process of chronic colitis. As we found, DSS application affected the cellular composition of the colon environment. Interestingly, treatment with ED amino acids significantly eliminated the colitis-mediated increase in the frequency of $CD4^+$, $CD8^+$, Th1, and Th17 cells in the colon mucosal LP of mice (Figures 4(a)–4(d)). However, ED amino acid application was unable to restore the frequency of Tregs in the colitis mice (Figure 4(e)), and these cells play a key role in the maintenance of immune homeostasis to prevent IBD [16, 17]. Our novel findings indicated that ED amino acids have a potential regulatory effect on Th1 and Th17 responses in this model.

3.5. An Elemental Diet Enriched in Amino Acids Prevents Colonic Epithelial Barrier Dysfunction Induced by DSS, Maintaining the Expression of Tight Junction Proteins. We next investigated whether ED amino acids have an effect on intestinal permeability by detecting the expression levels of three major tight junction proteins. Immunofluorescence analysis of claudin-1, occludin, and ZO-1 showed ample staining in the membrane, which was found mainly between epithelial cells of normal control mouse colons (Figure 5). We found that occludin, ZO-1, and claudin-1 expression levels in the colon were significantly reduced in colitis mice, and especially claudin-1. Interestingly, treatment with ED amino acids markedly decreased epithelial architectural derangements and restored the expression of occludin, ZO-1, and claudin-1 protein levels in colitis mice (Figure 5). Together, ED amino acids have a protective effect, repairing the disrupted barrier function.

4. Discussion

Recently, several probable mechanisms underlying the use of EN for IBD therapy were proposed. It was suggested that EN could decrease hypermetabolism in active CD patients [18]. Further, EN was found to ameliorate mesenteric fat alterations in adult CD by restoring adipocyte morphology and diminishing the inflammatory environment of mesenteric fat [19]. Moreover, EN changes the intestinal microbiota in IL-$10^{-/-}$ cell-transferred colitis mice, and especially the lactic acid bacteria composition [20]. Modification of the microbiota by EN might be responsible for the inhibition of colitis. However, whether it manages the disordered mucosal immune response during the process of colitis is not clearly understood.

In the present study, we employed a mouse model of DSS-induced chronic colitis to investigate the therapeutic effect of EN on disease severity, the cellular composition of the colon environment, and epithelial barrier functions. First, we demonstrated that three EN formulas effectively attenuated colitis severity and mucosal lesions in experimental chronic colitis mice, consistent with previous reports [10, 21]. Interestingly, we found that ED amino acids restored the body weight reductions better than the other two EN formulas. Among the three EN formulas, amino acid-based ED has the lowest fat content (0.17 g/100 kcal vs. 3.53 g/100 kcal vs. 1.70 g/100 kcal, Table 1). Fat can cause intestinal peristalsis; therefore, it is believed that low-fat diets are beneficial for IBD patients, based on their ability to keep the intestinal tract still. It was proposed that the fat content and fat composition of EN are responsible for their therapeutic effect in active CD patients [22]. Moreover, Bamba et al. reported that active CD patients receiving the low-fat diet are associated with the highest remission rate. In

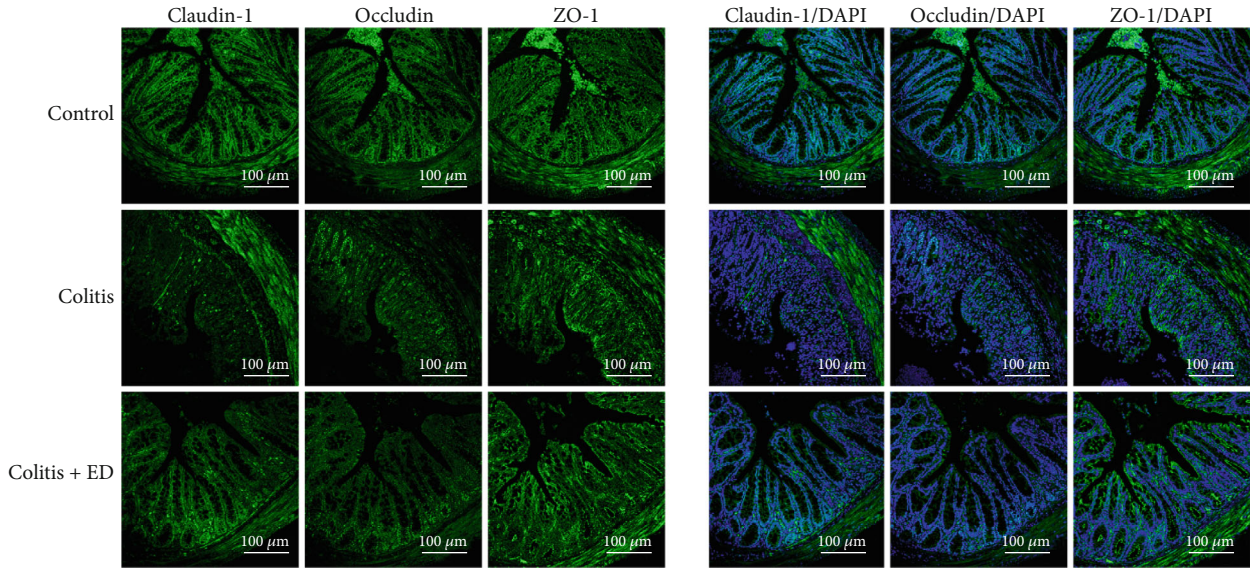


Figure 5: An elemental diet enriched in amino acids prevents colonic epithelial barrier dysfunction induced by dextran sulfate sodium (DSS), maintaining the expression of tight junction proteins. Immunofluorescence staining of claudin-1, occludin, and ZO-1 in the intestinal tissues from different groups of mice with positive expression (green) determined by confocal microscopy. Cell nuclei were stained with DAPI (blue). Scale bar, 100 μm.

contrast, when the fat content consisted of a high amount of long-chain triglycerides, the therapeutic effect against active CD decreased [23]. In the case of exclusive enteral nutrition, only ED amino acid formulas can provide a low-fat diet at lower than 20 g/day. Therefore, it is reasonable to assume that the outstanding anti-inflammatory effect of ED amino acids might be due to the low fat content.

Although a previous study by Souza et al. revealed that the consumption of amino acid diets can exacerbate experimental colitis [24], clinical outcomes suggest the opposite. Exclusive enteral nutrition with ED amino acids has been used extensively for the application of IBD therapy, and this has been proven effective in achieving and maintaining remission [25, 26]. During an inflammatory flare, IBD patients are at a high risk of nutrient depletion, and this is particularly true for children [27]. An improvement in malnutrition in IBD patients was observed after ED amino acid treatment [28]. In addition, the amino acid composition of ED is considered hypoallergenic [12], and some amino acids such as histidine, tryptophan, methionine, glutamine, cysteine, and arginine have beneficial effects [29]. Dietary interventions with specific amino acids in IBD can reduce inflammation, oxidative stress, and apoptosis in the gut, which is responsible for their anti-inflammatory activity, as reviewed by Zhang et al. [30]. Moreover, Faure et al. reported that intestinal tissue repair processes increase the host's need for specific nutrients, and diets supplemented with specific amino acids containing L-threonine, L-serine, L-proline, and L-cysteine strongly stimulate mucin synthesis in DSS-treated rats, suggesting the replenishment of mucins in the damaged mucosa [31]. Thus, increasing specific amino acid supplementation might affect epithelial protection and repair.

Next, the regulatory effects of ED amino acids on immune cell profiles in the local microenvironment were investigated. Although the pathogenic process of IBD is unclear, an imbalance in proinflammatory and regulatory $CD4^+$ T cell responses and damage to the epithelial barrier, which is the first-line defense of the mucosal immune system, are crucial for the development and progression of IBD [16, 32–34]. As a result, we subsequently analyzed the cellular composition of the colon environment (including $CD103^+CD11b^+$ DC, $CD3^-$ $NK1.1^+$ NK, $CD4^+$, and $CD8^+$ T cells) and found the significant infiltration of $CD4^+$ and $CD8^+$ T cells and the migration of DC cells into the colon LP of colitis mice. Interestingly, ED amino acids can reduce DC and $CD4^+$ and $CD8^+$ T cell accumulation, which is accompanied by the resolution of inflammation. More importantly, predominant Th1 and Th17 responses present as colitis-related abundant $CD4^+$ IFN-γ^+ Th1 and $CD4^+$ $IL17^+$ Th17 cells that infiltrate into the colon mucosa LP, and colitis-mediated upregulated *IFN-γ* and *IL-17A* mRNA transcripts in the intestinal tissues were found in colitis mice. Encouraging results also indicated that ED amino acids effectively inhibit the predominant Th1/Th17 responses by downregulating Th1 and Th17 cells and colonic *IFN-γ* and *IL-17A* mRNA transcripts. Such results provided supporting data to explain how EN regulates cytokines and the immune response disorders, which might reflect one aspect of the mucosal immune microenvironment in IBD.

In the current study, ED amino acids were deemed more effective with respect to their anti-inflammatory actions, rather than the restoration of immune homeostasis. ED amino acids reduced LPMC $CD4^+$, $CD8^+$ T cells, DC cells, and Th1 and Th17 cells and reduced the mRNAs transcripts of colonic upregulated proinflammatory cytokines in colitis mice but had no effect on the reduction of Treg frequency and the anti-inflammatory, colonic cytokine mRNA transcripts. ED amino acids reduced the colitis-mediated

upregulation of LPMC $CD103^+CD11b^+$ DCs, which are responsible for Th17 cell differentiation in the intestine [15], further indicating that ED amino acids might have an effect on innate and adaptive immune responses in the colon. However, it remains unclear whether ED amino acids only contributed to the reduction of $CD103^+CD11b^+$ DC cell-derived, mucosal Th17 cell differentiation or whether ED amino acids were simultaneously directly responsible for suppressing Th17 cell quantity. Because of the complex composition of EN, in vitro studies to clarify and differentiate its effect on immune cells are difficult to implement. Overall, we provide evidence to further support the outstanding anti-inflammatory effects of ED amino acids, which are not only due to the low fat content or amino acid composition, for the application to IBD.

Patients with IBD display increased intestinal epithelial permeability and disrupted barrier function [35]. In addition, correcting the epithelial barrier defect in IL-$10^{-/-}$ mice is beneficial to attenuate colitis [36]. Cytokines including IL-6, IL-1β, IFN-γ, and TNF-α promote disruption of the intestinal tight junctions [37], resulting in increased mucosal permeability and a vicious cycle of reactivation of the local inflammatory response [38, 39]. The intestinal epithelial barrier function relies on selective permeability, which depends on tight junctions [40]. Interestingly, we found that colitis results in the upregulation of *IL-1β*, *IFN-γ*, and *TNF-α* mRNA transcripts and the reduced expression of tight junction proteins. In this context, ED amino acids can prevent the reduction in tight junction protein expression; this effect might be partly due to the predominant inflammation-inhibiting action that occurs by reducing the generation of inflammatory mediators.

5. Conclusions

In conclusion, our data indicated that treatment with ED amino acids significantly mitigates DSS-induced colitis and improves mucosal lesions, maintaining the expression of tight junction proteins, suppressing the release of colonic proinflammatory mediators, and resulting in the bidirectional regulation of proinflammatory and anti-inflammatory cytokine release in the peripheral circulation and the suppression of Th1/Th17 responses. Our findings provide new evidence to support the outstanding anti-inflammatory effects of ED amino acids in a mouse model of colitis.

Data Availability

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

Conflicts of Interest

All authors declare no conflicts of interest.

Authors' Contributions

Di Guo and Jun Yang contributed equally to this work.

Acknowledgments

This work is supported by the National Natural Foundation Project of China (No. 81873558 and No. 81700490) and National Key Research and Development Program of China (No. 2018YFC0114604). We thank Troy Gharibani at the University of Maryland, USA, for editing and providing feedback for this manuscript.

Supplementary Materials

Table S1: the composition of three different EN formulas. *(Supplementary Materials)*

References

[1] J. K. Triantafillidis, C. Vagianos, and A. E. Papalois, "The role of enteral nutrition in patients with inflammatory bowel disease: current aspects," *BioMed Research International*, vol. 2015, Article ID 197167, 12 pages, 2015.

[2] C. Sarbagili-Shabat, R. Sigall-Boneh, and A. Levine, "Nutritional therapy in inflammatory bowel disease," *Current Opinion in Gastroenterology*, vol. 31, no. 4, pp. 303–308, 2015.

[3] B. Dupont, C. Dupont, A.-M. Justum, M.-A. Piquet, and J.-M. Reimund, "Enteral nutrition in adult Crohn's disease: present status and perspectives," *Molecular Nutrition & Food Research*, vol. 52, no. 8, pp. 875–884, 2008.

[4] N. Heerasing, B. Thompson, P. Hendy et al., "Exclusive enteral nutrition provides an effective bridge to safer interval elective surgery for adults with Crohn's disease," *Alimentary Pharmacology & Therapeutics*, vol. 45, no. 5, pp. 660–669, 2017.

[5] W. El-Matary, A. Otley, J. Critch, and A. M. Abou-Setta, "Enteral feeding therapy for maintaining remission in Crohn's disease: a systematic review," *Journal of Parenteral and Enteral Nutrition*, vol. 41, no. 4, pp. 550–561, 2017.

[6] F. M. Ruemmele, G. Veres, K. L. Kolho et al., "Consensus guidelines of ECCO/ESPGHAN on the medical management of pediatric Crohn's disease," *Journal of Crohn's & Colitis*, vol. 8, no. 10, pp. 1179–1207, 2014.

[7] D. Royall, G. R. Greenberg, J. P. Allard, J. P. Baker, and K. N. Jeejeebhoy, "Total enteral nutrition support improves body composition of patients with active Crohn's disease JPEN," *Journal of Parenteral and Enteral Nutrition*, vol. 19, pp. 95–99, 2016.

[8] J. M. Fell, "Control of systemic and local inflammation with transforming growth factor β containing formulas JPEN," *Journal of Parenteral and Enteral Nutrition*, vol. 29, pp. S126–S133, 2016.

[9] K. Bannerjee, C. Camacho-Hübner, K. Babinska et al., "Anti-inflammatory and growth-stimulating effects precede nutritional restitution during enteral feeding in Crohn disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 38, no. 3, pp. 270–275, 2004.

[10] A. Andou, T. Hisamatsu, S. Okamoto et al., "Dietary histidine ameliorates murine colitis by inhibition of proinflammatory cytokine production from macrophages," *Gastroenterology*, vol. 136, no. 2, pp. 564–574.e2, 2009.

[11] M. H. Giaffer, G. North, and C. D. Holdsworth, "Controlled trial of polymeric versus elemental diet in treatment of active Crohn's disease," *The Lancet*, vol. 335, no. 8693, pp. 816–819, 1990.

[12] S. Verma, S. Brown, B. Kirkwood, and M. H. Giaffer, "Polymeric versus elemental diet as primary treatment in active Crohn's disease: a randomized, double-blind trial," *The American Journal of Gastroenterology*, vol. 95, no. 3, pp. 735–739, 2000.

[13] H. S. Cooper, S. N. Murthy, R. S. Shah, and D. J. Sedergran, "Clinicopathologic study of dextran sulfate sodium experimental murine colitis," *Laboratory Investigation; a Journal of Technical Methods and Pathology*, vol. 69, no. 7, pp. 238–249, 1993.

[14] A. R. B. M. Muzaki, P. Tetlak, J. Sheng et al., "Intestinal $CD103^+$ $CD11b^-$ dendritic cells restrain colitis via IFN-γ-induced anti-inflammatory response in epithelial cells," *Mucosal Immunology*, vol. 9, pp. 336–351, 2016.

[15] E. K. Persson, H. Uronen-Hansson, M. Semmrich et al., "IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation," *Immunity*, vol. 38, no. 5, pp. 958–969, 2013.

[16] C. G. Mayne and C. B. Williams, "Induced and natural regulatory T cells in the development of inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 19, no. 8, pp. 1772–1788, 2013.

[17] D. Burzyn, C. Benoist, and D. Mathis, "Regulatory T cells in nonlymphoid tissues," *Nature Immunology*, vol. 14, no. 10, pp. 1007–1013, 2013.

[18] J. Zhao, J.-N. Dong, J.-F. Gong et al., "Impact of enteral nutrition on energy metabolism in patients with Crohn's disease," *World journal of Gastroenterology*, vol. 21, no. 4, pp. 1299–1304, 2015.

[19] Y. Feng, Y. Li, S. Mei et al., "Exclusive enteral nutrition ameliorates mesenteric adipose tissue alterations in patients with active Crohn's disease," *Clinical Nutrition*, vol. 33, no. 5, pp. 850–858, 2014.

[20] T. Kajiura, T. Takeda, S. Sakata et al., "Change of intestinal microbiota with elemental diet and its impact on therapeutic effects in a murine model of chronic colitis," *Digestive Diseases and Sciences*, vol. 54, no. 9, pp. 1892–1900, 2009.

[21] E. L. M. Vieira, A. J. Leonel, A. P. Sad et al., "Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis," *The Journal of Nutritional Biochemistry*, vol. 23, no. 5, pp. 430–436, 2012.

[22] M. A. Gassull, F. Fernandez-Banares, E. Cabre et al., "Fat composition may be a clue to explain the primary therapeutic effect of enteral nutrition in Crohn's disease: results of a double blind randomised multicentre European trial," *Gut*, vol. 51, no. 2, pp. 164–168, 2002.

[23] T. Bamba, T. Shimoyama, M. Sasaki et al., "Dietary fat attenuates the benefits of an elemental diet in active Crohn's disease: a randomized, controlled trial," *European Journal of Gastroenterology & Hepatology*, vol. 15, no. 2, pp. 151–157, 2003.

[24] A. L. Souza, S. L. F. Aguiar, M. C. G. Miranda et al., "Consumption of diet containing free amino acids exacerbates colitis in mice," *Frontiers in Immunology*, vol. 8, p. 1587, 2017.

[25] A. Tsertsvadze, T. Gurung, R. Court, A. Clarke, and P. Sutcliffe, "Clinical effectiveness and cost-effectiveness of elemental nutrition for the maintenance of remission in Crohn's disease: a systematic review and meta-analysis," *Health Technology Assessment*, vol. 19, no. 26, pp. 1–138, 2015.

[26] T. Ishige, T. Tomomasa, H. Tajiri, A. Yoden, and Japanese Study Group for Pediatric Crohn's Disease, "Japanese physicians' attitudes towards enteral nutrition treatment for pediatric patients with Crohn's disease: a questionnaire survey," *Intestinal Research*, vol. 15, no. 3, pp. 345–351, 2017.

[27] S. Vidal-Lletjós, M. Beaumont, D. Tomé, R. Benamouzig, F. Blachier, and A. Lan, "Dietary protein and amino acid supplementation in inflammatory bowel disease course: what impact on the colonic mucosa?," *Nutrients*, vol. 9, no. 3, p. 310, 2017.

[28] M. Nakano, K. Tominaga, A. Hoshino, T. Sugaya, K. Kanke, and H. Hiraishi, "Therapeutic efficacy of an elemental diet for patients with Crohn's disease and its association with amino acid metabolism," *Saudi Journal of Gastroenterology*, vol. 23, no. 1, pp. 20–27, 2017.

[29] X. Bao, Z. Feng, J. Yao, T. Li, and Y. Yin, "Roles of dietary amino acids and their metabolites in pathogenesis of inflammatory bowel disease," *Mediators of Inflammation*, vol. 2017, Article ID 6869259, 9 pages, 2017.

[30] H. Zhang, C.-A. A. Hu, J. Kovacs-Nolan, and Y. Mine, "Bioactive dietary peptides and amino acids in inflammatory bowel disease," *Amino Acids*, vol. 47, no. 10, pp. 2127–2141, 2015.

[31] M. Faure, C. Mettraux, D. Moennoz et al., "Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats," *The Journal of Nutrition*, vol. 136, no. 6, pp. 1558–1564, 2006.

[32] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease," *Nature*, vol. 448, no. 7152, pp. 427–434, 2007.

[33] I. Ordás, L. Eckmann, M. Talamini, D. C. Baumgart, and W. J. Sandborn, "Ulcerative colitis," *The Lancet*, vol. 380, no. 9853, pp. 1606–1619, 2012.

[34] D. C. Baumgart and W. J. Sandborn, "Crohn's disease," *The Lancet*, vol. 380, no. 9853, pp. 1590–1605, 2012.

[35] D. Corridoni, K. O. Arseneau, and F. Cominelli, "Inflammatory bowel disease," *Immunology Letters*, vol. 161, no. 2, pp. 231–235, 2014.

[36] M. C. Arrieta, K. Madsen, J. Doyle, and J. Meddings, "Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse," *Gut*, vol. 58, no. 1, pp. 41–48, 2008.

[37] Q. Li, Q. Zhang, C. Wang, X. Liu, N. Li, and J. Li, "Disruption of tight junctions during polymicrobial sepsis *in vivo*," *The Journal of Pathology*, vol. 218, no. 2, pp. 210–221, 2009.

[38] R. Al-Sadi, M. Boivin, and T. Ma, "Mechanism of cytokine modulation of epithelial tight junction barrier," *Frontiers in Bioscience*, vol. 14, pp. 2765–2778, 2009.

[39] T. Suzuki, "Regulation of intestinal epithelial permeability by tight junctions," *Cellular and Molecular Life Sciences*, vol. 70, no. 4, pp. 631–659, 2013.

[40] J. R. Turner, "Intestinal mucosal barrier function in health and disease," *Immunology*, vol. 9, no. 11, pp. 799–809, 2009.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 2640273, 8 pages
https://doi.org/10.1155/2020/2640273

Review Article

Diagnosis and Treatment of Rheumatic Adverse Events Related to Immune Checkpoint Inhibitors

Yan Xiao,[1] Lin Zeng,[1] Qinglin Shen,[1] Zhiyong Zhou,[1] Zhifang Mao,[1] Qin Wang,[1] Xiquan Zhang,[1] Yingliang Li,[2] and Weirong Yao[1]

[1]*Department of Oncology, Jiangxi Provincial People's Hospital Affiliated to Nanchang University, Jiangxi, China*
[2]*Department of General Surgery, The First Affiliated Hospital of Nanchang University, Jiangxi, China*

Correspondence should be addressed to Yingliang Li; liyingliang1977@163.com and Weirong Yao; 13907002901@126.com

Received 24 April 2020; Revised 14 June 2020; Accepted 1 July 2020; Published 4 August 2020

Guest Editor: Xiaoquan Rao

Immune checkpoint inhibitors (ICIs) have completely changed the treatment of cancer, and they also can cause multiple organ immune-related adverse reactions (irAEs). Among them, rheumatic irAE is less common, mainly including inflammatory arthritis, rheumatic myalgia/giant cell arteritis, inflammatory myopathy, and Sjogren's syndrome. For oncologists, rheumatism is a relatively new field, and early diagnosis and treatment is very important, and we need to work closely with experienced rheumatologists. In this review, we focused on the incidence, clinical characteristics, and treatment strategies of rheumatic irAE.

1. Introduction

In recent years, immune checkpoint inhibitors (ICIs) have made significant breakthroughs in cancer treatment. A large number of clinical trials at home and abroad have confirmed that ICIs is a broad-spectrum, long-lasting, safe, and effective antitumor drug [1]. It can inhibit and kill tumor cells by enhancing the antitumor immune function of the body. It has shown a remarkable clinical effect in the treatment of many kinds of malignant tumors. At present, it has been approved by FDA for melanoma, lung cancer, renal cell carcinoma, Hodgkin's lymphoma, head and neck tumor, and urothelial carcinoma [2]. According to its mechanism of action, ICIs can be divided into three categories: programmed cell death protocol 1 (PD-1), programmed cell death protocol ligand 1 (PD-L1) inhibitors, and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) inhibitors [3, 4].

ICIs can enhance the antitumor effect of T cells by blocking the negative regulatory signals of T cells and also affect the immune tolerance of human normal tissues, resulting in immune-related adverse events (irAEs) [5, 6]. irAEs are very common in clinic, and they can occur in almost any organ during or after the treatment of ICIs, generally involving the skin, digestive tract, and endocrine system, but rheumatic irAEs seem to be less common [7–9]. In clinical practice, the common terminology criteria adverse events (CTCAE) are usually used to grade irAEs [10, 11] (for details, see Table 1). The severity is divided into G1: mild or asymptomatic; no intervention is needed; G2: moderate, affecting instrumental activities of daily living (ADL), such as shopping; limited intervention is needed; G3: serious, medical events, limiting self-care ADL, requiring hospitalization; G4: life-threatening events, requiring emergency treatment; and G5: deaths related to adverse reactions. Although most of the irAEs are levels 1 to 2, there is still 0.5%-18.0% in more than level 3 of adverse reactions, even life-threatening [12].

Rheumatic irAEs are different from other organs' irAEs and traditional autoimmune diseases. Rheumatic irAEs can persist for a long time even after termination of treatment [13, 14]. Rheumatic irAEs have a wide range of manifestations, mainly including inflammatory arthritis (IA), polymyalgia rheumatica (PMR)/giant cell arteritis (GCA), inflammatory myopathy (IM), and Sjogren's syndrome (SS). These irAEs are mainly described in patients without autoimmune diseases in the past. However, it has also been reported recently that patients with underlying rheumatic diseases have relapsed and developed new irAEs after receiving ICI treatment.

Table 1: General terminology standard for adverse event (CTCAE) version 5.0.

Level	Clinical description
1	Mild: asymptomatic or mild; only clinical or diagnostic; no treatment required
2	Moderate: requires minor, local, or noninvasive treatment; age equivalent instrumental ADL limitation
3	Serious or medically significant but not immediately life-threatening Causes hospitalization or prolongation of hospitalization; disability; self-care ADL limitation
4	Life threatening; in need of urgent treatment
5	Death-related AE

At present, clinicians have a relatively poor understanding of rheumatic irAEs. Although there are few fatal complications, it will significantly affect the functional activities of cancer patients and limit the use of ICIs [13]. Therefore, this review will focus on the pathogenesis, incidence, clinical characteristics, and treatment strategies of rheumatic irAEs.

2. Pathogenesis of Rheumatic irAEs

CTLA-4 and PD-1/PD-L1 are important immunosuppressive molecules in the immune system, which can inhibit the activation of effector T cells and maintain the balance between the activation and inhibition of T cells. CTLA-4 acts on the early stage of T cell activation, while PD-1/PD-L1 inhibits the activated T cells in the effective stage [15]. ICIs can enhance the antitumor activity by blocking CTLA-4 and PD-1/PD-L1 to activate effector T cells, especially $CD8^+$ T cells, and destroying the signal transduction pathway that inhibits tumor immunity [16]. At present, there are few studies on the pathogenesis of irAEs. The possible mechanisms can be summarized as follows: (1) overactivation of effector T cells, (2) reduction of regulatory T cell function, (3) large-scale release of IFN-γ and TNF, (4) toxic effects of macrophages and neutrophils, and (5) antibody produced by B cells. Therefore, further research is needed to explore the potential mechanism of rheumatic irAEs.

3. Incidence of Rheumatic irAEs

The mechanism of action of different types of ICIs is different, which leads to different incidence of irAEs. In general, the incidence of irAEs of CTLA-4 mAb was higher than that of PD-1/PD-L1 mAb [17]. A meta-analysis showed that the prevalence of irAEs with CTLA-4 monoclonal antibody could be as high as 75% [18], while the prevalence of irAEs with PD-1 and/or PD-L1 monoclonal antibody was about 30% [19]. In addition, CTLA-4 mAb has more serious toxicity than PD-1/PD-L1 mAb. It has been found that 43% of the patients treated with ipilimumab have level 3 or more toxic events, while less than 20% of the patients are treated with PD-1/PD-L1 mAb [20].

At present, the description of rheumatic irAEs mainly comes from case reports, which is easy to be ignored in clinical practice [21]. So far, the most common symptoms of rheumatic irAEs are arthralgia/arthritis and myalgia/myositis, with the prevalence of 1-43% and 2-20%, respectively [22]. In a phase III clinical trial for melanoma, the incidence of joint pain secondary to ipilimumab was about 5%, the incidence of joint pain secondary to pembrolizumab was 9%-20%, the incidence of joint pain secondary to nivolumab was 5%-16% [23], and the incidence of joint pain treated by ipilimumab and nivolumab combination was 10.5% [24]. According to reports, the incidence of myositis caused by ICIs is 0.15-1.28%, and the probability of concomitant myocarditis is as high as 32.0% [25]. However, in the relevant literature of ICI clinical trials, few studies describe PMR and GCA, and one retrospective study reported that the incidence of PMR caused by ICI treatment was 0.2-2.0% [26, 27]. A case of giant cell arteritis was reported in a phase I clinical trial of ipilimumab $\pm$ bevacizumab in the treatment of metastatic melanoma. Dry syndrome caused by ICIs has also been reported recently [28]. In the clinical trial of nivolumab in the treatment of metastatic melanoma, 24% of the patients had dry mouth. In the clinical trial of nivolumab in the treatment of renal cancer, 3.0-11.0% of patients also have dry mouth [29].

In fact, the authenticity of the incidence of rheumatic irAEs is usually limited [13, 14]. On the one hand, the codes of rheumatism/musculoskeletal adverse events used in clinical trials are inconsistent. For example, arthritis can be encoded as joint pain, joint effusion, and musculoskeletal pain. If the AE code is not strictly standardized, the coding of the same symptom may be different. Therefore, the similar encoding can be integrated to better reflect the real incidence rate of rheumatic irAEs. On the other hand, the reason is the classification of CTCAE used in current clinical trials. In many clinical trials, only level 3 adverse events are reported, while rheumatic irAEs are usually mild, so they are easy to be missed. CTCAE classifies many rheumatic adverse events requiring corticosteroid or ICI treatment into level 1 or level 2. The rCTCAE compiled by OMERACT evaluates the applicability of CTCAE used in the field of oncology in the field of rheumatology, reclassifies the coded adverse events, and classifies similar symptoms into higher categories [30, 31]. Therefore, the incidence of rheumatic/musculoskeletal irAEs may be higher if this rCTCAE is used.

4. Clinical Characteristics of Rheumatic irAEs

4.1. Inflammatory Arthritis (IA). IA is a group of diseases characterized by arthritis [31]. The clinical manifestations of inflammatory arthritis caused by ICIs are various, which can be divided into two categories: one is similar to rheumatoid arthritis (RA), which mainly affects small joints (metacarpophalangeal joint, proximal interphalangeal joint, and the wrist and knee), which is different from the traditional RA. This kind of patients is not common in

Table 2: Management of immunology-related informational arthritis.

Level	Description	NCCN/CSCO guideline	ESMO guideline
G1	Mild pain with inflammatory symptoms (improved by exercise or heating), erythema, and joint swelling	Continue ICIs; NSAIDs (such as naproxen, 500 mg, twice a day, 4-6 weeks); if NSAIDs are not effective, consider using prednisone 10-20 mg/D for 2-4 weeks. If symptoms do not improve, upgrade to level 2 management treatment; consider steroid use in the affected joint based on the location of the joint and the number of affected joints.	Continue ICIs; acetaminophen and/or NSAIDs were used.
G2	Moderate pain with inflammatory symptoms, erythema, joint swelling; affect the ability to use tools of daily living (ADL)	Suspend ICIs; prednisone was used for 4-6 weeks, 0.5 mg/kg/d. If the symptoms did not improve, it was upgraded to level 3 management; if symptoms do not improve after 4 weeks, rheumatology consultation is recommended.	When the symptoms were improved and prednisone ≤ 10 mg/D, the use of ICIs could be resumed; larger doses of NSAIDs can be used as needed; consider intraarticular steroid injection; if the symptoms were not well-controlled, prednisone was used for 4-6 weeks, 10-20 mg/d; if the symptoms improve, gradually reduce within 4 to 6 weeks; if the symptoms do not improve, upgrade to level 3 management treatment; if the corticosteroid dose cannot be reduced to <10 mg/d after 3 months, consider DMARD.
G3	Severe pain with severe inflammatory pain, skin erythema or joint swelling; irreversible joint injury; disease; self-care ADL limitation	Suspend or permanently stop ICIs; prednisone for 4-6 weeks, 1 mg/kg/D; if the symptoms do not improve within 2 weeks, rheumatology consultation is recommended; according to the clinical phenotype of inflammatory arthritis, DMARD is considered to be used additionally. The available drugs include methotrexate, sulfasalazine, azathioprine, leflunomide, infliximab, and tuozhumab.	Suspend or permanently stop ICIs; if the symptom recovers to G1, continue the use of ICIs after consultation with the rheumatologist; prednisone was used for 4 weeks, 0.5-1 mg/kg/d; if symptoms do not improve or worsen after 4 weeks, consider using synthetic DMARD (methotrexate, leflunomide, and sulfasalazine) or biological DMARDs (TNF-α or IL-6 inhibitors).

women. In the early stage of the disease, tendon involvement is more prominent, and rheumatoid factor (RF) and anti-citrulline peptide antibody (ACPA) are usually negative [32]. Another is similar to spinal arthritis (SPA), which is characterized by oligoarthritis, mainly involving in large joints, such as inflammatory back pain, adhesion point inflammation, and phalangitis [14, 31, 33, 34]. A few patients also have reactive arthritis (conjunctivitis with oligoarthritis, asymptomatic urethritis) and psoriatic arthritis [26, 35, 36]. However, HLA-B27 was not positive in these patients [20].

A number of research results show that the time of joint pain after the application of ICIs is from 2 to 24 months, and the median time of joint pain disappearance is 9.2 ± 6.1 months, but musculoskeletal symptoms may last for more than one year [37–41].

4.2. PMR (Polymyalgia Rheumatica)/GCA (Giant Cell Arteritis). PMR is an inflammatory disease, mostly in the elderly, which mainly occurs in the age of more than 50 years. Its typical clinical features are persistent pain and morning stiffness in the neck, scapula, and pelvic belt, sometimes with systemic symptoms, such as mild peripheral arthritis and dorsal edema [23, 29, 41–43]. The auxiliary examination showed that the inflammatory index was significantly increased, RF and ACPA were generally negative, and low dose hormone (prednisone < 15 mg/D) was effective. The imaging manifestations of bursitis of deltoid muscle and tenosynovitis of the biceps under the shoulder may be the characteristics of RA or PMR [44, 45]. GCA is a kind of vasculitis, which mainly invades the major arteries, such as one or more branches of the carotid artery (especially the temporal artery), accompanied by granuloma formation, which is relatively rare in China [20]. PMR and GCA represent different clinical manifestations in the same disease process. Patients with GCA often have PMR, so the two diseases are often discussed together [46].

After the use of ICIs, the clinical and imaging manifestations of PMR are almost the same as those of traditional PMR. In most cases, RF and ACPA are negative [23, 39, 47, 48]. It can be seen that there is a significant increase in the inflammatory index, but some studies have found that there is no increase in CRP in this part of patients with typical clinical characteristics of PMR [26]. And some patients did not respond to low-dose prednisone [13]. Only a few patients will cause GCA after using ICIs, mainly manifested as headache,

Table 3: Management of immunotherapy-related polymyalgia rheumatica.

Level	Description	NCCN guideline
G1	Mild pain and/or stiffness, no limitation of ADL	Continue immunotherapy; prednisone, the initial dose of 5-20 mg/D ×6 weeks, then decreased in 4-6 weeks.
G2	Moderate pain and/or stiffness, affecting instrumental ADL	Stop immunotherapy; prednisone 10-20 mg/D, decreased in 8-12 weeks; if there is no improvement, please consult with the rheumatology department.
G3	Severe pain and/or stiffness, affecting self-care ADL	

Table 4: Management of immunotherapy-related myositis.

Level	Description	NCCN guideline	CSCO guideline
G1	Mild symptoms with or without pain	Consider stopping ICIs; consider PMR/GCA (see Table 3 for treatment principle); continuous monitoring of aldolase and creatine kinase; if indicated, treat pain (e.g., NSAIDs).	Continue ICIs; overall evaluation of patients' muscle strength; creatine kinase, aldolase, transaminase (AST, ALT), and lactate dehydrogenase (LDH) were monitored; if the level of creatine kinase increases and the muscle strength decreases, glucocorticoid can be given; after eliminating the related contraindications, acetaminophen or NSAIDs can be given for pain relief.
G2	Moderate symptoms with or without pain, affecting instrumental ADL	If the level rises, stop ICIs; muscle MRI and EMG were performed; prednisone 1-2 mg/kg/D; consider muscle biopsy, especially in severe or refractory cases; aldolase and creatine kinase were monitored continuously until symptoms disappeared or steroids were stopped.	ICI was suspended until the related symptoms were controlled, creatine kinase returned to the normal level, and prednisone dosage was less than 10 mg; NSAIDs can be given to relieve pain after removing related taboos; if creatine kinase ≥ 3 times of the upper limit of the normal value, prednisone (or equivalent dose of other drugs) was given for treatment.
G3	Severe symptoms with or without pain, affecting self-care ADL	If there are indications, treat the pain; please consult with the rheumatology department or neurology department; intravenous immunoglobulin (IVIG), 2G/kg, should be used for administration according to the instructions; if steroid is difficult to treat, plasma exchange may be considered and infliximab or mycophenolate mofetil may be given.	Suspend ICIs until G1; consider admission; please consult with the rheumatology department or neurology department; use 1 mg/kg/D methylprednisolone (or equivalent dose of other drugs); IVIG and plasma exchange were considered.

tenderness of temporal artery, intermittent lameness of mandible, and transient diplopia. The pathological biopsy findings are consistent with the traditional GCA [47]. It has been reported that the attack time of PMR/GCA secondary to ICIs is 10 days to 1 year [23, 26, 47].

4.3. Inflammatory Myopathy (IM). IM is a group of heterogeneous diseases; the main pathological features are inflammatory cell infiltration and myofibrillar necrosis of skeletal muscle [49]. Different types of IM can involve different target organs such as the skin and muscle, so the clinical manifestations are complex. Among them, idiopathic inflammatory myopathies (IIMS), including polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM), have unknown etiology and are related to autoimmunity. The IM caused by ICIs is relatively rare, and its clinical manifestations are consistent with PM. It is often manifested as myalgia, weakness of proximal muscle, ptosis of the upper eyelid, and elevation of muscle enzyme. Compared with IIMS, the symptoms of such patients are usually sudden attack, usually within two months of ICI treatment, and the symptoms generally last for 5 days to 115 weeks [25, 50].

Different from the traditional IM, the classic dermatomyositis rash caused by ICIs is rare in IM patients. The response of intravenous immunoglobulin may be poor. The specific antibody of serum myositis is usually negative, and the axial part or facial muscles may be involved [51–54]. And the frequency of overlap with myocarditis and myasthenia gravis in these patients was higher than that of traditional IM [55]. Therefore, the diagnosis should be differentiated from tumor-related myocarditis and myasthenia gravis. A small number of patients will also have myositis syndrome with fasciitis [56]. Up to now, there is no case of IM caused by ICIs with the unique histological characteristics of inclusion body myositis. Although the overall incidence of myositis is not high, the mortality rate can reach 17.0%, second only to myocarditis [55]. At the same time, the mortality rate of myocarditis or other neuromuscular diseases is higher, which is usually caused by heart failure or respiratory failure [50, 57]. It is worth noting that there may be cases with normal muscle enzymes or muscle involvement,

but no myasthenia or myalgia [58–63]. In view of the high mortality rate of myositis, we should pay attention to the dynamic monitoring of muscle enzyme changes in patients with clinical high suspicion of IM and carry out muscle biopsy if necessary.

4.4. Sjogren's Syndrome (SS). SS is a chronic inflammatory autoimmune disease involving mainly exocrine glands such as salivary glands and lacrimal glands [28]. The main clinical manifestations are dry mouth, dry eyes, rampant caries, and mumps. The serum autoantibody is anti-SSA or anti-SSB (+), and the dry mouth is responsive to sialidase treatment. Warner [64] et al. discussed the clinicopathological characteristics of SS related to ICI treatment, evaluated 20 patients with xerostomia, and found that the median interval between ICI treatment and xerostomia attack was 70 days, mainly manifested as more serious xerostomia symptoms at night or after exercise, hoarseness of voice, change of taste, sensitivity to spicy or acid food, and abnormal parotid swelling or tenderness. Only 2 of them were positive for RA and anti-SSA. Labial salivary gland biopsy (LSGB) showed mild to severe sialitis, which was different from traditional SS, with diffuse lymphocytic infiltration and acinar injury. There were T lymphocyte aggregation in 8 patients, mainly $CD3^+$ T cells; PD-1/PD-L1 was positive. In addition, it has been reported that 4 cases of severe salivary hypofunction after treatment with nivolumab, ipilimumab, or combination therapy have been described. Anti-Ro antibody is negative, only 1 case has parotitis, parotid ultrasound shows hypoechoic focus, and anti-LA/SSB antibody indicates positive [28].

5. Treatment of Rheumatic irAEs

For the whole process management of rheumatic irAEs, the European Society for Medical Oncology (EMSO), the National Comprehensive Cancer Network (NCCN), and the Chinese Society of Clinical Oncology (CSCO) have developed immune-related toxicity management guidelines according to the consensus of experts. These guidelines were issued by consensus of experts from various disciplines, and no prospective test was conducted [65, 66]. Recent studies have shown that patients with rheumatic irAEs have better prognosis in tumor treatment than other irAEs [26]. Therefore, it is very important for oncologists to properly manage these rheumatic irAEs.

5.1. Treatment Strategy of Inflammatory Arthritis. IA is the most common clinical manifestation in rheumatic irAEs. In consideration of this diagnosis, it is necessary to evaluate the joint function and improve the laboratory tests such as ESR/CRP, ANA, RF, and ACPA, and it is feasible to determine the number of joint involvements by joint X-ray, MRI, or musculoskeletal ultrasound. ESMO, NCCN, and CSCO guidelines all provide specific recommendations for the treatment of IA, and the overall treatment principles are basically the same (Table 2). For patients above the G2 level, patients whose symptoms last more than 6 weeks or need more than 20 mg of prednisone per day and cannot decrease to less than 10 mg/D within 4 weeks should be considered to be transferred to rheumatology department [31].

5.2. Treatment Strategy of RA and GCA. The incidence rate of PMR and GCA is low. For patients with suspected PMR, ESR and CRP are necessary. Ultrasound examination of the shoulder and buttocks is also necessary. If visual symptoms or headache occurs, then temporal artery ultrasound and biopsy are considered. At present, only suggestions for PMR/GCA treatment are put forward in NCCN guidelines. The treatment principle of PMR is shown in Table 3. The difference between the treatment strategy of GCA and PMR lies in stopping immunotherapy immediately after diagnosis, giving 1 mg/kg/D prednisone and decreasing in quantity within 8-12 weeks. If accompanied by visual symptoms, consider steroid pulse therapy (methylprednisolone 500-1000 mg) and consult with the eye. For GCA, the rheumatology department should be consulted even for mild cases to prevent permanent organ damage [31].

5.3. Treatment Strategy of Myositis or Myalgia. IM is also one of the common clinical manifestations of rheumatic irAEs. Once it occurs, the mortality is high. During the diagnosis, we should perfect the examination of creatine kinase/aldolase, troponin, myositis antibody, muscle MRI, and electromyogram; take muscle biopsy if necessary; and focus on the possibility of myasthenia gravis or myocarditis. The ESMO guidelines do not mention the treatment of ICI-related myositis. See Table 4 for the treatment principles of NCCN and CSCO compass for the disease. The treatment principles of the two are slightly different. For G1 patients, NCCN guidelines consider the need to stop immunotherapy, while CSCO guidelines consider whether to continue to use ICIs through a comprehensive assessment of patients' muscle strength. In the actual clinical work, for all suspected cases of myositis, myasthenia, and creatine kinase elevation, we should keep a high vigilance and timely refer to the rheumatology department or neurology department to avoid life-threatening situations [31].

5.4. Treatment Strategy of Sjogren's Syndrome and Other Connective Tissue Diseases. There are no specific and detailed treatment suggestions for SS and other connective tissue diseases. The treatment of these diseases mainly refers to the treatment principle of traditional rheumatism and adjusts the dosage of glucocorticoid and immune checkpoint inhibitors according to the severity of organ involvement. Most of the patients with SS described in the case reports were treated with systemic glucocorticoids, with an average dose of prednisone of 40 mg/D, and a few patients with severe disease were treated with IVIG and other immunosuppressive therapy [41, 67, 68]. Pilocarpine, as a muscarinic receptor agonist, can also effectively improve the symptoms of xerostomia. Similarly, other connective tissue diseases can be treated with systemic hormone. For example, in one patient with lupus nephritis after treatment with ipilimumab, renal function was significantly improved after treatment with 1 mg/kg prednisone [69].

6. Summary

With the wide application of ICIs, the emergence of irAEs in clinical work has brought many challenges to oncologists but also opened up new research areas. Among them, rheumatic irAEs are relatively rare, mainly including inflammatory arthritis, rheumatic myalgia/giant cell arteritis, inflammatory myopathy, and Sjogren's syndrome. These diseases should be distinguished from traditional rheumatic diseases. At present, irAEs are generally classified by CTCAE and managed according to the standard international guidelines. The therapeutic drugs mainly include NSAIDs, glucocorticoids, and DMARDs. For oncologists, rheumatism is a relatively new field, we must improve the understanding of rheumatic irAEs, understand its pathogenesis, clinical characteristics, and treatment methods.

Although the overall incidence of rheumatic irAEs is not high, we still need to be vigilant. Oncologists need to actively cooperate with experienced rheumatologists to improve the diagnosis rate of rheumatic irAEs, timely referral so as not to delay the treatment opportunity, and at the same time, weigh the advantages and disadvantages to decide whether to continue the treatment of ICIs.

Conflicts of Interest

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

Authors' Contributions

Yan Xiao, Lin Zeng, and Qinglin Shen contributed equally to this work.

References

[1] D. S. Chen and I. Mellman, "Elements of cancer immunity and the cancer-immune set point," *Nature*, vol. 541, no. 7637, pp. 321–330, 2017.

[2] W. Pönisch, R. Alten, and C. Baerwald, "Autoimmune reactions to immune checkpoint inhibitors," *Zeitschrift Für Rheumatologie*, vol. 76, Supplement 2, pp. 57–62, 2017.

[3] A. Hoos, "Development of immuno-oncology drugs – from CTLA4 to PD1 to the next generations," *Nature Reviews Drug Discovery*, vol. 15, no. 4, pp. 235–247, 2016.

[4] M. A. Postow, M. K. Callahan, and J. D. Wolchok, "Immune checkpoint blockade in cancer therapy," *Journal of Clinical Oncology*, vol. 33, no. 17, pp. 1974–1982, 2015.

[5] M. Z. Afzal, R. Mabaera, and K. Shirai, "Metastatic uveal melanoma showing durable response to anti-CTLA-4 and anti-PD-1 combination therapy after experiencing progression on anti-PD-1 therapy alone," *Journal for Immunotherapy of Cancer*, vol. 6, no. 1, p. 13, 2018.

[6] A. Inno, G. Metro, P. Bironzo et al., "Pathogenesis, clinical manifestations, and management of immune checkpoint inhibitors toxicity," *Tumori Journal*, vol. 103, no. 5, pp. 405–421, 2017.

[7] L. Calabrese and X. Mariette, "The evolving role of the rheumatologist in the management of immune-related adverse events (irAEs) caused by cancer immunotherapy," *Annals of the Rheumatic Diseases*, vol. 77, no. 2, pp. 162–164, 2018.

[8] J. S. Weber, F. S. Hodi, J. D. Wolchok et al., "Safety profile of nivolumab monotherapy: a pooled analysis of patients with advanced melanoma," *Journal of Clinical Oncology*, vol. 35, no. 7, pp. 785–792, 2017.

[9] V. M. Thomas, P. Bindal, S. A. Alexander, and K. McDonald, "Nivolumab-induced hepatitis: a rare side effect of an immune check point inhibitor," *Journal of Oncology Pharmacy Practice*, vol. 26, no. 2, pp. 459–461, 2020.

[10] A. Trotti, A. D. Colevas, A. Setser, and E. Basch, "Patient-Reported outcomes and the evolution of adverse event reporting in oncology," *Journal of Clinical Oncology*, vol. 25, no. 32, pp. 5121–5127, 2007.

[11] M. W. Schoen, E. Basch, L. L. Hudson et al., "Software for administering the National Cancer Institute's patient-reported outcomes version of the common terminology criteria for adverse events: usability study," *JMIR Human Factors*, vol. 5, no. 3, article e10070, 2018.

[12] S. Champiat, O. Lambotte, E. Barreau et al., "Management of immune checkpoint blockade dysimmune toxicities: a collaborative position paper," *Annals of Oncology*, vol. 27, no. 4, pp. 559–574, 2016.

[13] L. H. Calabrese, C. Calabrese, and L. C. Cappelli, "Rheumatic immune-related adverse events from cancer immunotherapy," *Nature Reviews Rheumatology*, vol. 14, no. 10, pp. 569–579, 2018.

[14] L. C. Cappelli, A. A. Shah, and C. O. Bingham III, "Immune-related adverse effects of cancer immunotherapy— implications for rheumatology," *Rheumatic Diseases Clinical North America*, vol. 43, no. 1, pp. 65–78, 2017.

[15] S. Jamal, M. Hudson, A. Fifi-Mah, and C. Ye, "Immune-related adverse events associated with cancer immunotherapy: a review for the practicing rheumatologist," *The Journal of Rheumatology*, vol. 47, no. 2, pp. 166–175, 2020.

[16] L. Dyck and K. H. G. Mills, "Immune checkpoints and their inhibition in cancer and infectious diseases," *Europe Journal of Immunology*, vol. 47, no. 5, pp. 765–779, 2017.

[17] D. Day and A. R. Hansen, "Immune-related adverse events associated with immune checkpoint inhibitors," *Biodrugs Clinical Immunotherapeutics Biopharmaceuticals & Gene Therapy*, vol. 30, no. 6, pp. 571–584, 2016.

[18] S. Bowyer, P. Prithviraj, P. Lorigan et al., "Efficacy and toxicity of treatment with the anti-CTLA-4 antibody ipilimumab in patients with metastatic melanoma after prior anti-PD-1 therapy," *British Journal of Cancer*, vol. 114, no. 10, pp. 1084–1089, 2016.

[19] S. Baxi, A. Yang, R. L. Gennarelli et al., "Immune-related adverse events for anti-PD-1 and anti-PD-L1 drugs: systematic review and meta-analysis," *BMJ Clinical Research*, vol. 360, no. 14, p. 793, 2018.

[20] K.-A. Lee, H. R. Kim, and S. Y. Yoon, "Rheumatic complications in cancer patients treated with immune checkpoint inhibitors," *The Korean Journal of Internal Medicine*, vol. 34, no. 6, pp. 1197–1209, 2019.

[21] L. C. Cappelli, A. A. Shah, and C. O. Bingham III, "Cancer immunotherapy-induced rheumatic diseases emerge as new clinical entities," *RMD Open*, vol. 2, no. 2, article e000321, 2016.

[22] L. C. Cappelli, A. K. Gutierrez, C. O. Bingham III, and A. A. Shah, "Rheumatic and musculoskeletal immune-related adverse events due to immune checkpoint inhibitors: a systematic review of the literature," *Arthritis Care and Research*, vol. 69, no. 11, pp. 1751–1763, 2017.

[23] R. Belkhir, S. L. Burel, L. Dunogeant et al., "Rheumatoid arthritis and polymyalgia rheumatica occurring after immune checkpoint inhibitor treatment," *Annals of the Rheumatic Diseases*, vol. 76, no. 10, pp. 1747–1750, 2017.

[24] J. Larkin, V. Chiarion-Sileni, R. Gonzalez et al., "Combined nivolumab and ipilimumab or monotherapy in untreated melanoma," *New England Journal Medicine*, vol. 373, no. 1, pp. 23–34, 2015.

[25] A. Moreira, C. Loquai, C. Pföhler et al., "Myositis and neuromuscular side-effects induced by immune checkpoint inhibitors," *Euroupe Journal of Cancer*, vol. 106, pp. 12–23, 2019.

[26] M. Kostine, L. Rouxel, T. Barnetche et al., "Rheumatic disorders associated with immune checkpoint inhibitors in patients with cancer-clinical aspects and relationship with tumour response: a single-centre prospective cohort study," *Annals of the Rheumatic Diseases*, vol. 77, no. 3, pp. 393–398, 2018.

[27] B. Le Burel, S. Champiat, C. Mateus et al., "Prevalence of immune-related systemic adverse events in patients treated with anti-programmed cell death 1/anti-programmed cell death-ligand 1 agents: a single-centre pharmacovigilance database analysis," *Europe Jounal of Cancer*, vol. 82, pp. 34–44, 2017.

[28] V. Capozzi, S. Makhoul, E. Aprea et al., "PTR-MS characterization of VOCs associated with commercial aromatic bakery yeasts of wine and beer origin," *Molecules*, vol. 21, no. 4, p. 483, 2016.

[29] R. J. Motzer, B. I. Rini, D. F. McDermott et al., "Nivolumab for metastatic renal cell carcinoma: results of a randomized phase II trial," *Journal of Clinical Oncology*, vol. 33, no. 13, pp. 1430–1437, 2015.

[30] T. Woodworth, D. E. Furst, R. Alten et al., "Standardizing assessment and reporting of adverse effects in rheumatology clinical trials II: the rheumatology common toxicity criteria v. 2.0," *Journal of Rheumatology*, vol. 34, no. 6, pp. 1401–1414, 2007.

[31] I. Puzanov, A. Diab, K. Abdallah et al., "Managing toxicities associated with immune checkpoint inhibitors: consensus recommendations from the Society for Immunotherapy of Cancer (SITC) Toxicity Management Working Group," *Journal for Immunotherapy of Cancer*, vol. 5, no. 1, p. 95, 2017.

[32] A. Haikal, E. Borba, T. Khaja, G. Doolittle, and P. Schmidt, "Nivolumab-induced new-onset seronegative rheumatoid arthritis in a patient with advanced metastatic melanoma: a case report and literature review," *Avicenna Journal of Medicine*, vol. 8, no. 1, pp. 34–36, 2018, 8.

[33] M. Tocut, R. Brenner, and G. Zandman-Goddard, "Autoimmune phenomena and disease in cancer patients treated with immune checkpoint inhibitors," *Autoimmunity Reviews*, vol. 17, no. 6, pp. 610–616, 2018.

[34] K. Ohnuma, R. Hatano, N. H. Dang, and C. Morimoto, "Rheumatic diseases associated with immune checkpoint inhibitors in cancer immunotherapy," *Modern Rheumatology*, vol. 29, no. 5, pp. 721–732, 2018.

[35] S. Law-Ping-Man, A. Martin, E. Briens, L. Tisseau, and G. Safa, "Psoriasis and psoriatic arthritis induced by nivolumab in a patient with advanced lung cancer," *Rheumatology*, vol. 55, no. 11, pp. 2087–2089, 2016.

[36] B. Ruiz-Bañobre, E. Pérez-Pampín, J. García-González et al., "Development of psoriatic arthritis during nivolumab therapy for metastatic non-small cell lung cancer, clinical outcome analysis and review of the literature," *Lung Cancer*, vol. 108, pp. 217–221, 2017.

[37] M. H. Smith and A. R. Bass, "Arthritis after cancer immunotherapy: symptom duration and treatment response," *Arthritis Care Research*, vol. 71, no. 3, pp. 362–366, 2019.

[38] M. J. Mooradian, M. Nasrallah, J. F. Gainor et al., "Musculoskeletal rheumatic complications of immune checkpoint inhibitor therapy: a single center experience," *Seminars in Arthritis and Rheumatism*, vol. 48, no. 6, pp. 1127–1132, 2019.

[39] M. Lidar, E. Giat, D. Garelick et al., "Rheumatic manifestations among cancer patients treated with immune checkpoint inhibitors," *Autoimmunity Reviews*, vol. 17, no. 3, pp. 284–289, 2018.

[40] L. C. Cappelli, A. K. Gutierrez, A. N. Baer et al., "Inflammatory arthritis and sicca syndrome induced by nivolumab and ipilimumab," *Annals of the Rheumatic Diseases*, vol. 76, no. 1, pp. 43–50, 2016.

[41] C. Calabrese, E. Kirchner, K. Kontzias, V. Velcheti, and L. H. Calabrese, "Rheumatic immune-related adverse events of checkpoint therapy for cancer: case series of a new nosological entity," *RMD Open*, vol. 3, no. 1, article e000412, 2017.

[42] M. L. Gauci, B. Baroudjian, P. Laly et al., "Remitting seronegative symmetrical synovitis with pitting edema (RS3PE) syndrome induced by nivolumab," *Seminars in Arthritis and Rheumatism*, vol. 47, no. 2, pp. 281–287, 2017.

[43] K. Nakamagoe, T. Moriyama, H. Maruyama et al., "Polymyalgia rheumatica in a melanoma patient due to nivolumab treatment," *Journal Cancer Research Clinical Oncology*, vol. 143, no. 7, pp. 1357-1358, 2017.

[44] C. Salvarani, F. Cantini, L. Boiardi, and G. G. Hunder, "Polymyalgia rheumatica and giant-cell arteritis," *The New England Journal of Medicine*, vol. 347, no. 4, pp. 261–271, 2002.

[45] B. Dasgupta, M. A. Cimmino, H. Maradit-Kremers et al., "2012 provisional classification criteria for polymyalgia rheumatica: a European League Against Rheumatism/American College of Rheumatology collaborative initiative," *Annals of the Rheumatic Diseases*, vol. 71, no. 4, pp. 484–492, 2012.

[46] M. Gonzalez-Gay, E. Matteson, and S. Castaneda, "Polymyalgia rheumatica," *The Lancet*, vol. 390, no. 10103, pp. 1700–1712, 2017.

[47] B. L. Goldstein, L. Gedmintas, and D. J. Todd, "Drug-associated polymyalgia rheumatica/giant cell arteritis occurring in two patients after treatment with ipilimumab, an antagonist of ctla-4," *Arthritis Rheumatology*, vol. 66, no. 3, pp. 768-769, 2014.

[48] M. Bernier, C. Guillaume, N. Leon et al., "Nivolumab causing a polymyalgia rheumatica in a patient with a squamous non-small cell lung cancer," *Journal of Immunotherapy*, vol. 40, no. 4, pp. 129–131, 2017.

[49] A. Selva-O'Callaghan, I. Pinal-Fernandez, E. Trallero-Araguás, J. C. Milisenda, J. M. Grau-Junyent, and A. L. Mammen, "Classification and management of adult inflammatory myopathies," *The Lancet Neurology*, vol. 17, no. 9, pp. 816–828, 2018.

[50] M. Touat, T. Maisonobe, S. Knauss et al., "Immune checkpoint inhibitor-related myositis and myocarditis in patients with cancer," *Neurology*, vol. 91, no. 10, pp. 985–994, 2018.

[51] M. Shah, J. H. Tayar, N. Abdel-Wahab, and M. E. Suarez-Almazor, "Myositis as an adverse event of immune checkpoint blockade for cancer therapy," *Seminars in Arthritis and Rheumatism*, vol. 48, no. 4, pp. 736–740, 2019.

[52] H. Kadota, T. Gono, Y. Shirai, Y. Okazaki, M. Takeno, and M. Kuwana, "Immune checkpoint inhibitor-induced myositis: a case report and literature review," *Current Rheumatology Reports*, vol. 21, no. 4, pp. 1747–1750, 2019.

[53] T. Liewluck, J. C. Kao, and M. L. Mauermann, "PD-1 inhibitor-associated myopathies," *Journal of Immunotherapy*, vol. 41, no. 4, pp. 208–211, 2018.

[54] E. L. Mitchell, P. K. H. Lau, C. Khoo et al., "Rheumatic immune-related adverse events secondary to anti-programmed death-1 antibodies and preliminary analysis on the impact of corticosteroids on anti- tumour response: A case series," *Europe Journal of Cancer*, vol. 105, no. 10, pp. 88–102, 2018.

[55] S. Suzuki, N. Ishikawa, F. Konoeda et al., "Nivolumab-related myasthenia gravis with myositis and myocarditis in Japan," *Neurology*, vol. 89, no. 11, pp. 1127–1134, 2017.

[56] J. Narváez, P. Juarez-López, J. LLuch et al., "Rheumatic immune-related adverse events in patients on anti-PD-1 inhibitors: Fasciitis with myositis syndrome as a new complication of immunotherapy," *Autoimmunity Reviews*, vol. 17, no. 10, pp. 1040–1045, 2018.

[57] J. Bourgeois-Vionnet, B. Joubert, E. Bernard et al., "Nivolumab-induced myositis: a case report and a literature review," *Journal of the Neurological Sciences*, vol. 387, no. 10, pp. 51–53, 2018.

[58] G. Hunter, C. Voll, and C. A. Robinson, "Autoimmune inflammatory myopathy after treatment with ipilimumab," *The Canadian Journal of Neurological Sciences*, vol. 36, no. 4, pp. 518–520, 2009.

[59] M. Yoshioka, N. Kambe, Y. Yamamoto, K. Suehiro, and H. Matsue, "Case of respiratory discomfort due to myositis after administration of nivolumab," *The Journal of Dermatology*, vol. 42, no. 10, pp. 1008-1009, 2015.

[60] L. Saini and N. Chua, "Severe inflammatory myositis in a patient receiving concurrent nivolumab and azacitidine," *Leukemia Lymphoma*, vol. 58, no. 8, pp. 2011–2013, 2016.

[61] Y. H. Chen, F. C. Liu, C. H. Hsu, and C. F. Chian, "Nivolumab induced myasthenia gravis in a patient with squamous cell lung carcinoma: case report," *Medicine*, vol. 96, no. 27, article e7350, 2017.

[62] H. Vallet, A. Gaillet, N. Weiss et al., "Pembrolizumab-induced necrotic myositis in a patient with metastatic melanoma," *Annals of Oncology*, vol. 27, no. 7, pp. 1352-1353, 2016.

[63] B. Koh, K. Tuite, A. Khattak, and J. M. Dyke, "Lymphocyte involvement in nivolumab-induced autoimmune myositis," *Pathology*, vol. 51, no. 5, pp. 555–557, 2019.

[64] B. M. Warner, A. N. Baer, E. J. Lipson et al., "Sicca syndrome associated with immune checkpoint inhibitor therapy," *The Oncologist*, vol. 24, no. 9, pp. 1259–1269, 2019.

[65] J. B. A. G. Haanen, F. Carbonnel, C. Robert et al., "Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up[†]," *Annals of Oncology*, vol. 29, no. 4, pp. 264–266, 2017.

[66] J. R. Brahmer, C. Lacchetti, B. J. Schneider et al., "Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American Society of Clinical Oncology Clinical Practice Guideline," *Journal of Clinical Oncology*, vol. 36, no. 17, pp. 1714–1768, 2018.

[67] S. Le Burel, S. Champiat, E. Routier et al., "Onset of connective tissue disease following anti-PD1/PD-L1 cancer immunotherapy," *Annals of the Rheumatic Diseases*, vol. 77, no. 3, pp. 468–470, 2018.

[68] E. I. Dumbrava, V. Smith, R. Alfattal, A. K. El-Naggar, M. Penas-Prado, and A. M. Tsimberidou, "Autoimmune granulomatous inflammation of lacrimal glands and axonal neuritis following treatment with ipilimumab and radiation therapy," *Journal of Immunotherapy*, vol. 41, no. 7, pp. 336–339, 2018.

[69] F. Fadel, K. Karoui, and B. Knebelmann, "Anti-CTLA4 antibody-induced lupus nephritis," *The New England Journal of Medicine*, vol. 361, no. 2, pp. 211-212, 2009.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 3067273, 20 pages
https://doi.org/10.1155/2020/3067273

Research Article

Integrated Bioinformatics Analysis Identifies ELAVL1 and APP as Candidate Crucial Genes for Crohn's Disease

Heli Li,[1] Qianru Li,[1,2,3] Shiran Sun,[3] Ping Lei,[1] Xiong Cai,[3] and Guanxin Shen[1]

[1]*Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China 430030*
[2]*Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*
[3]*Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

Correspondence should be addressed to Xiong Cai; caixiong@live.com and Guanxin Shen; shenguanxin@hust.edu.cn

Received 20 April 2020; Accepted 12 June 2020; Published 17 July 2020

Academic Editor: Lihua Duan

Immune imbalance and barrier destruction of intestinal mucosa are the central pathogenic factors of Crohn's disease (CD). In this study, three independent microarray studies of CD were integrated and 9912 differentially expressed genes (DEGs) were analysed by NetworkAnalyst to screen candidate crucial genes. NetworkAnalyst identified ELAV-like RNA binding protein 1 (ELAVL1) as the most crucial upregulated gene and amyloid-β precursor protein (APP) as the most crucial downregulated gene in peripheral blood of CD patients. By computing significance with hypergeometric test based on the KEGG pathway database, upregulated DEGs highlight the pathways of T cell receptor signaling and the differentiation of T helpers. Downregulated DEGs were found enriched in pathways in multiple cancers, MAPK signaling, Rap1 signaling, and PI3K-AKT signaling. Further taking all DEGs together, Gene Set Enrichment Analysis (GSEA) brought out the NOD-like receptor (NLR) signaling pathway which could be regulated by ELAVL1. xCell found decreased naïve and differentiated T cell proportions in the peripheral blood of CD patients suggesting T cell migration to the intestinal tissue and/or exhaustion. Further, ELAVL1 expression correlating with multiple T cell proportions suggests that ELAVL1 may regulate T cell activation. These findings illustrated that ELAVL1 and APP were candidate crucial genes in the peripheral blood of CD patients. ELAVL1 possibly acts as a key regulator of T cell activation via the NLR signaling pathway. APP might be a downstream effector of infliximab treatment connecting with MAPK signaling.

1. Introduction

Crohn's disease (CD), as a systemic inflammatory disease, mainly influences the gastrointestinal tract with a wide range of contributing factors including host genetics, immune system, environmental exposures, and the gut microbiome [1]. Although the pathogenesis is complex, the decades of studies have illustrated that CD is caused by environmental factors that broke the mucosal barrier and increased the luminal antigens into the lamina propria [2]. Different innate immune cells, like dendritic cells, recognize the antigens via pattern recognition receptors, such as Toll-like receptors (TLR) and NLR, and then regulate the activation of T cells [3].

Among the factors associated with CD in immune system, T cells were highlighted in CD pathology because about 200 CD risk loci are involved in T cell signaling [4, 5]. Furthermore, the $CD4^+$ T cell, including Th1, Th17, and regulatory T (Treg) cells, were associated with the severity of CD, particularly with active inflammation [2]. In addition, $CD8^+$ T cell transcriptional signatures were identified as reliable prognostic biomarkers in the blood of CD patients [6, 7]. Likewise, numerous studies are devoted to the development of diagnostic methods related to T cells for CD. For example, four CD-related differentially methylated regions were identified in the whole blood [8]. In addition, one T cell subtype from the blood of CD patients is enriched for the CD-risk gene [9]. Nevertheless, the key regulators and mechanisms

regulating T cell activation in the blood of CD have not been fully described. Thus, we integrated three independent microarray studies to screen crucial genes in the blood of CD patients.

We performed an integrated bioinformatics analysis to find key regulators by NetworkAnalyst [10], a web-based visual analytics platform. Indeed, the utility of NetworkAnalyst to identify DEGs and the pathway has been demonstrated recently. For example, network analyses identified HNF4A and PTBP1 as effective biomarkers for Parkinson's disease [11] and 873 DEGs genetically related to insulin resistance [12].

In this study, we launched a network-based bioinformatics analysis in an attempt to screen DEGs in the blood of CD patients, followed by the KEGG pathway enrichment analysis, GSEA, and interaction network of DEGs. We identified ELAVL1 and APP, previously implicated in regulating the activation of innate immunity [13, 14] and T cells [15], the most significant up- and downregulated genes. Furthermore, GSEA data show that NOD-like receptor signaling-associated gene signatures are enriched and the xCell analysis indicated that most T cells are significantly decreased in the three microarray studies, which could help in understanding the role of ELAVL1 and APP in the blood of CD patients.

2. Materials and Methods

2.1. Microarray Source. The microarray studies of CD were downloaded from the Gene Expression Omnibus (GEO) by using the terms "Crohn's disease" and "blood". Three microarray studies were selected for subsequent integrative analysis. GSE86434 included 23 newly diagnosed CD patients and 24 healthy controls (HC). GSE94648 included 22 healthy controls, 9 inactive CD patients and 41 active CD patients. GSE119600 included 47 adult healthy controls, 48 adult CD patients, and 47 child CD patients. PRJEB28822 was chosen to be the verified datasets from the European Nucleotide Archive (ENA), which included 102 pediatric IBD patients and 51 controls, and 95 adult IBD patients and 46 controls, separately.

2.2. Gene Expression and Connectivity Correlation Assay. To assess the comparability of two datasets, measures of average gene expression and overall connectivity between two datasets were correlated. The higher the correlations, the better the chance of finding similarities between the two datasets at subsequent stages of the analysis. All enrolled datasets with or without batch effect adjustment were examined for average expression correlation (correlation between expression ranks of genes in two studies) and connectivity correlation (correlation between connectivity ranks of genes in two studies) by R package WGCNA [16]. Better comparable datasets were indicated by greater positive correlations and more significant p values.

2.3. Integrated Network-Based Bioinformatics Analysis. We performed integrated bioinformatics analysis using NetworkAnalyst [10] in accordance with the protocol [17]. After annotating the gene probes to a common Entrez ID, these datasets were normalized per platform requirement and uploaded to the website. DEGs of each dataset were defined by $p < 0.05$ and log2 fold change > 1. The Venn diagram of DEGs was generated by the FunRich tool (version 3.1).

Network-based bioinformatics analysis was performed by NetworkAnalyst according to the pipeline described. The whole blood-specific protein-protein interaction (PPI) of the top 20 DEGs and up- or downregulated genes was constructed per protocol [17]; then, the KEGG pathway enrichment analysis was performed by applying hypergeometric test.

2.4. Gene Set Enrichment Analysis. Preranked GSEA was analysed using a NetworkAnalyst module powered by R package fgsea [10]. All DEGs ranked by fold changes were put into the analysis, and the results was visualized as interactive heatmaps.

2.5. In Silico Immune Cell Type Enrichment Analysis. xCell, a gene signature-based method reliably portraying the cellular heterogeneity landscape of tissue expression profiles, was performed to explore immune cell types [18], and the proportion of T cells were obtained for each CD and HC sample per instruction.

2.6. Statistical Analysis. All basic statistical analyses, including the Mann–Whitney test, Pearson correlation, and Spearman correlation were calculated by R software. A p value < 0.05 was considered to be statistically significant. Data were represented by mean and standard deviation (SD) or median and quantile depending on distributions.

3. Results

3.1. Screening DEGs by Integrated Bioinformatics Analysis. Three microarray studies (Table 1) were analysed using NetworkAnalyst to screen DEGs in the blood of CD patients. Firstly, these datasets were preprocessed to ensure they are comparable enough. The correlations were positive and the p values were significant in all cases before batch effect adjustments (Figures 1(a)–1(c)). The correlations (cor) for the gene expression and connectivity of GSE86434 and GSE119600 (expression, cor = 0.97, $p < 1e-200$; connectivity, cor = 0.74, $p < 1e-200$) were better than GSE86434 and GSE94648 (expression, cor = 0.76, $p < 1e-200$; connectivity, cor = 0.34, $p < 1.6e-135$) or GSE94648 and GSE119600 (expression, cor = 0.75, $p < 1e-200$; connectivity, cor = 0.34, $p < 1.6e-135$). GSE86434 and GSE119600 were from the same platform (Illumina HumanHT-12 V4.0 expression bead chip). Thus, it is consistent with the notion that datasets from the same platform are more comparable than datasets from different platforms. To remove this batch effect, parametric empirical Bayes frameworks provided by ComBat function were applied by NetworkAnalyst. And the results after batch effect moving were shown in Figures 1(d)–1(f), indicating that the processed data are more comparable. PCA plots with or without batch effect adjustment were visualized in Figure 1(g).

Table 1: Gene expression datasets used in this study.

Disease	Datasets	Platform	Cases	Controls	References
Exploration					
CD	GSE86434	GPL10558	23	24	[8]
CD	GSE94648	GPL19109	50	22	[19]
CD	GSE119600	GPL10558	95	47	[20]
Validation					
IBD	PRJEB28822	Ion AmpliSeq Transcriptome Human Gene Expression Panel	102 Pediatric 95 Adult	51 Pediatric 46 Adult	[21]

Integrated bioinformatics analysis identified 9912 DEGs in the three microarray studies, in which 4705 genes were upregulated and 5207 were downregulated in CD compared with HC. A Venn diagram of integrative analysis DEGs and individual DEGs are shown in Figure 1(h). There were 1361 genes specifically classified by integrated bioinformatics analysis that are weakly but consistently expressed among the three datasets. 2511 genes were identified as lost genes, which are expressed in individual datasets but not in the integrated datasets.

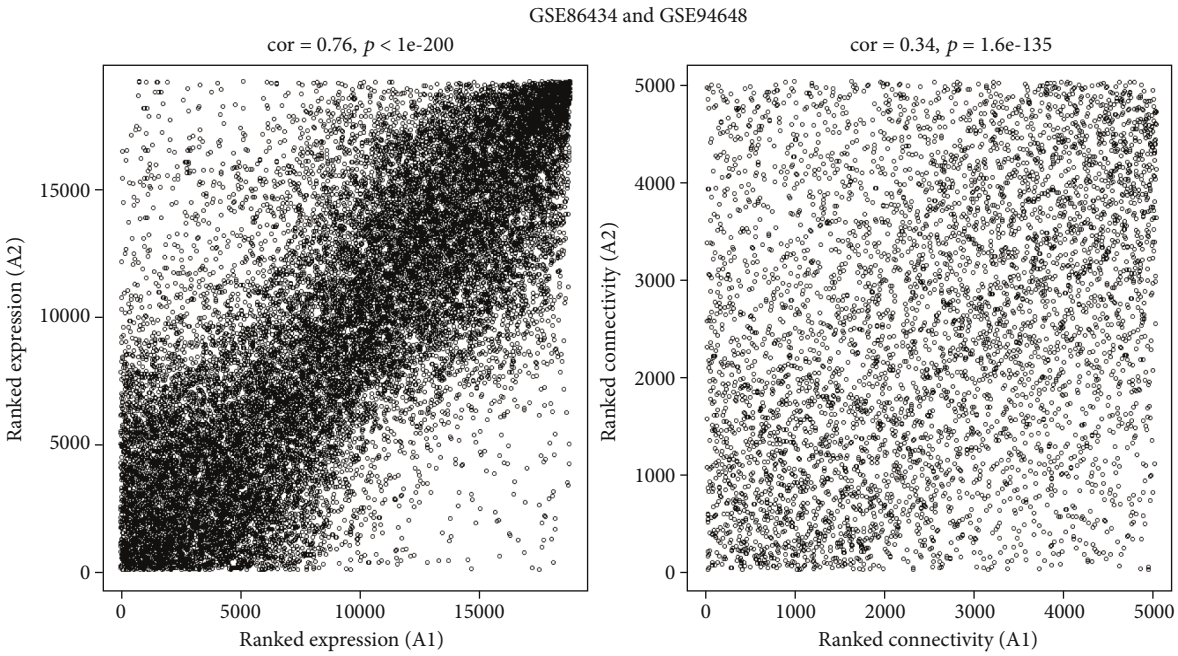
3.2. Top 20 Hub Genes Are Identified by Network-Based Analysis. Firstly, we put all DEGs into network-based analysis per instruction [10]. According to the degree of centrality (DC) and betweenness (BC), the most highly ranked node was APP (DC = 1056; BC = 4730653.29) followed by EGFR (DC = 580; BC = 1711464.65) and ELAVL1 (DC = 574; BC = 1999915.6) and the top 20 hub genes were presented (Table 2). The results of the KEGG pathway enrichment analysis of these 20 genes were shown in Table 3. The whole blood-specific PPI (Figure 2(a)) and enrichment network (Figure 2(b)) were generated by NetworkAnalyst. The zero-order interaction network of these genes contained 20 nodes and 38 edges (Figure 2(a)). The results of pathway enrichment indicated that variables in the blood transcriptome of CD were closely connected to several cancers including colorectal cancer, and also with the PI3K-AKT and MAPK signaling pathways.

3.3. ELAVL1 and APP Are Hub Genes Measured by Network Analysis. Then, we analysed pathway enriched in up- or downregulated DEGs separately to explore key regulators in the blood of CD patients, by NetworkAnalyst [10]. Upregulated genes in the blood of CD were enriched in the pathways, including T cell receptor signaling pathway, primary immunodeficiency, Th1 and Th2 cell differentiation, Th17 cell differentiation, and NF-kappa B signaling pathway (Figure 3(a)). The most crucial gene among upregulated DEGs was ELAVL1 (DC = 357; BC = 709523.17) (Figure 3(b)). Downregulated genes in the blood of CD were enriched in the pathways, including pathways in cancer, MAPK signaling pathway, Rap1 signaling pathway, and PI3K-AKT signaling pathway (Figure 3(c)). The most crucial gene among downregulated DEGs was APP (BC = 1244517.75; DC = 492) (Figure 3(d)).

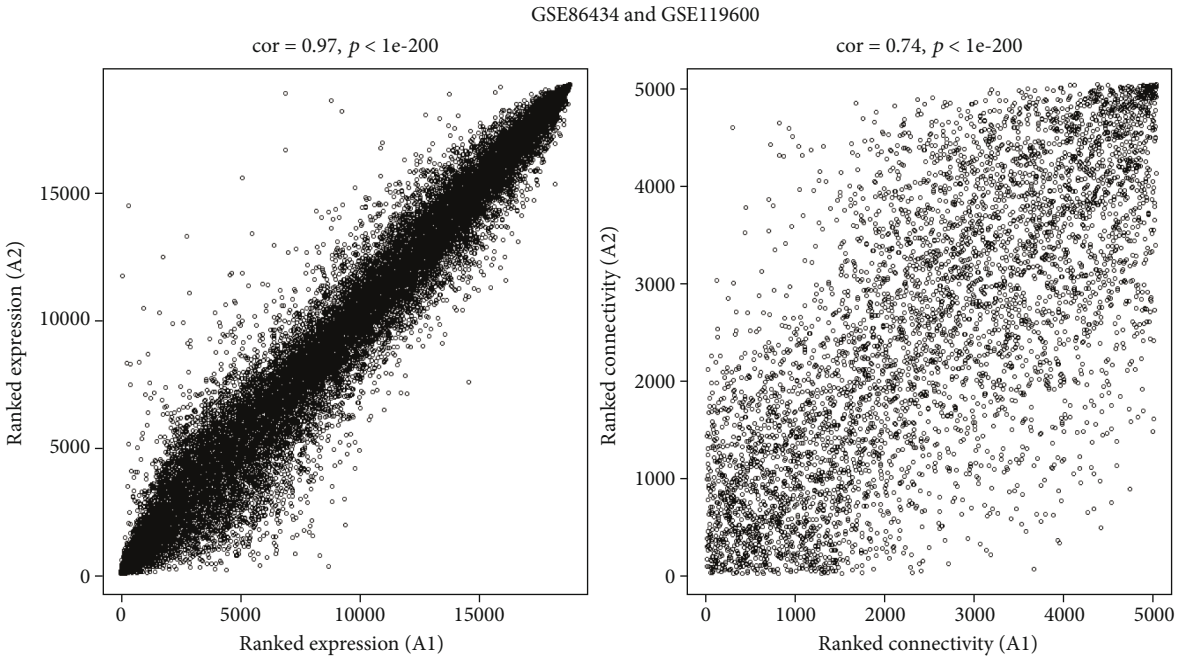
Furthermore, we analysed an AmpliSeq study (PRJEB28822) in the whole peripheral blood of IBD patients [21]. APP expression was significantly reduced in pediatric IBD patients compared with HC (FC = −0.54, $p < 0.001$), while ELAVL1 was significantly elevated (FC = 0.15, $p < 0.05$).

3.4. NOD-like Receptor Signaling Pathway Is Enriched in the Blood of CD by GSEA Analysis. To further clarify the possible mechanism of CD, preranked GSEA was performed to analyse the three microarray datasets separately or integrally selecting fold change as the gene ranking methods (Table 4). As shown in Table 4 and Figures 4(a)–4(d), GSEA analysis suggested that NOD-like receptor signaling pathway is the only enriched gene set we can find in the top 10 enriched gene sets of all three datasets. In addition, the integrated dataset analysed by GSEA also indicated that NOD-like receptor signaling pathway was enriched in CD patients (Figures 4(e) and 4(f)). These results revealed that NOD-like receptor signaling may act as a crucial role in the development of CD.

3.5. ELAVL1 Expression Correlates with Genes in NLR Signaling Pathway, and APP Expression Is Associated with MAPK Signaling Cross Multiple Datasets. To further explore and validate the association between candidate crucial genes and top enriched signaling pathways in CD patients, we analysed the direct correlation between ELAVL1 and major genes in the NLR pathway and correlation between APP and major genes in MAPK signaling by the Pearson correlation. As shown in Figure 5(a), genes in the NLR signaling pathway, including NLRP1, NLRP3, CXCL8, TRAK4, and TNF, were correlated with the expression of ELAVL1 at least in one of the datasets. Additionally, GRB2, FOS, MYD88, MAP3K3, and EGF were found associated with the expression of APP (Figure 5(b)). These results strongly supported the direct correlation between ELAVL1 and NLR signaling, as well as correlations between APP and MAPK signaling in the peripheral blood of CD patients. A slight inconsistency of correlations and significances among three datasets may be due to varied sample sizes and markable heterogeneity of the disease.

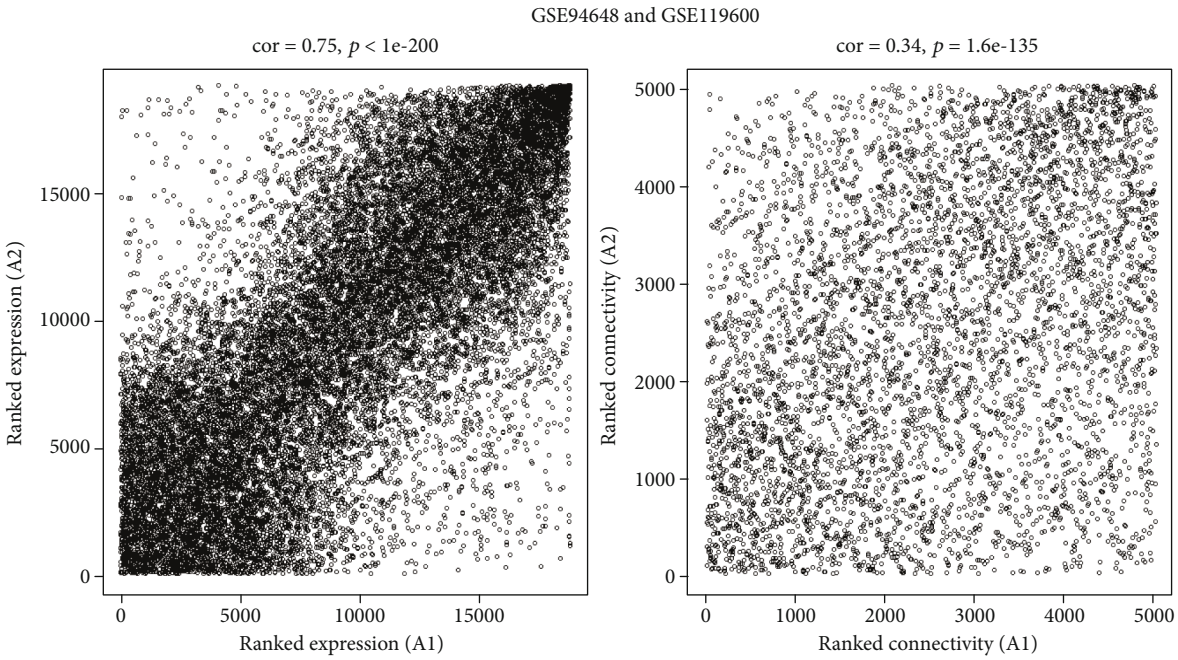


(a)

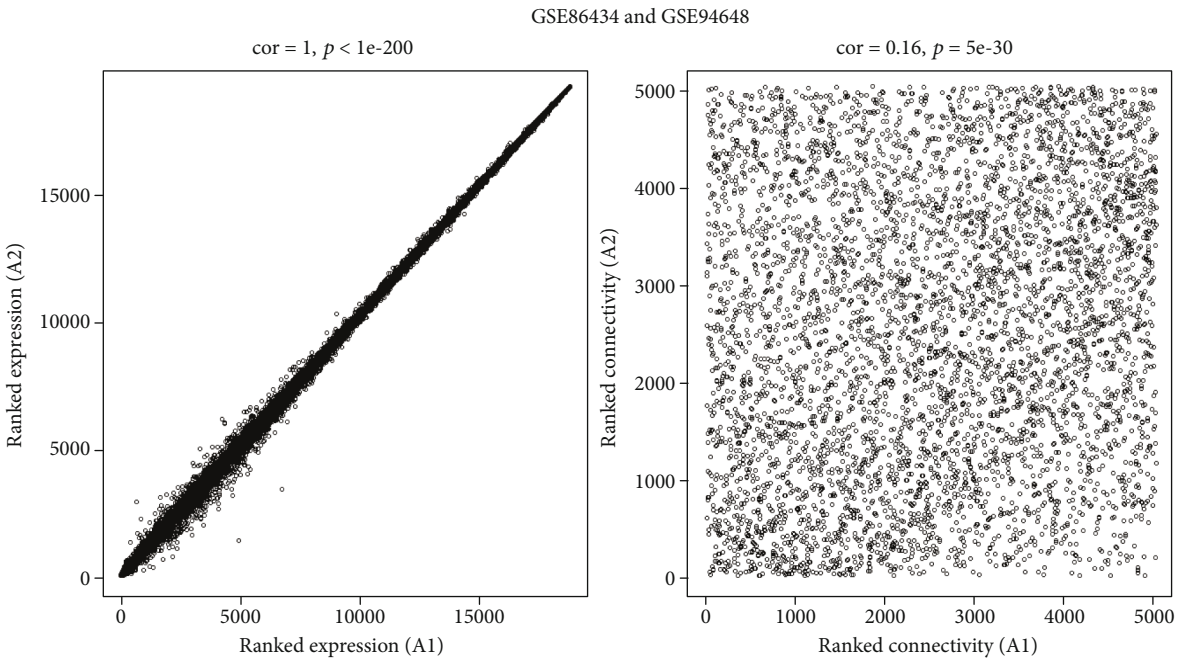


(b)

Figure 1: Continued.

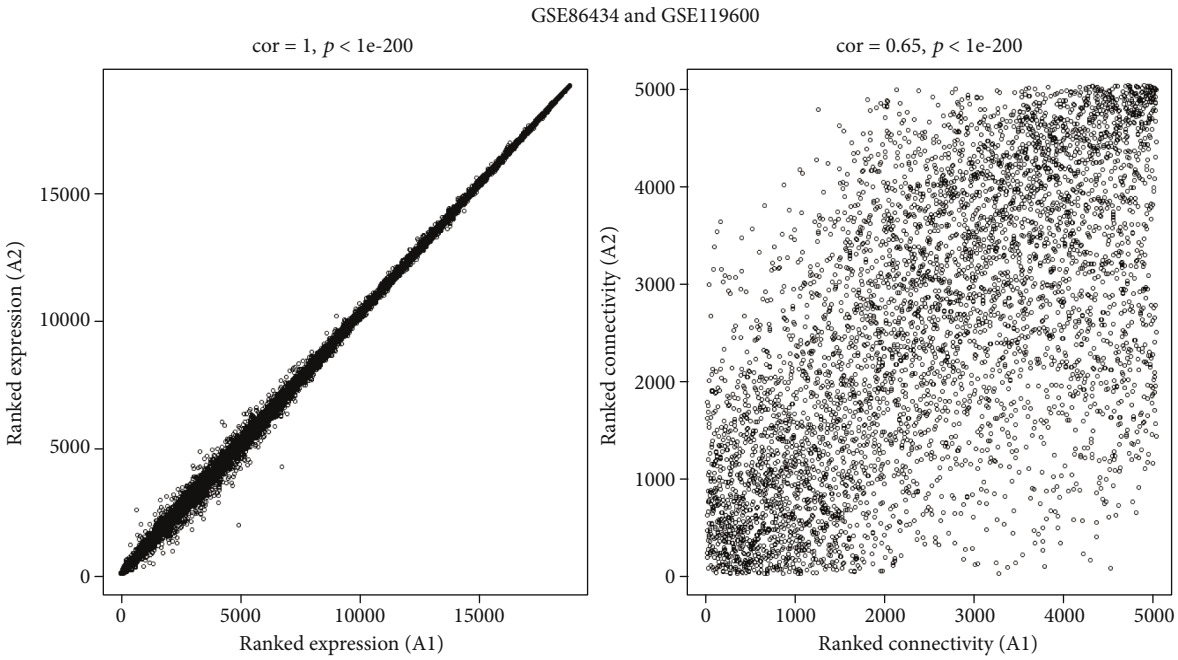


(c)

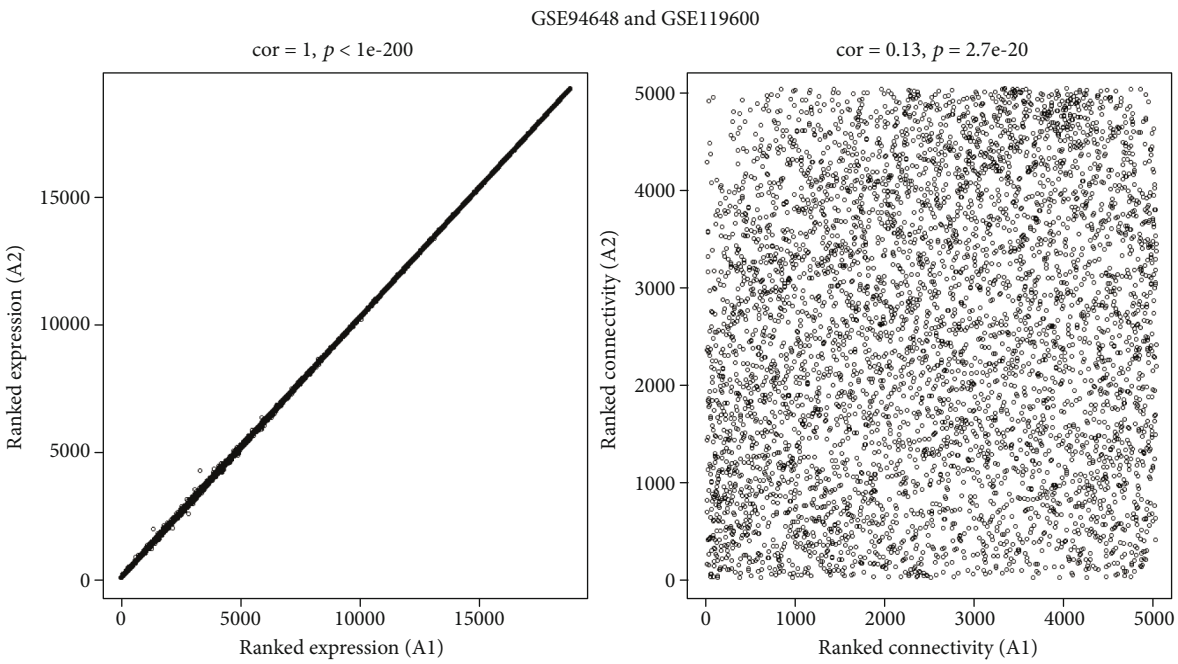


(d)

Figure 1: Continued.



(e)



(f)

Figure 1: Continued.

Figure 1: Screening DEGs by integrated bioinformatics analysis. The gene expression and connectivity of GSE86434 and GSE94648 (a), GSE86434 and GSE119600 (b), and GSE94648 and GSE119600 (c). After moving batch effects with parametric empirical Bayes frameworks provided by the ComBat function in R package sva, the gene expression and connectivity of GSE86434 and GSE94648 (d), GSE86434 and GSE119600 (e), and GSE94648 and GSE119600 (f). (g) PCA plot for sample clustering of all datasets without batch effect adjustment (A) and with batch effect adjustment (B). (h) Venn diagram of integrative analysis DEGs (Integrated-DE) and DEGs from each individual dataset (Individual-DE).

3.6. Naïve and Differentiated T Cell Proportions in the Peripheral Blood of CD Patients Are Decreased Compared to Health Controls. Previous studies have indicated that distinct T cell subsets, such as Th1/Th17, Tregs, memory, and naïve T cells, are activated in the gut of CD patients. Therefore, we performed xCell to explore the signature of T cells in the peripheral blood of CD from the three datasets and found that the altered T cell proportions were one of the hallmarks in the peripheral blood of CD (Figure 6). Specifically, $CD4^+$ T cells were obviously decreased in GSE86434 (Figure 6(a)) and GSE94648 (Figure 6(b); similar trends were also shown in GSE119600 (Figure 6(c)). Because of the essential function of $CD4^+$ T cells in helping the activation of $CD8^+$ T cells, these data also show the decrease of $CD8^+$ T cells in GSE86434 (Figure 6(a)) and GSE94648 (Figure 6(b)), as well as in GSE119600 (Figure 6(b). Similarly, the central memory $CD8^+$ T (Tcm) cells were clearly decreased in all three datasets. Of note, the effector memory $CD8^+$ T (Tem) cells were apparently decreased in the three datasets, which may be a new hallmark in the blood of CD patients. In addition, Th1 cells, the cell type mostly relevant to CD, were disordered in the three datasets. Since T cells are activated and recruited

Table 2: Top 20 hub genes with DC and BC.

Gene	DC	BC	Expression
APP	1056	4730653	-33.853
EGFR	580	1711465	-15.619
ELAVL1	574	1999916	34.987
CAND1	381	578227.8	24.477
ITGA4	370	457397.4	-15.926
SIRT7	357	744489.5	-45.873
FBXO6	352	760217.7	-53.7
CCDC8	322	504043	-15.191
GRB2	303	630311.8	-26.82
TP53	281	621198.7	20.389
MOV10	260	573433.5	-20.408
NXF1	241	496040.6	-23.619
MYC	239	458057	21.06
HSP90AA1	232	486480.7	28.182
HUWE1	232	242665.7	26.081
LRRK2	223	216252.1	-31.578
COPS5	220	313401.8	48.805
ARRB2	219	230981.9	-42.185
PAN2	217	168810.6	30.93
CUL5	208	174910.9	22.703

Table 3: KEGG analysis of the 20 hub genes identified by network-based analysis.

Term	Count	Gene	*p* value
Endometrial cancer	4	20%	3.65*e*-6
Colorectal cancer	4	20%	1.77*e*-5
Prostate cancer	4	20%	2.85*e*-5
PI3K-Akt signaling pathway	6	30%	3.1*e*-5
Bladder cancer	3	15%	6.01*e*-5
Breast cancer	4	20%	1.45*e*-4
MAPK signaling pathway	5	25%	1.71*e*-4

to the intestinal tissue in CD to resist bacterial infections and continuously activated T cells can be exhausted, most T cell subsets decreased in the peripheral blood of CD can be interpreted by T cell migration to the intestinal tissue and/or exhaustion.

3.7. ELAVL1 Expression Correlates with Various T Cell Subset Proportions in the Peripheral Blood of CD Patients. Since we have found that ELAVL1 is closely associated to NLR signaling, which has an important impact on priming the activation of T cells, we further investigate the association between ELAVL1 expression and the xCell scores of T cells in CD patients by the Spearman correlation. As shown in Figures 7(a)–7(c), different T cell subsets, including $CD4^+$ naive T cells, $CD4^+$ Tcm, $CD4^+$ Tem, $CD8^+$ naive T cells, $CD8^+$ Tcm, and $CD8^+$ Tem, were found positively correlated with ELAVL1 expression in dataset GSE119600 including 95 CD patients (Figure 7(c)). These results suggest that the upregulated expression of ELAVL1 is associated with T cell activation, probably through promoting the NLR signaling pathway. While in GSE86434 and GSE94648, the absence of a significant correlation may due to limited CD sample sizes of the two datasets.

4. Discussion

Up to date, the immunological pathogenesis of CD continues to be complex, and finding the key regulators of the immune response is essential for knowing the disease and improving the clinical management of CD. Specifically, investigations focusing on exploring CD pathogenesis and identifying therapeutic targets are warranted for the discovery of effective drugs. Here, we identified the candidate crucial genes in the peripheral blood of CD by a network-based analysis of NCBI GEO datasets GSE86434, GSE94648, and GSE119600. Integrative analysis identified 9912 DEGs among these datasets. ELAVL1 and APP were confirmed as the most significant up- and downregulated genes by NetworkAnalyst. Furthermore, the differential expression significances of the two hub genes were validated by an AmpliSeq dataset PRJEB28822, indicating that ELAVL1 and APP may be potential biomarkers for CD patients.

ELAVL1, also known as Hu antigen R (HuR), is an abundant RNA binding protein that can affect the stability and translation of many RNAs and participate in the regulation of chronic inflammation and cancer progression [22, 23]. It has been identified that ELAVL1 was increased after cellular stress with protective activities [24], which is positively regulated by NF-κB and Smads [25]. Furthermore, the increase of ELAVL1 was confirmed to suppress inflammatory responses in mice [23], suggesting the important role of ELAVL1 as a posttranscriptional mediator for inflammation. However, it is worth noting that the increased expression of ELAVL1 is identified to promote the overexpression of COX-2, and thus contributing to the growth of colon cancer, whose risk is increased in the setting of CD [26, 27]. Moreover, the influence of ELAVL1 in promoting malignant transformation has been well documented in multiple cancers [28–32]. That is in consistent with our pathway enrichment of top 20 hub genes indicating that variables in blood transcriptome of CD were closely connected to several cancers including colorectal cancer. The pathway enrichment of top 20 hub genes also shed light on the PI3K-AKT and MAPK signaling pathways, that is in line with investigations that phosphorylation by p38 MAPK results in the accumulation of ELAVL1 in the cytoplasm [33] and the elevation of ELAVL1 is essential for enhancing the proliferation of gastric cancer cells, which depends on the activation of PI3K-AKT and NF-κB signaling [34]. Our results highlight the crucial role of ELAVL1 in connecting inflammatory meditation with tumorigenesis, suggesting the potential role of ELAVL1 in carcinogenesis of colorectal cancer in the background of CD.

APP has been known as central to the pathogenesis of Alzheimer's disease (AD) and has been confirmed as the potential biomarker in predicting brain amyloid-β burden [35]. Although the specific mechanism coexisting in AD and CD is still unclear, it has been demonstrated that there

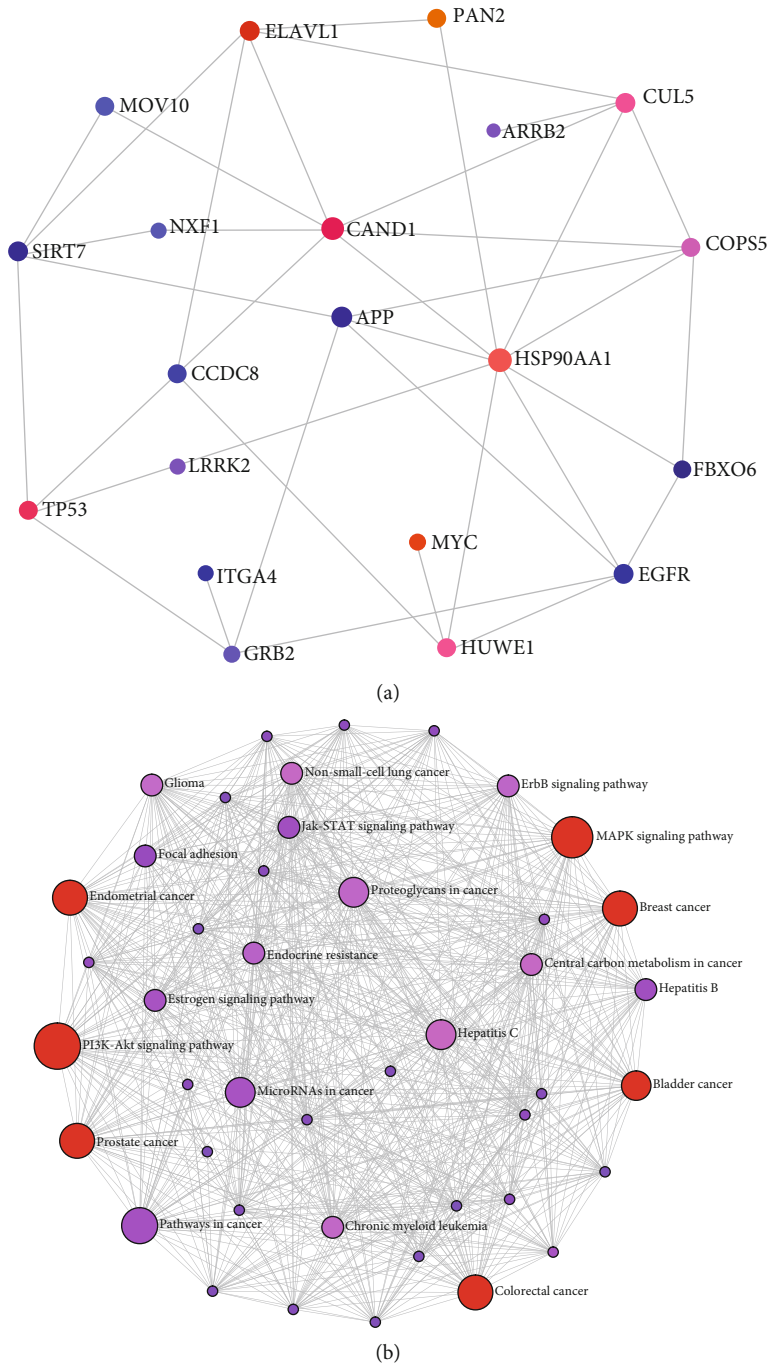
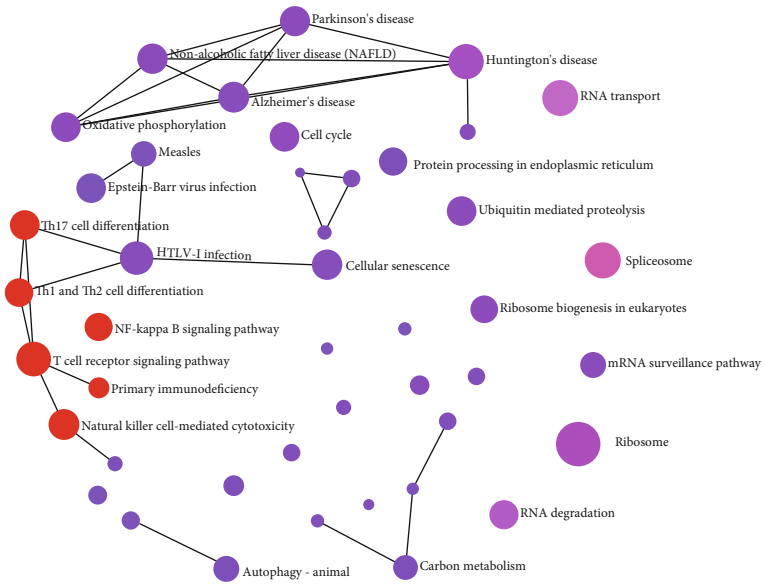
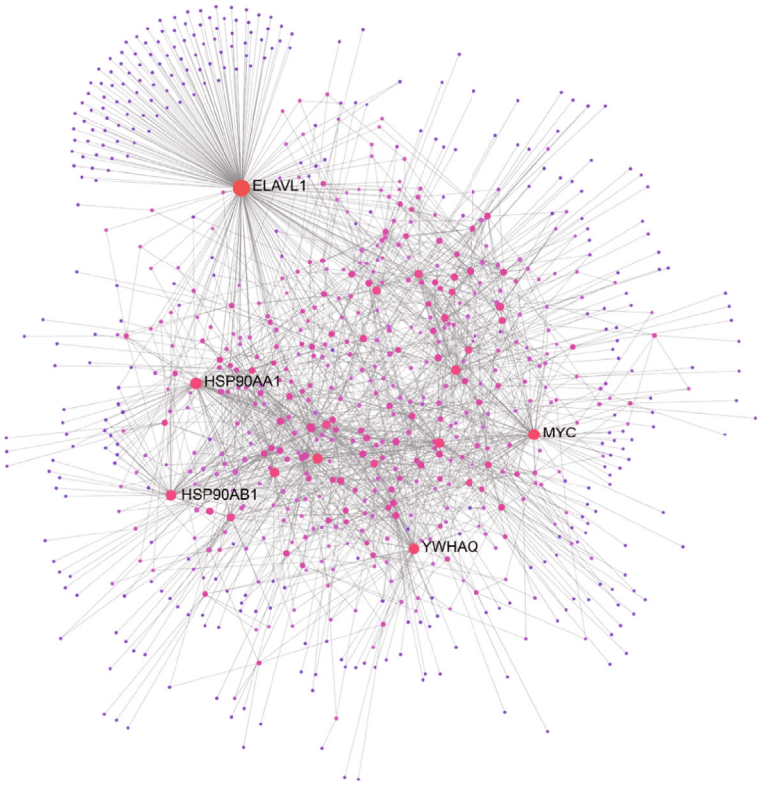


Figure 2: Top 20 hub genes are identified by network-based analysis. (a) Zero-order interaction network and (b) enrichment network of top 20 hub genes.

are genetic factors overlapping between AD and CD [36]. Besides, the immune response is considered one of major contributors in both AD and CD. A recent review highlights the influence of the peripheral immune system in AD [37], such as monocytes and lymphocytes, which have been linked to CD in a number of studies [38]. This overlap in the pathogenesis between the two diseases may suggest the potential central role of APP in CD pathogenesis. Though literature about APP in CD is very limited, we can still note that Apolipoprotein E (APOE), widely found increased in serum of CD patients who were primary nonrespondents or had responded clinically and serologically after infliximab treatment of CD [39], can increase transcription of APP significantly [40]. Next, APP can active the MAPK signaling pathway [41, 42], which is one of the top enriched gene sets in our pathway enrichment analysis of top 20 hub genes. Since detailed mechanism about infliximab treatment in CD has not been fully elucidated, APP may be predicted as a downstream effector of the treatment which is worthy of further investigation.

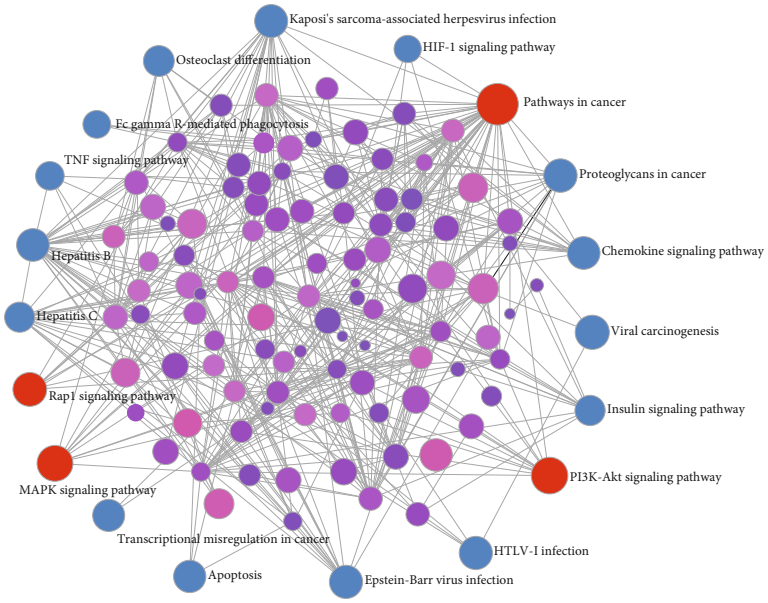


(a)

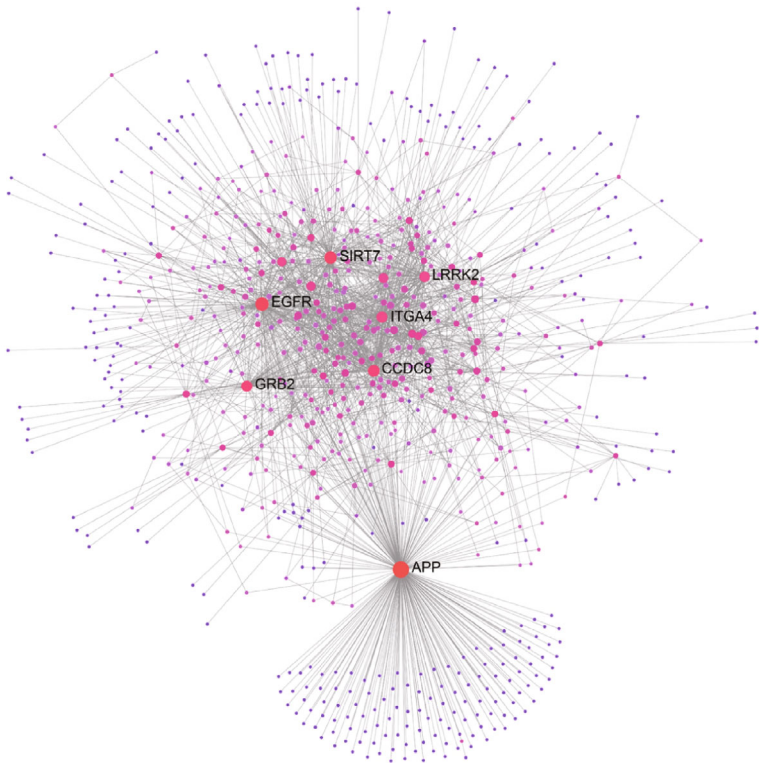


(b)

Figure 3: Continued.



(c)



(d)

Figure 3: ELAVL1 and APP are hub genes measured by network analysis. (a) Enrichment network and (b) zero-order interaction network of upregulated genes identified by network-based analysis. (c) Enrichment network and (d) zero-order interaction network of downregulated genes identified by network-based analysis.

The results from GSEA of the individual and overall databases highlight the NOD-like receptor signaling pathway in the blood of CD patients. NOD1 and NOD2, members of the NOD-like receptor family, are two important mediators of inflammation induced by endoplasmic reticulum stress, which is a major contributor to CD [43, 44]. In addition to the roles in regulating innate immune responses, there are plenty of evidences that NOD1 and NOD2 signaling have an impact on adaptive immune responses. In mice, NOD1 and NOD2 signaling are involved in the activation of Th1, Th2, and Th17 cells [45]. NOD2 also can drive $CD8^+$ T cell activation via the cross-presentation pathway [46]. Our

Table 4: GSEA analysis of the three microarray datasets.

GSE86434		GSE94648		GSE119600		Integrated	
Name	Enrichment score	Name	Enrichment score	Name	Enrichment score	Name	Enrichment score
NOD-like receptor signaling pathway	0.64066	Huntington's disease	0.453565	Pathways in cancer	0.404973	MAPK signaling pathway	0.446547
Kaposi's sarcoma-associated herpesvirus infection	0.473815	Alzheimer's disease	0.486581	Endocytosis	0.426882	Kaposi's sarcoma-associated herpesvirus infection	0.499763
Tuberculosis	0.48882	Alcoholism	0.509264	MAPK signaling pathway	0.462268	Rap1 signaling pathway	0.517768
Chemokine signaling pathway	0.474491	NOD-like receptor signaling pathway	0.492519	Proteoglycans in cancer	0.46461	NOD-like receptor signaling pathway	0.633331
Influenza A	0.504921	Nonalcoholic fatty liver disease (NAFLD)	0.487171	Kaposi's sarcoma-associated herpesvirus infection	0.454893	Tuberculosis	0.549604
Alcoholism	0.597721	Parkinson's disease	0.573813	Regulation of actin cytoskeleton	0.48289	Influenza A	0.505989
Necroptosis	0.526145	Lysosome	0.453183	Rap1 signaling pathway	0.524153	Hepatitis B	0.536561
Phagosome	0.515787	Oxidative phosphorylation	0.614682	Tuberculosis	0.47918	Alcoholism	0.488690
Hepatitis C	0.558024	Systemic lupus erythematosus	0.625444	Focal adhesion	0.501669	Necroptosis	0.525488
Osteoclast differentiation	0.615345	Huntington's disease	0.517828	NOD-like receptor signaling pathway	0.500948	Phagosome	0.513988

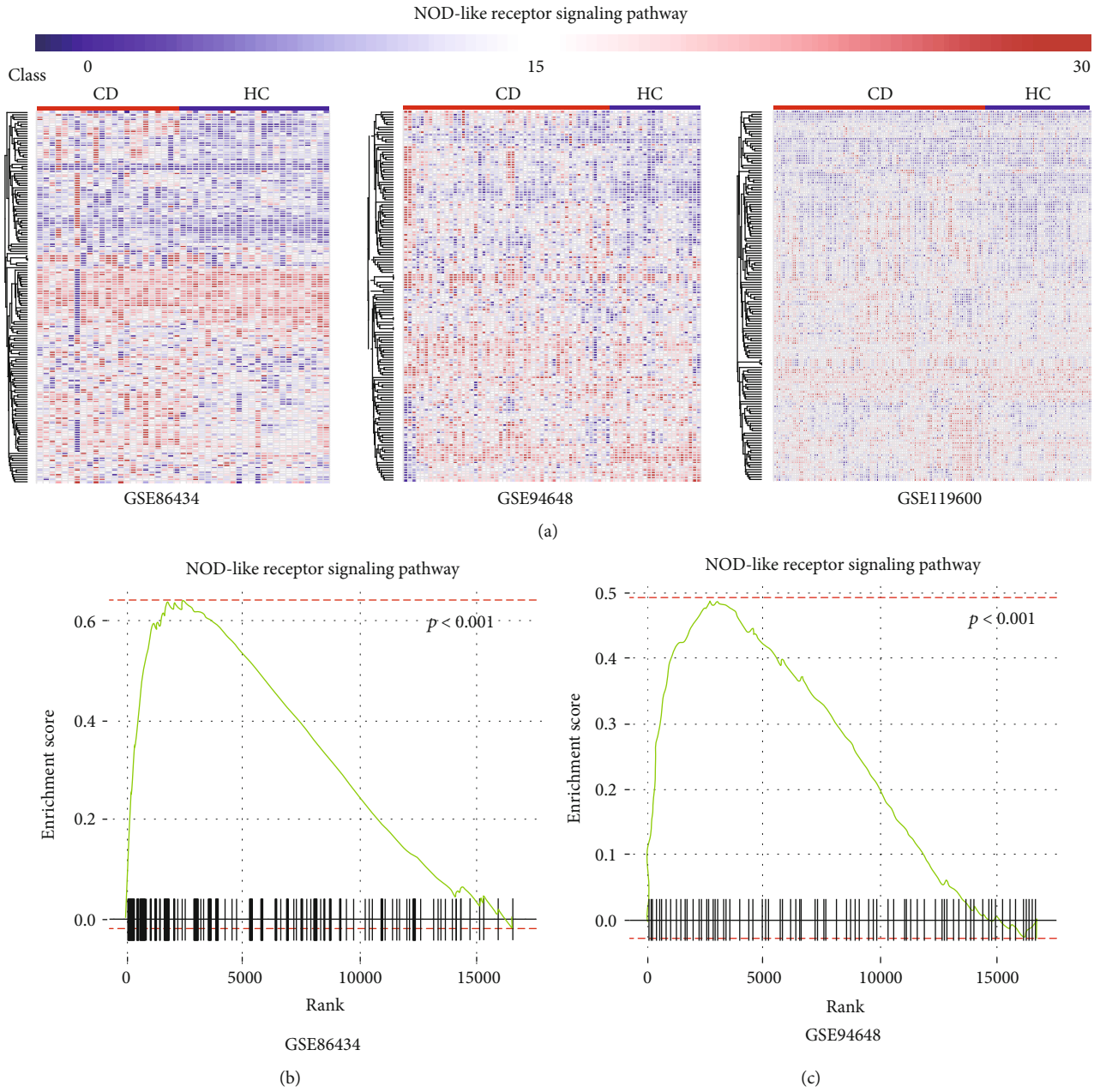


Figure 4: Continued.

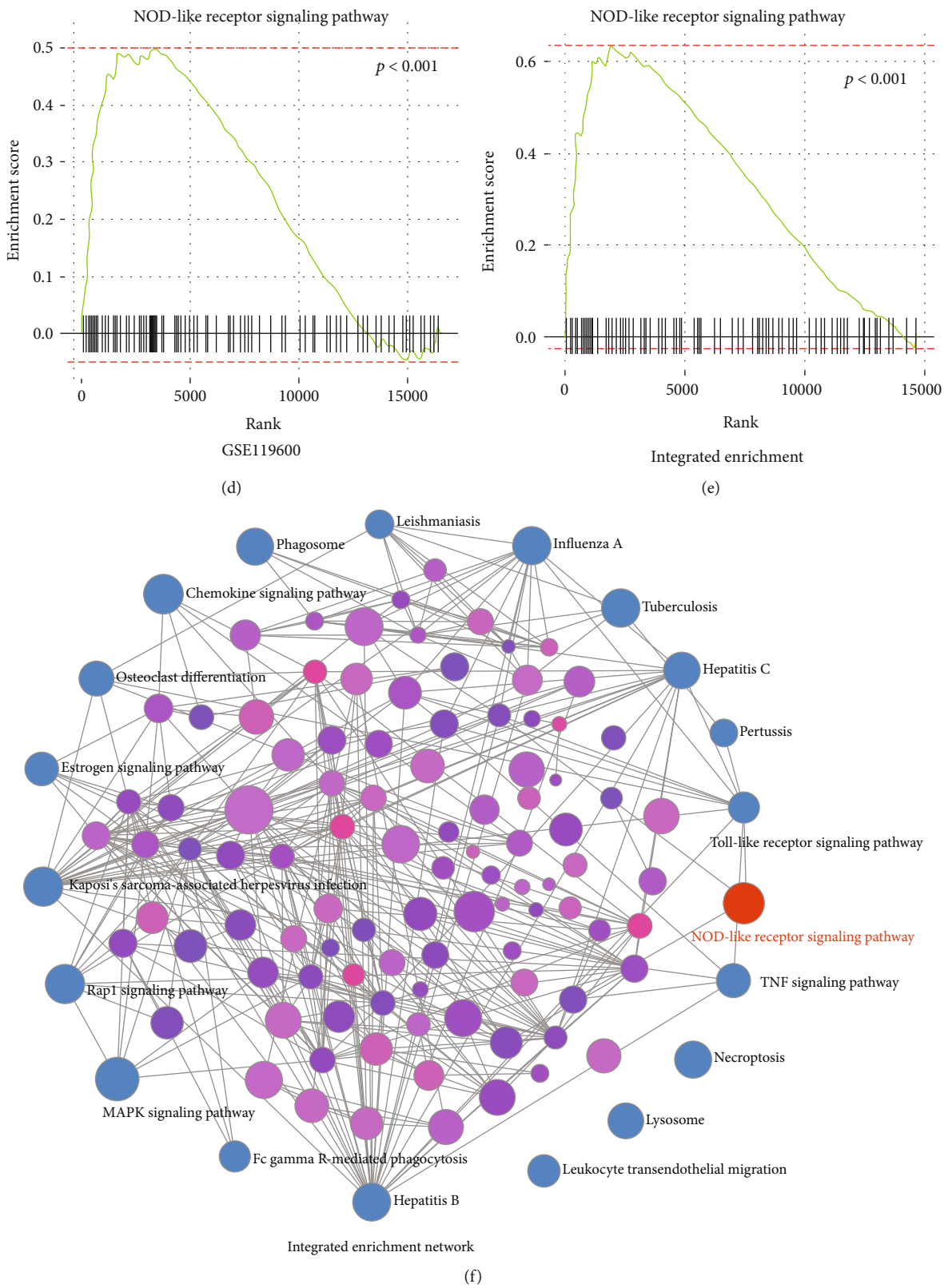


Figure 4: NOD-like receptor signaling pathway is enriched in the blood of CD by GSE analysis. (a) GSEA-generated heatmaps of core enrichment genes in the NOD-like receptor signaling pathway upregulated in CD patients of three datasets. (b–d) Enrichment maps were used for the visualization of NOD-like receptor signaling pathway enrichment results separately. (e) Integrated enrichment network of the three microarray datasets was generated by NetworkAnalyst. (f) Enrichment map visualizes NOD-like receptor signaling pathway enrichment results from integrated data.

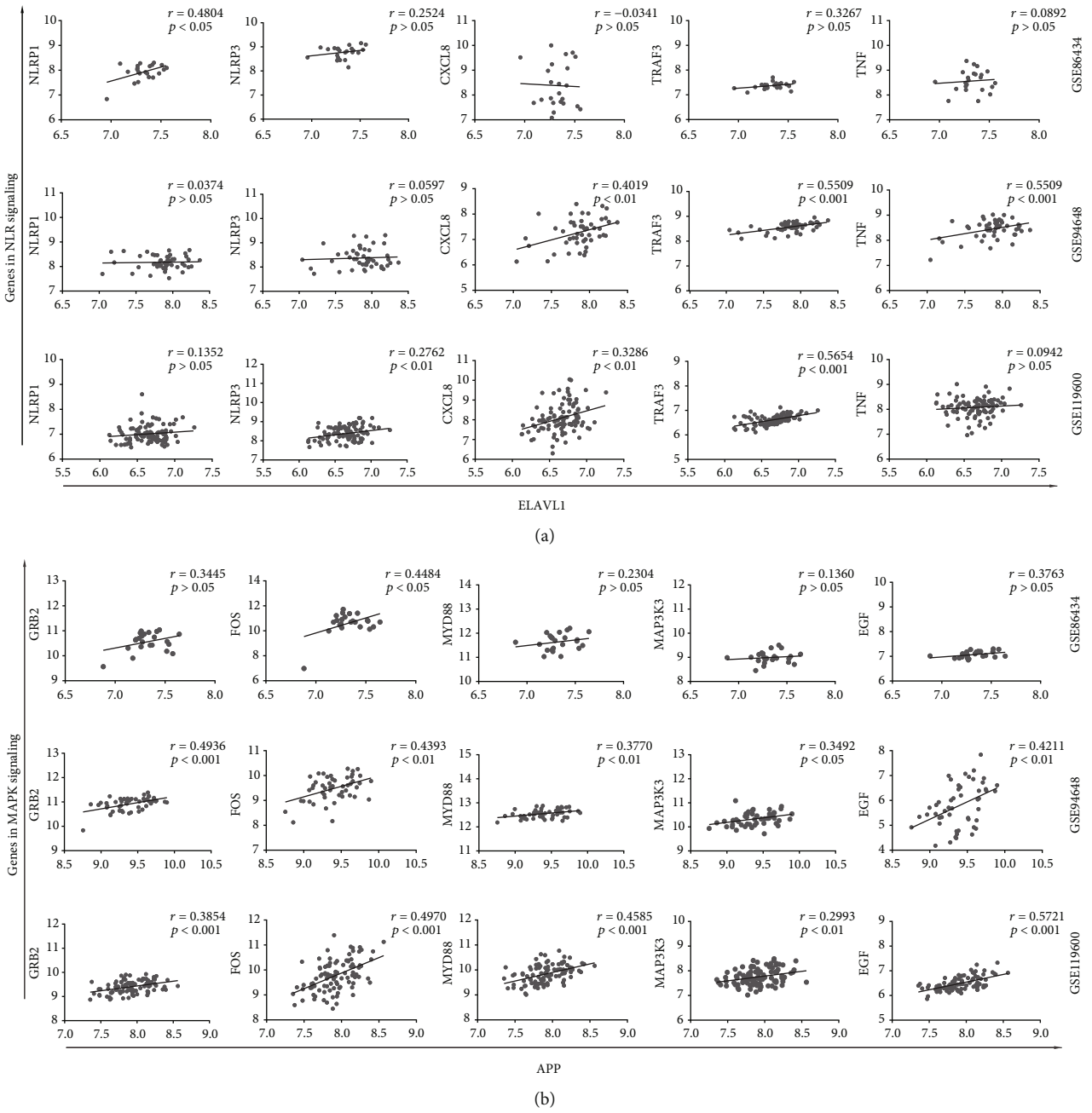


Figure 5: The transcriptome association between candidate crucial genes and top enriched signaling pathways in the peripheral blood of CD patients. The gene expression correlation between ELAVL1 and selected genes in NLR signaling (a) and correlation between APP and selected genes in MAPK signaling (b) in CD patients analysed using the expression data from the three datasets were represented by scatterplots with regression lines. Pearson correlation coefficients (r) and p values were calculated and shown.

integrative analysis also highlights the pathway of the T cell receptor signaling pathway and the differentiation of Th1, Th2, and Th17, which act essential roles in the pathogenesis of CD [38, 47, 48]. It is reasonable for us to assume that the NLR signaling pathway may work as a key regulator of CD pathology by activating T cells. Moreover, the highest ranking of the integrated dataset analysed by GSEA is the MAPK signaling pathway, followed by the Rap1 signaling pathway. As known, Rap1 plays an essential role in regulating the activation of MAPK [49], which is definitely involved in the development of CD [50] and is an effective target for the treatment of CD [51]. Therefore, it will be interesting to focus linkage between APP and MAPK and investigation of their role in the progression of CD seems to be promising.

The adaptive immune system is considered the key regulator of the pathogenesis of CD [3]. When the intestinal barrier is broken, pattern recognition receptors, such as TLR [52] and NLR [53], recognize the microbe-associated molecular patterns, thus promoting the activation and differentiation of T cells [54]. Especially, $CD4^+$ T cells are activated and differentiated into Th1/Th17, help the development of memory T cells, and then recruited to the gut to fight against

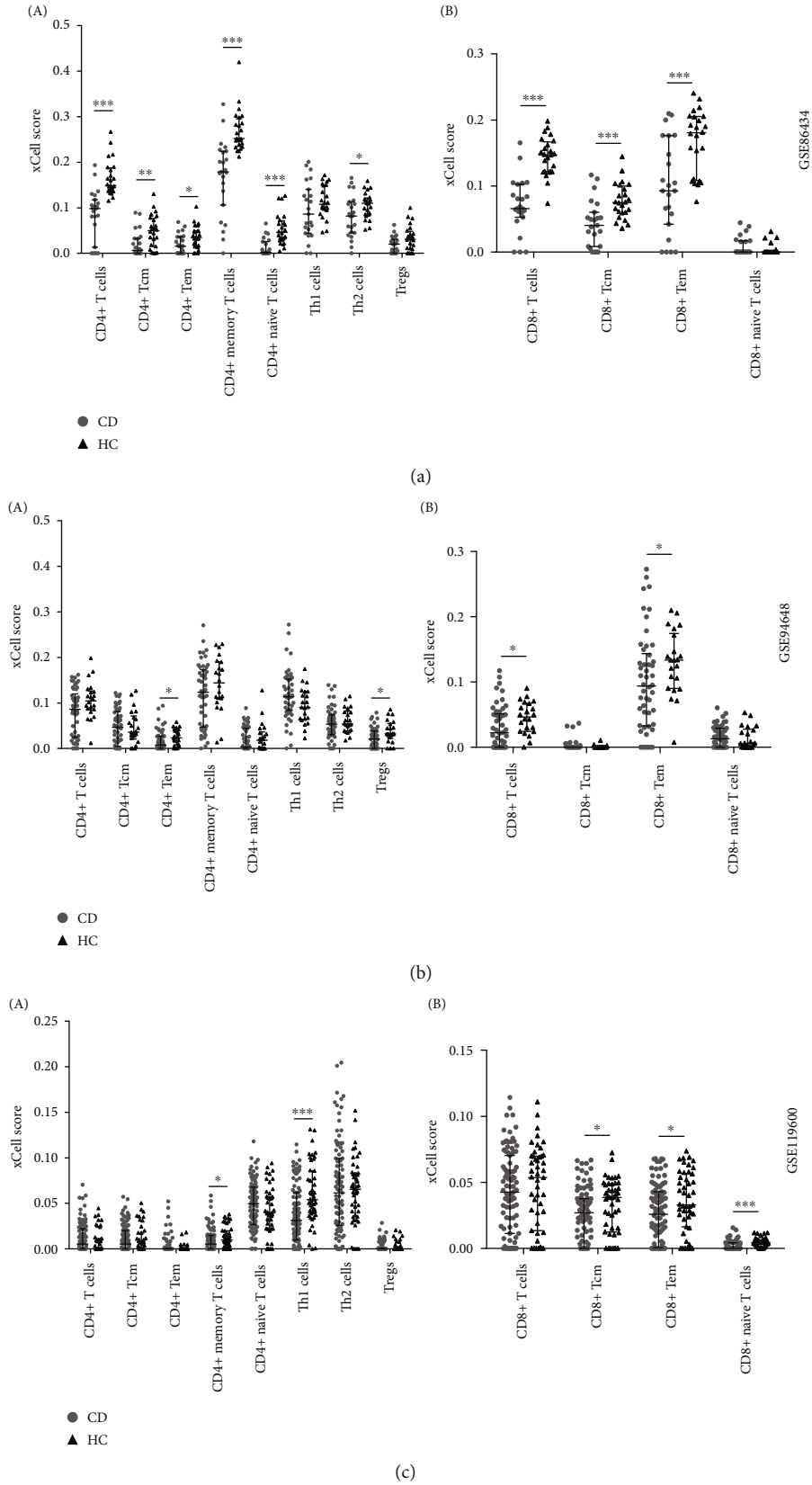


Figure 6: T cell proportions are significantly altered in the peripheral blood of CD comparing to health control. The scatterplots show the xCell scores for $CD4^+$ (A) and $CD8^+$ (B) T cells of CD and HC from GSE86434 (a), GSE94648 (b) and GSE119600 (c). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

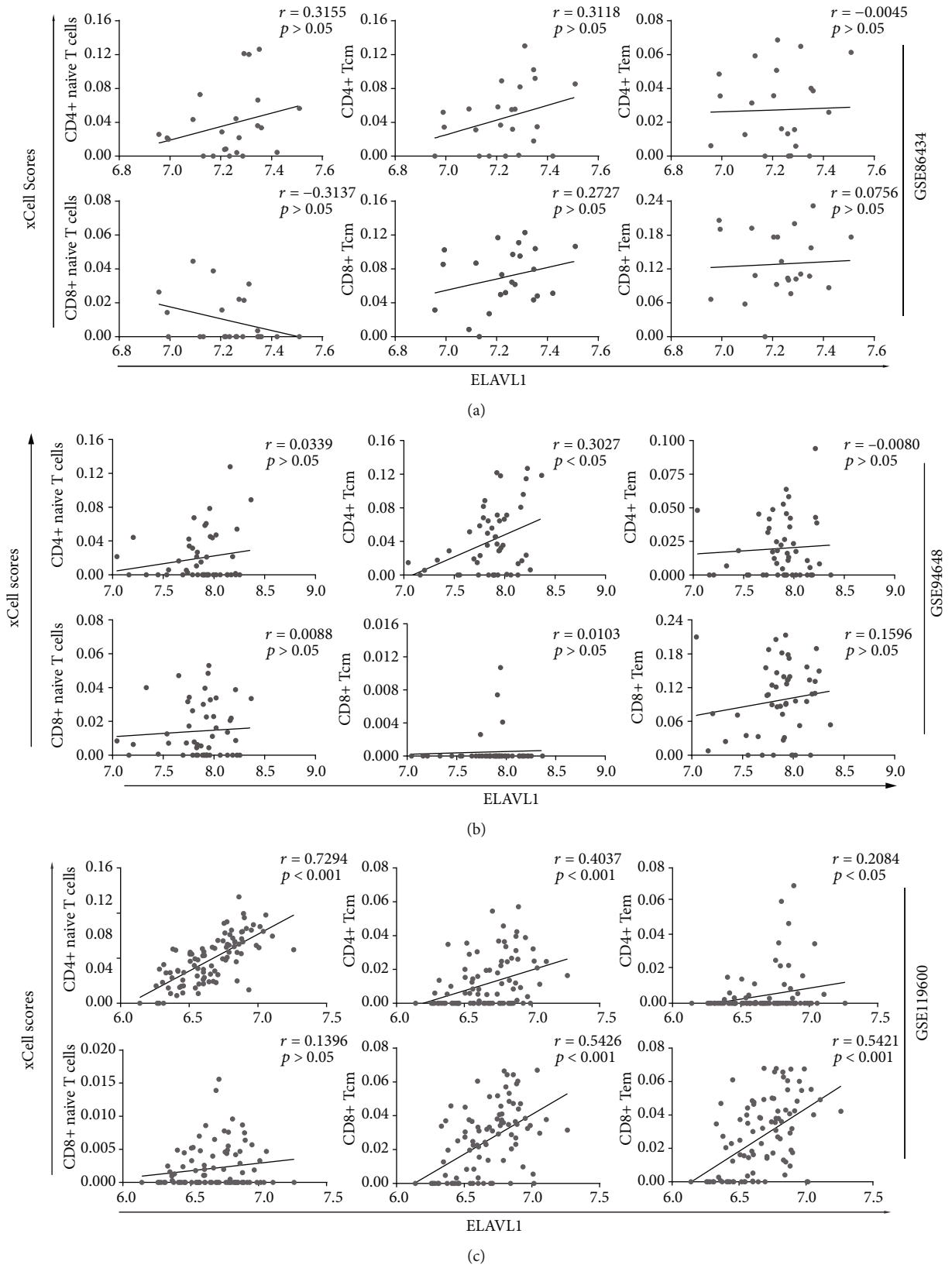


Figure 7: ELAVL1 expression correlates with multiple T cell subset activation in the peripheral blood of CD patients. The association between ELAVL1 and the xCell scores of selected T cell subsets of CD patients were analysed using the expression data from GSE86434 (a), GSE94648 (b), and GSE119600 (c). Scatterplots were represented with regression lines. The Spearman correlation coefficients (r) and p values were calculated and shown.

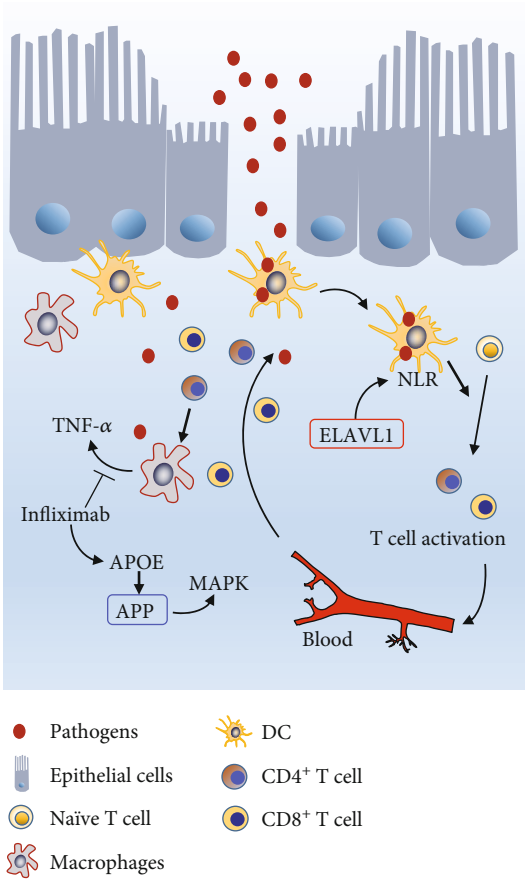


Figure 8: The schematic diagram illustrating ELAVL1/NLR, APP/MAPK signaling, and T cell migration and/or exhaustion in CD pathogenesis.

the infection of bacteria, fungi, and viruses [55]. However, continuous T cell activation will aggravate inflammatory damage [2] and will even lead to the exhaustion of T cells [56–58]. Of note, the exhaustion of T cells regularly impedes the ability to defeat viral infection [59] and promote the occurrence of cancer [60]. Interestingly, the data of xCell indicated that $CD4^+$ and $CD8^+$ T cell proportions are obviously reduced in the blood, which may be caused by the increased migration of T cells into the gut and/or the exhaustion of T cells. Consistent with FACS data from other literatures [61–63], our xCell analysis highlight the decrease of $CD8^+$ Tem as a general signature in the blood of CD, which is conventionally considered a cytotoxic cell type to defect the infection of virus. Although little is known about the detail mechanism, these phenomena suggest that the decrease of T cells, especially the $CD8^+$ Tem, in the peripheral blood may be a novel feature for certain CD patients.

As known, ELAVL1/HuR acts as a central posttranscriptional regulator of NOD2 expression, and HuR silencing can reduce NOD2 expression and mRNA stability [64]. In addition, HuR stimulated by integrin engagement and the level of HuR nuclear export are definitely involved in the activation of T cells [65]. Interestingly, we found that ELAVL1 expression showed strong positive correlations with multiple T cell subset proportions in GSE119600. The association supports the idea that ELAVL1 can modulate the immune response by activating the T cells. However, we cannot demonstrate the regulatory roles of ELAVL1 on T cell activation in the current transcriptome analysis; further investigation in an animal model will be meaningful and straightforward.

In conclusion, our study suggests that ELAVL1 and APP are candidate crucial genes in the blood of CD and highlights the function of the NLR signaling pathway in priming the activation of T cells of CD. ELAVL1 may modulate the immune response of CD via the NLR signaling pathway and in turn regulate T cells status. APP could be a downstream effector of infliximab treatment connecting with MAPK signaling (a schematic diagram representing a possible mechanism is shown in Figure 8). Our analysis will be helpful for further investigation and understanding of the mechanism of ELAVL1 and APP in CD pathogenesis.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Heli Li and Xiong Cai designed the study, analysed the data, and wrote the manuscript. Qianru Li and Shiran Sun analysed the data. Xiong Cai, Ping Lei, and Guanxin Shen provided funding.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (Nos. 81302130 and 81871307). Additional support was provided by Health Commission of Hubei Province (WJ2019M171).

References

[1] N. S. Seyed Tabib, M. Madgwick, P. Sudhakar, B. Verstockt, T. Korcsmaros, and S. Vermeire, "Big data in IBD: big progress for clinical practice," *Gut*, p. gutjnl-2019-320065, 2020.

[2] R. Boyapati, J. Satsangi, and G.-T. Ho, "Pathogenesis of Crohn's disease," *F1000prime reports*, vol. 7, p. 44, 2015.

[3] V. A. Petruo, E. Krauss, A. Kleist et al., "Perceived distress, personality characteristics, coping strategies and psychosocial impairments in a national German multicenter cohort of patients with Crohn's disease and ulcerative colitis," *Zeitschrift für Gastroenterologie*, vol. 57, no. 4, pp. 473–483, 2019.

[4] J. Z. Liu, International Multiple Sclerosis Genetics Consortium, S. van Sommeren et al., "Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations," *Nature Genetics*, vol. 47, no. 9, pp. 979–986, 2015.

[5] K. K.-H. Farh, A. Marson, J. Zhu et al., "Genetic and epigenetic fine mapping of causal autoimmune disease variants," *Nature*, vol. 518, no. 7539, pp. 337–343, 2015.

[6] J. C. Lee, P. A. Lyons, E. F. McKinney et al., "Gene expression profiling of CD8$^+$ T cells predicts prognosis in patients with Crohn disease and ulcerative colitis," *The Journal of Clinical Investigation*, vol. 121, no. 10, pp. 4170–4179, 2011.

[7] D. Biasci, J. C. Lee, N. M. Noor et al., "A blood-based prognostic biomarker in IBD," *Gut*, vol. 68, no. 8, pp. 1386–1395, 2019.

[8] N. T. Ventham et al., "Integrative epigenome-wide analysis demonstrates that DNA methylation may mediate genetic risk in inflammatory bowel disease," *Nature Communications*, vol. 7, no. 1, p. 13507, 2016.

[9] W. T. Uniken Venema, M. D. Voskuil, A. V. Vila et al., "Single-cell RNA sequencing of blood and ileal T cells from patients with Crohn's disease reveals tissue-specific characteristics and drug targets," *Gastroenterology*, vol. 156, no. 3, pp. 812–815.e22, 2019.

[10] G. Zhou, O. Soufan, J. Ewald, R. E. W. Hancock, N. Basu, and J. Xia, "NetworkAnalyst 3.0: a visual analytics platform for comprehensive gene expression profiling and meta-analysis," *Nucleic acids research*, vol. 47, no. W1, pp. W234–W241, 2019.

[11] J. A. Santiago and J. A. Potashkin, "Network-based metaanalysis identifies HNF4A and PTBP1 as longitudinally dynamic biomarkers for Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 7, pp. 2257–2262, 2015.

[12] M. K. Pujar, B. Vastrad, and C. Vastrad, "Integrative analyses of genes associated with subcutaneous insulin resistance," *Biomolecules*, vol. 9, no. 2, p. 37, 2019.

[13] P. Chakrabarty, C. Ceballos-Diaz, A. Beccard et al., "IFN-γ promotes complement expression and attenuates amyloid plaque deposition in amyloid β precursor protein transgenic mice," *The Journal of Immunology*, vol. 184, no. 9, pp. 5333–5343, 2010.

[14] E. Christodoulou-Vafeiadou, F. Ioakeimidis, M. Andreadou et al., "Divergent innate and epithelial functions of the RNA-binding protein HuR in intestinal inflammation," *Frontiers in Immunology*, vol. 9, p. 2732, 2018.

[15] C. Stellato, M. M. Gubin, J. D. Magee et al., "Coordinate regulation of *GATA-3* and Th2 cytokine gene expression by the RNA-binding protein HuR," *The Journal of Immunology*, vol. 187, no. 1, pp. 441–449, 2011.

[16] J. A. Miller, S. Horvath, and D. H. Geschwind, "Divergence of human and mouse brain transcriptome highlights Alzheimer disease pathways," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 28, pp. 12698–12703, 2010.

[17] J. Xia, E. E. Gill, and R. E. W. Hancock, "NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data," *Nature Protocols*, vol. 10, no. 6, pp. 823–844, 2015.

[18] D. Aran, Z. Hu, and A. J. Butte, "xCell: digitally portraying the tissue cellular heterogeneity landscape," *Genome biology*, vol. 18, no. 1, p. 220, 2017.

[19] N. Planell, M. C. Masamunt, R. F. Leal et al., "Usefulness of transcriptional blood biomarkers as a non-invasive surrogate marker of mucosal healing and endoscopic response in ulcerative colitis," *Journal of Crohn's & Colitis*, vol. 11, no. 11, pp. 1335–1346, 2017.

[20] J. Ostrowski, The Polish PBC study Group, K. Goryca et al., "Common functional alterations identified in blood transcriptome of autoimmune cholestatic liver and inflammatory bowel diseases," *Scientific reports*, vol. 9, no. 1, p. 7190, 2019.

[21] J. Ostrowski, M. Dabrowska, I. Lazowska et al., "Redefining the practical utility of blood transcriptome biomarkers in inflammatory bowel diseases," *Journal of Crohn's & Colitis*, vol. 13, no. 5, pp. 626–633, 2019.

[22] R. Liu, K. Wu, Y. Li, R. Sun, and X. Li, "Human antigen R: a potential therapeutic target for liver diseases," *Pharmacological Research*, vol. 155, p. 104684, 2020.

[23] V. Katsanou, O. Papadaki, S. Milatos et al., "HuR as a negative posttranscriptional modulator in inflammation," *Molecular Cell*, vol. 19, no. 6, pp. 777–789, 2005.

[24] S. C. Jeyaraj, M. Singh, D. A. Ayupova, S. Govindaraju, and B. S. Lee, "Transcriptional control of human antigen R by bone morphogenetic protein," *The Journal of Biological Chemistry*, vol. 285, no. 7, pp. 4432–4440, 2010.

[25] S. Srikantan, "HuR function in disease," *Frontiers in bioscience*, vol. 17, no. 1, p. 189, 2012.

[26] D. D. Weedon, R. G. Shorter, D. M. Ilstrup, K. A. Huizenga, and W. F. Taylor, "Crohn's disease and cancer," *The New England Journal of Medicine*, vol. 289, no. 21, pp. 1099–1103, 1973.

[27] S. N. Gyde, P. Prior, J. C. Macartney, H. Thompson, J. A. Waterhouse, and R. N. Allan, "Malignancy in Crohn's disease," *Gut*, vol. 21, no. 12, pp. 1024–1029, 1980.

[28] I. L. de Silanes, A. Lal, and M. Gorospe, "HuR: post-transcriptional paths to malignancy," *RNA Biology*, vol. 2, no. 1, pp. 11–13, 2014.

[29] W. Peng, N. Furuuchi, L. Aslanukova et al., "Elevated HuR in pancreas promotes a pancreatitis-like inflammatory microenvironment that facilitates tumor development," *Molecular and cellular biology*, vol. 38, no. 3, 2018.

[30] J. M. García-Cárdenas, S. Guerrero, A. López-Cortés et al., "Post-transcriptional regulation of colorectal cancer: a focus on RNA-binding proteins," *Frontiers in Molecular Biosciences*, vol. 6, p. 65, 2019.

[31] W. Kakuguchi, T. Nomura, T. Kitamura et al., "Suramin, screened from an approved drug library, inhibits HuR functions and attenuates malignant phenotype of oral cancer cells," *Cancer Medicine*, vol. 7, no. 12, pp. 6269–6280, 2018.

[32] S. K. Lee, K.-K. Park, H.-J. Kim et al., "Human antigen R-regulated CCL20 contributes to osteolytic breast cancer bone metastasis," *Scientific reports*, vol. 7, no. 1, 2017.

[33] V. Lafarga, A. Cuadrado, I. Lopez de Silanes, R. Bengoechea, O. Fernandez-Capetillo, and A. R. Nebreda, "p38 mitogen-activated protein kinase- and HuR-dependent stabilization of p21^{Cip1} mRNA mediates the G_1/S checkpoint," *Molecular and Cellular Biology*, vol. 29, no. 16, pp. 4341–4351, 2009.

[34] M.-J. Kang, B.-K. Ryu, M.-G. Lee et al., "NF-κB Activates Transcription of the RNA-Binding Factor HuR, via PI3K-AKT Signaling, to Promote Gastric Tumorigenesis," *Gastroenterology*, vol. 135, no. 6, pp. 2030–2042.e3, 2008.

[35] A. Nakamura, N. Kaneko, V. L. Villemagne et al., "High performance plasma amyloid-β biomarkers for Alzheimer's disease," *Nature*, vol. 554, no. 7691, pp. 249–254, 2018.

[36] J. S. Yokoyama, Y. Wang, A. J. Schork et al., "Association between genetic traits for immune-mediated diseases and Alzheimer disease," *JAMA Neurology*, vol. 73, no. 6, pp. 691–697, 2016.

[37] S. Jevtic, A. S. Sengar, M. W. Salter, and J. McLaurin, "The role of the immune system in Alzheimer disease: etiology and treatment," *Ageing Research Reviews*, vol. 40, pp. 84–94, 2017.

[38] M. F. Neurath, "Targeting immune cell circuits and trafficking in inflammatory bowel disease," *Nature Immunology*, vol. 20, no. 8, pp. 970–979, 2019.

[39] M. Gazouli, A. K. Anagnostopoulos, A. Papadopoulou et al., "Serum protein profile of Crohn's disease treated with infliximab," *Journal of Crohn's & Colitis*, vol. 7, no. 10, pp. e461–e470, 2013.

[40] Y.-W. A. Huang, B. Zhou, M. Wernig, and T. C. Südhof, "ApoE2, ApoE3, and ApoE4 differentially stimulate APP transcription and Aβ secretion," *Cell*, vol. 168, no. 3, pp. 427–441.e21, 2017.

[41] J. Xu, Y. Ying, G. Xiong, L. Lai, Q. Wang, and Y. Yang, "Amyloid β precursor protein silencing attenuates epithelial-mesenchymal transition of nasopharyngeal carcinoma cells via inhibition of the MAPK pathway," *Molecular Medicine Reports*, vol. 20, no. 1, pp. 409–416, 2019.

[42] X. Wu, S. Chen, and C. Lu, "Amyloid precursor protein promotes the migration and invasion of breast cancer cells by regulating the MAPK signaling pathway," *International Journal of Molecular Medicine*, vol. 45, no. 1, pp. 162–174, 2020.

[43] A. M. Keestra-Gounder, M. X. Byndloss, N. Seyffert et al., "NOD1 and NOD2 signalling links ER stress with inflammation," *Nature*, vol. 532, no. 7599, pp. 394–397, 2016.

[44] R. Caruso, N. Warner, N. Inohara, and G. Núñez, "NOD1 and NOD2: signaling, host defense, and inflammatory disease," *Immunity*, vol. 41, no. 6, pp. 898–908, 2014.

[45] J. H. Fritz, L. L. Bourhis, G. Sellge et al., "Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity," *Immunity*, vol. 26, no. 4, pp. 445–459, 2007.

[46] D. Corridoni, S. Shiraishi, T. Chapman et al., "NOD2 and TLR2 signal via TBK1 and PI31 to direct cross-presentation and CD8 T cell responses," *Frontiers in Immunology*, vol. 10, p. 958, 2019.

[47] X. R. Xu, C. Q. Liu, B. S. Feng, and Z. J. Liu, "Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 20, no. 12, pp. 3255–3264, 2014.

[48] A. Kaistha and J. Levine, "Inflammatory bowel disease: the classic gastrointestinal autoimmune disease," *Current Problems in Pediatric and Adolescent Health Care*, vol. 44, no. 11, pp. 328–334, 2014.

[49] T. Nagai, S. Nakamuta, K. Kuroda et al., "Phosphoproteomics of the dopamine pathway enables discovery of Rap1 activation as a reward signal in vivo," *Neuron*, vol. 89, no. 3, pp. 550–565, 2016.

[50] D. Hommes, B. van den Blink, T. Plasse et al., "Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease," *Gastroenterology*, vol. 122, no. 1, pp. 7–14, 2002.

[51] B. Van Den Blink, T. Ten Hove, G. R. Van Den Brink, M. P. Peppelenbosch, and S. J. Van Deventer, "From extracellular to intracellular targets, inhibiting MAP kinases in treatment of Crohn's disease," *Annals of the New York Academy of Sciences*, vol. 973, no. 1, pp. 349–358, 2002.

[52] T. Watanabe, A. Kitani, P. J. Murray, Y. Wakatsuki, I. J. Fuss, and W. Strober, "Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis," *Immunity*, vol. 25, no. 3, pp. 473–485, 2006.

[53] W. Strober, N. Asano, I. Fuss, A. Kitani, and T. Watanabe, "Cellular and molecular mechanisms underlying NOD2 risk-associated polymorphisms in Crohn's disease," *Immunological Reviews*, vol. 260, no. 1, pp. 249–260, 2014.

[54] A. Geremia, P. Biancheri, P. Allan, G. R. Corazza, and A. Di Sabatino, "Innate and adaptive immunity in inflammatory bowel disease," *Autoimmunity Reviews*, vol. 13, no. 1, pp. 3–10, 2014.

[55] A. N. Hegazy, N. R. West, M. J. T. Stubbington et al., "Circulating and tissue-resident $CD4^+$ T cells with reactivity to intestinal microbiota are abundant in healthy individuals and function is altered during inflammation," *Gastroenterology*, vol. 153, no. 5, pp. 1320–1337.e16, 2017.

[56] D. E. Speiser, D. T. Utzschneider, S. G. Oberle, C. Münz, P. Romero, and D. Zehn, "T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion," *Nature Reviews. Immunology*, vol. 14, no. 11, pp. 768–774, 2014.

[57] M. Kurachi, "$CD8^+$ T cell exhaustion," *Seminars in Immunopathology*, vol. 41, no. 3, pp. 327–337, 2019.

[58] J. M. Angelosanto, S. D. Blackburn, A. Crawford, and E. J. Wherry, "Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection," *Journal of Virology*, vol. 86, no. 15, pp. 8161–8170, 2012.

[59] E. F. McKinney, J. C. Lee, D. R. W. Jayne, P. A. Lyons, and K. G. C. Smith, "T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection," *Nature*, vol. 523, no. 7562, pp. 612–616, 2015.

[60] A. I. Yu, L. Zhao, K. A. Eaton et al., "Gut microbiota modulate CD8 T cell responses to influence colitis-associated tumorigenesis," *Cell reports*, vol. 31, no. 1, p. 107471, 2020.

[61] B. Roosenboom, P. J. Wahab, C. Smids et al., "Intestinal $CD103^+CD4^+$ and $CD103^+CD8^+$ T-cell subsets in the gut of inflammatory bowel disease patients at diagnosis and during follow-up," *Inflammatory Bowel Diseases*, vol. 25, no. 9, pp. 1497–1509, 2019.

[62] S. Fernandes, N. Srivastava, R. Sudan et al., "SHIP1 deficiency in inflammatory bowel disease is associated with severe Crohn's disease and peripheral T cell reduction," *Frontiers in Immunology*, vol. 9, p. 1100, 2018.

[63] F. Ma, Y. Zhang, J. Xing et al., "Fecal bacteria from treatment-naive Crohn's disease patients can skew helper T cell responses," *Experimental Cell Research*, vol. 361, no. 1, pp. 135–140, 2017.

[64] J. Shang, Q. Wan, X. Wang et al., "Identification of NOD2 as a novel target of RNA-binding protein HuR: evidence from NADPH oxidase-mediated HuR signaling in diabetic nephropathy," *Free Radical Biology & Medicine*, vol. 79, pp. 217–227, 2015.

[65] J. G. Wang, M. Collinge, V. Ramgolam et al., "LFA-1-dependent HuR nuclear export and cytokine mRNA stabilization in T cell activation," *The Journal of Immunology*, vol. 176, no. 4, pp. 2105–2113, 2006.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 7523914, 7 pages
https://doi.org/10.1155/2020/7523914

Research Article

The Increased Ratio of Blood CD56^{bright} NK to CD56^{dim} NK Is a Distinguishing Feature of Primary Sjögren's Syndrome

Bingxia Ming,[1] **Tong Wu**,[1] **Shaozhe Cai**,[1] **Peng Hu**,[1] **Jungen Tang**,[1] **Fang Zheng**,[2,3] **Cong Ye**,[1] **and Lingli Dong**[1]

[1]*Department of Rheumatology and Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China*
[2]*Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*
[3]*Key Laboratory of Organ Transplantation, Ministry of Education; NHC Key Laboratory of Organ Transplantation; Key Laboratory of Organ Transplantation, Chinese Academy of Medical Sciences, Wuhan, Hubei, China*

Correspondence should be addressed to Cong Ye; yecong2011@163.com and Lingli Dong; tjhdongll@163.com

Received 20 April 2020; Accepted 13 June 2020; Published 9 July 2020

Academic Editor: Lihua Duan

Objective. The aim of this study was to characterize the subsets of circulating CD56^{+} NK cells in pSS patients and their potential value in the diagnosis and/or prediction of prognosis in patients with pSS. *Methods*. We included 52 pSS patients fulfilling the 2002 AECG criteria or 2012 ACR criteria and 20 age- and gender-matched healthy volunteers. The frequency and absolute number of NK cells and CD56 NK cell subsets in peripheral blood samples were detected by flow cytometry. Other laboratory parameters such as the IgG level and complement protein levels were extracted from the clinical system. *Results*. Both the frequency and the absolute number of peripheral blood NK cells were reduced in pSS patients compared to healthy controls. The proportion of CD56^{bright} NK cell subset was increased, and the proportion of CD56^{dim} NK cell subset was decreased among NK cells, resulting in the ratio of CD56^{bright} NK to CD56^{dim} NK which was significantly elevated in pSS patients. ROC analysis indicated that the AUC of CD56^{bright} NK/CD56^{dim} NK ratio was 0.838, and the best diagnostic cut-off point was 0.0487 for pSS patients. Furthermore, this CD56^{bright} NK/CD56^{dim} NK ratio was positively correlated with the IgG level and negatively correlated with the complement protein C3 and C4 levels. More importantly, the CD56^{bright}/CD56^{dim} NK ratio was either slightly increased or not changed in other autoimmune diseases such as SLE and IgG4-related disease. *Conclusion*. Our findings suggest that the ratio of blood CD56^{bright} NK to CD56^{dim} NK might have a diagnostic value relatively specific for pSS.

1. Introduction

Primary Sjögren's syndrome (pSS) is a slowly progressed autoimmune disorder characterized by lymphocytic infiltration of exocrine glands and subsequent significant loss of secretory function with oral or eye dryness [1–3]. The diagnosis of pSS is based on the focal infiltration of mononuclear cells (mainly T and B cells) in glands and the presence of serum autoantibodies and hyperglobulinemia [4–8]. The above characteristics emphasize the role of abnormal adaptive immune responses in the pathogenesis of pSS. However, few studies have explored the role of innate immune indicators in the identification of pSS patients.

Natural killer (NK) cells are innate lymphoid cells that exhibit the capacity to secrete cytokines and possess natural cytotoxicity [9]. Although animal models of pSS have not directly implicated NK cells in disease pathogenesis, recent work implicates a regulatory role of NK cells in exocrine gland tissues and peripheral blood. For example, NK cells expressing NKp30 were proposed to interact with epithelial cells and subsequently mediate the enhancement of the inflammatory state in the salivary gland through secretion of interferon-γ (IFN-γ) [3]. Another study found that activated NK/NKT cells from pSS patients stimulated by interleukin-33 (IL-33) and IL-12/IL-23 could produce IFN-γ thereby perpetuating cellular damage [10]. In addition,

increasing evidence has shown that NK cells play a critical role in both type I and type II IFN biologic functions resulting from their interaction with various dendritic cell (DC) subsets in pSS progression [11–14]. Taken together, these data suggest that NK cells play an important role in the pathogenesis of pSS.

NK cells are characterized conventionally by the expression of the CD56 surface marker [15, 16]. Based on the expression of CD56, human NK cells are divided into $CD56^{bright}$ and $CD56^{dim}$ subsets [17]. It is commonly recognized that $CD56^{bright}$ NK cells account for about 10% of human peripheral blood NK cells mainly producing various cytokines and chemokines, whereas $CD56^{dim}$ NK cells account for about 90% of human peripheral blood NK cells with higher cytotoxic [9, 18, 19]. $CD56^{bright}$ and $CD56^{dim}$ NK cells are successive stages in the development of NK cells. The circulating $CD56^{bright}$ NK cells are generally considered to be the precursors of the $CD56^{dim}$ NK cells [20]. A recent study has found $CD56^{high}$ cells in the peripheral blood of newly diagnosed pSS patients were significantly reduced [21]. In contrast, another study published in 2013 showed that the proportion of circulating $CD56^{bright}$ NK cells relative to the total NK cells was increased among pSS patients compared to controls [3]. The role of $CD56^{bright}$ and $CD56^{dim}$ NK subpopulations and their clinical significance in pSS is poorly understood. We hypothesize that a shifted balance between the CD56 NK cell subsets may reflect the immune status of pSS.

In this study, we analyzed the characteristics of peripheral blood $CD56^{bright}$ NK cell subset and $CD56^{dim}$ NK cell subset in patients with pSS and found that the ratio of $CD56^{bright}$ NK to $CD56^{dim}$ NK was elevated, and the area under the curve (AUC) of this ratio was 0.838 by receiver operator characteristic (ROC) curve analysis. Furthermore, this ratio was positively associated with serum IgG level and negatively associated with complement C3 and C4 levels, though not associated with EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) in pSS patients.

2. Materials and Methods

2.1. Patients and Healthy Volunteers. Independent identification and newly diagnosed patients with pSS, SLE, and IgG4-related disease (IgG4-RD) as well as healthy controls (HC) were included in this study. Samples of patients were obtained from the clinic of the Department of Rheumatology and Immunology, Tongji Hospital from November 2017 to November 2018. Patients with pSS (52), SLE [7], and IgG4-RD [22] fulfilled their respective classification criteria. Disease activity was scored by measuring the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) in pSS. All subjects had neither a medical history of virus hepatitis, lymphoma, human immunodeficiency virus (HIV) infection, and diabetes nor a history of smoking, antiacetylcholine drugs, and radiation therapy for head and neck. Medical records were reviewed, and information regarding age, sex, and laboratory parameters was collected.

The approval of the ethical committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology was obtained before the study. The ethical institutional review board (IRB) ID is TJ-C20151109. All subjects have provided written informed consent to participate in the study.

2.2. Cell Isolation and Flow Cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples from all patients and controls by Ficoll-Paque (GE Healthcare, Amersham, UK) density gradient centrifugation. Fluorochrome-conjugated antibodies specific for the following cell surface molecules were used: anti-human-CD3 (HIT3a)-PE and anti-human-CD56 (HCD56)-FITC (antibody reagents were purchased from Biolegend, UK). PBMCs were incubated with fluorochrome-conjugated antibodies diluted in PBS for cell surface staining at 4°C. Cells were subsequently washed and resuspended in PBS prior to analysis. Flow cytometry was carried out by using a Calibur flow cytometer (BD Biosciences, San Diego, CA), and data were analyzed using FlowJo 7.6 software (Tree Star, Inc., Ashland, OR). The lymphocyte population was identified by assessment of the size and granularity of cells using forward scatter (FSC) and side scatter (SSC). NK cell percentage expressed as a proportion of total gated lymphocytes. Among NK cells, $CD56^{bright}$ and $CD56^{dim}$ subsets were identified according to the level of CD56 expression. Gates were set by using isotype control antibodies.

2.3. Statistical Methods. GraphPad Prism 5.0 software was used for all statistical analyses. The Mann-Whitney U test was used to compare the independent groups. The ROC curve was used to analyze the diagnostic value of the ratio of $CD56^{bright}$ NK to $CD56^{dim}$ NK. Spearman's rank correlation test was applied to analyze the relationship between the two factors. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Demographic Data of pSS Patients and Healthy Controls. The demographic characteristics and clinical features of pSS patients and healthy controls are described in Table 1. There was no significant difference in ages and sex between the groups.

3.2. Blood CD56 NK Cell Subset Levels in pSS Patients. In accordance with previous literature [3], human NK cells defined as $CD3^{-}CD56^{+}$ lymphocytes in this study could be separated into $CD56^{bright}$ and $CD56^{dim}$ subsets in all pSS patients and HC (Figure 1(a)). The proportion of peripheral blood NK cells in total lymphocytes was reduced in pSS patients compared to healthy controls (Figure 1(b)). Among NK cells, the proportion of the $CD56^{bright}$ NK cell subset was increased while the $CD56^{dim}$ NK cell subset was decreased in patients of pSS (Figure 1(b)). The absolute number of NK cells and CD56 NK cell subsets was decreased, and the $CD56^{dim}$ NK cell subset decreased more (Figure 1(c)). We have also detected the expression of CD107a and intracellular IFN-γ, which reflect the function of $CD56^{+}$ NK cell subsets in pSS patients. $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were presented with an increased percentage of $CD107a^{+}$ cells and decreased IFN-γ expression (supplementary figure 1). The

Table 1: Overview of individuals at baseline used in this study.

	pSS	HC
Characteristics		
Number	52	20
Age in years (mean ± SD)	47.7 ± 11.3	46.8 ± 12.0
Range	20-68	24-77
Female : male	49 : 3	19 : 1
Clinical features		
ANA+ (%)	44 (84.6)	0 (0)
Ro/SSA+ (%)	44 (84.6)	NA
La/SSB+ (%)	23 (44.2)	NA
ESR, high levels (>20 mm/h) (%)	25 (48.1)	NA
CRP, high levels (>5 mg/l) (%)	7 (13.5)	NA
IgG, elevated levels (>16 g/l) (%)	26 (50)	NA
Focus score ≥ 1 (%)	42 (87.5)[a]	NA
ESSDAI range (median)	0-15 (4)	NA

Categorical data are expressed as the absolute frequency, with percentages in parenthesis. ANA: antinuclear antibodies; Ro/SSA+: antibodies against Ro/Sjögren's syndrome A antigen; La/SSB+: antibodies against La/Sjögren's syndrome B antigen; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; focus score indicates the number of inflammatory foci containing more than 50 mononuclear cells per 4 mm^2 biopsy tissue. ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; NA: not applicable. [a]$n = 48$.

above results imply that the imbalance of CD56 NK cell subsets existed in pSS patients.

3.3. The CD56^{bright} NK to CD56^{dim} NK Ratio in the Identification of pSS Patients. We further calculated the ratio of CD56^{bright} NK cell subset to CD56^{dim} NK cell subset as described above and found that the ratio of CD56^{bright}NK to CD56^{dim}NK was significantly elevated in pSS patients than that in healthy controls ($p < 0.0001$, Figure 1(d)). Furthermore, the ratio of CD56^{bright} NK to CD56^{dim} NK was also analyzed in other autoimmune diseases, such as SLE and IgG4-RD. The ratio of CD56^{bright} NK to CD56^{dim} NK in peripheral blood of SLE patients was slightly increased ($p = 0.041$) and not different from those in healthy controls in patients with IgG4-RD (Figure 1(d)).

3.4. Sensitivity and Specificity of CD56^{bright} NK to CD56^{dim} NK Ratio in pSS Patients. ROC curve analysis indicated that the area under the curve (AUC) was 0.838, and the best diagnostic cut-off point of the ratio of CD56^{bright} NK to CD56^{dim} NK was 0.0487 for the patients with pSS (Figure 2). Using 0.0487 as the threshold value of the ratio, the sensitivity, specificity, and Youden index were 84.6%, 75%, and 0.596, respectively.

3.5. The Correlation of CD56^{bright} NK to CD56^{dim} NK Ratio with Disease Activity in pSS Patients. We next analyzed the correlation of CD56^{bright} NK to CD56^{dim} NK ratio with disease activity in pSS patients. Spearman rank correlation did not identify a significant correlation of the ratio with the ESSDAI (supplementary figure 2). However, there was a significant correlation between the ratio and immunological features often associated with pSS, including IgG and complement C3 and C4 levels (Figures 3(a)–3(c)). These data suggest that the CD56^{bright} NK to CD56^{dim} NK ratio might be suitable for reflecting the immune status in pSS patients.

3.6. The Correlation of CD56^{bright} NK to CD56^{dim} NK Ratio with the Therapeutic Effect of pSS Patients. We have compared the CD56^{bright} NK, CD56^{dim} NK, and CD56^{bright} NK to CD56^{dim} NK ratio between before and after treatment in pSS patients. The drugs of treatment are mainly glucocorticoid and hydroxychloroquine. As shown in supplementary figure 1, ESSDAI and serum IgG level were decreased after treatment (supplementary figure 3D-E). The ratio and CD56^{bright} NK cell count were unchanged between before and after treatment, though the decreased CD56^{dim} NK cell count was upregulated after treatment in pSS patients (supplementary figure 3A-C).

4. Discussion

Increasing experimental evidence has shown a direct involvement of NK cells and CD56 NK cell subsets in some human immunopathologies such as psoriatic arthritis, SLE, multiple sclerosis, and Behcet's disease [19, 23–25]. However, as innate lymphoid cells, the role and clinical significance of CD56 NK cell subsets in pSS patients are poorly understood. In the present study, we compared the change of circulating CD56 NK cell subsets, firstly evaluated the value of CD56^{bright} NK to CD56^{dim} NK ratio in the diagnosis of pSS, and analyzed its association with clinical parameters.

As expected, we observed that the CD56^{bright} NK to CD56^{dim} NK ratio had the potential to diagnose pSS in autoimmune diseases. Using a cut-off value of 0.0487, we found that the sensitivity and specificity of the ratio were 84.6% and 75%, respectively. In order to further determine the significance of the CD56^{bright} NK to CD56^{dim} NK ratio in the identification of pSS patients, the changes of this ratio in other autoimmune diseases were analyzed. Accordingly, we found that the CD56^{bright} NK to CD56^{dim} NK ratio was also increased in SLE patients and unchanged in IgG4-RD. These distinct changes of the ratio in different autoimmune diseases may be attributed to their different pathological mechanisms. It has been reported that much similar pathogenesis have existed between patients with pSS and SLE [2, 22, 26], including the type I and type II IFN system in the innate immune phase, and autoantibodies such as anti-SSA/SSB and antinuclear antibody consistently appeared in both diseases, but not for IgG4-RD.

Due to the limited supply of fresh exocrine gland tissues, this study focused on the circulating NK cells in the peripheral blood of pSS patients. Both CD56^{bright} NK cells and CD56^{dim} NK cells were found to be depleted within the peripheral blood compartment of pSS patients compared to healthy controls, while the latter decreased more. We also found that among NK cells, the percentage of CD56^{bright} NK cells was increased and of CD56^{dim} NK cells was decreased, resulting from the greater loss of CD56^{dim} NK cell subset. Further analysis showed that the ratio of CD56^{bright}

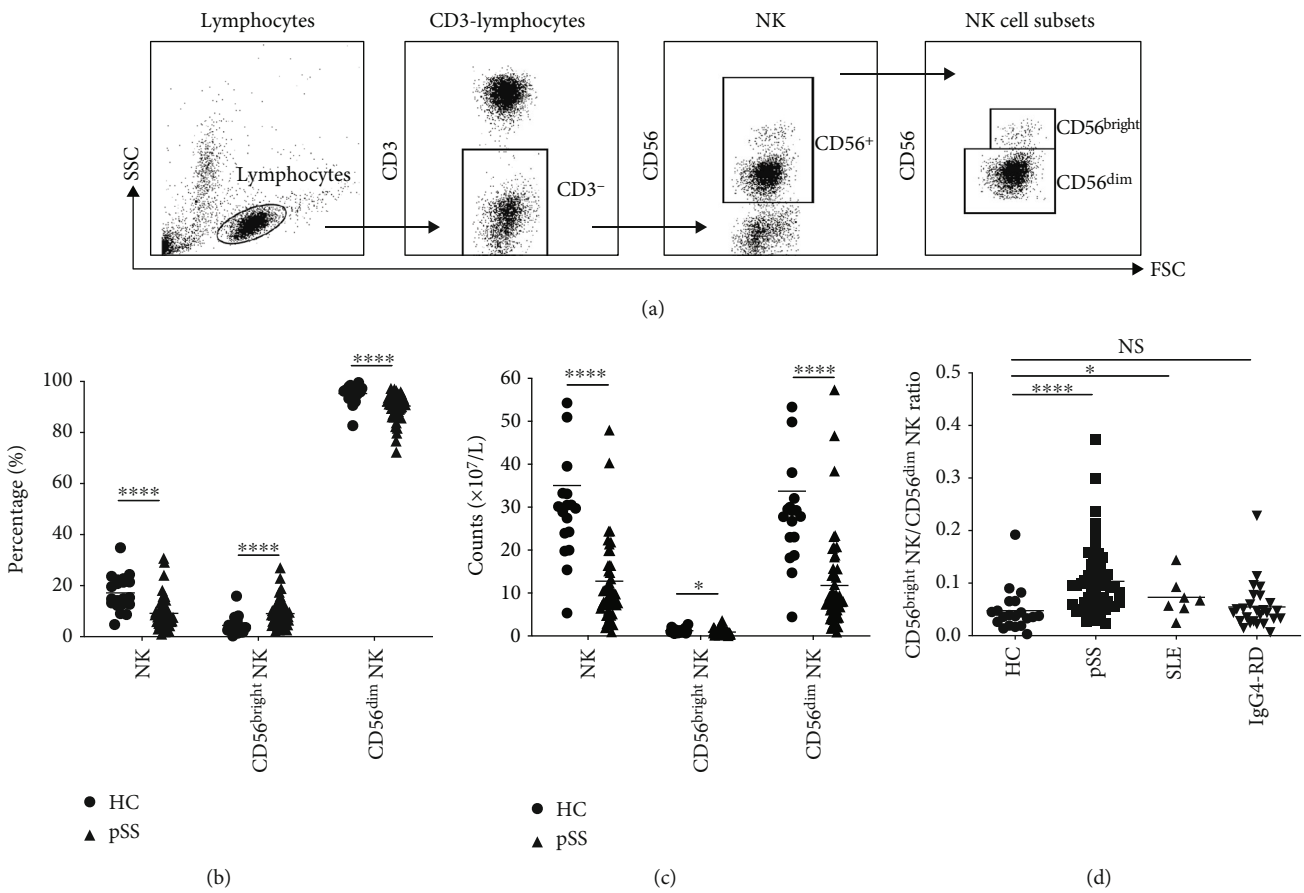


Figure 1: Comparison of percentage and counts of peripheral blood NK cells and CD56 NK cell subsets between pSS patients and HC. (a) The gating strategy is shown for the analysis of NK population. (b) The percentage of peripheral blood NK cells and their constituent CD56 subsets. (c) The absolute number of peripheral blood NK cells and their constituent CD56 NK cell subsets. (d) The ratio of $CD56^{bright}$ NK/$CD56^{dim}$ NK in autoimmune diseases. The horizontal line represents the mean. Definitions of abbreviations: HC = healthy controls; pSS = primary Sjögren's syndrome.

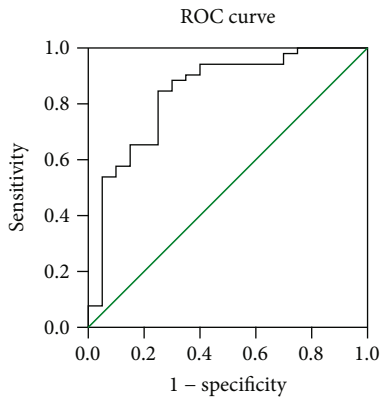


Figure 2: ROC curve of the ratio of $CD56^{bright}$ NK to $CD56^{dim}$ NK. The curve plots the relationship between sensitivity and 1 – specificity for different cut-off levels. When 0.0487 was used as the cut-off point for the diagnostic score of suspected pSS patients, the maximum value of the Youden index was achieved as 0.596.

NK cells relative to the total NK population to $CD56^{dim}$ NK cells was significantly elevated. Taken together, these results imply an imbalance of CD56 NK cell subsets in pSS, and this imbalance may reflect the immune status to some extent in pSS patients.

It is commonly recognized that serum IgG level, complement C3 and C4 consumption, and high ESSDAI are usually used to evaluate the disease activity in pSS patients. Goules et al. found that $CD56^{+}$ NK cell incidence in the minor salivary glands of pSS patients was slightly increased, but not associated with the grade of inflammation [27]. Another previous study reported that $CD56^{+}$ NK cell number in salivary glands was positively correlated to RF and serum C4 levels, and not correlated with the anti-SSA/SSB levels [28]. Recent research observed that the proportion of circulating $CD56^{bright}$ NK cells was increased in pSS patients, though it was not associated with disease activity [3]. To confirm whether the $CD56^{bright}$ NK to $CD56^{dim}$ NK ratio could reflect the disease activity, we analyzed the correlation between this ratio and disease activity indexes. In agreement with the previously reported data about the $CD56^{+}$ NK cells, we found that there is no significant correlation between the ratio and

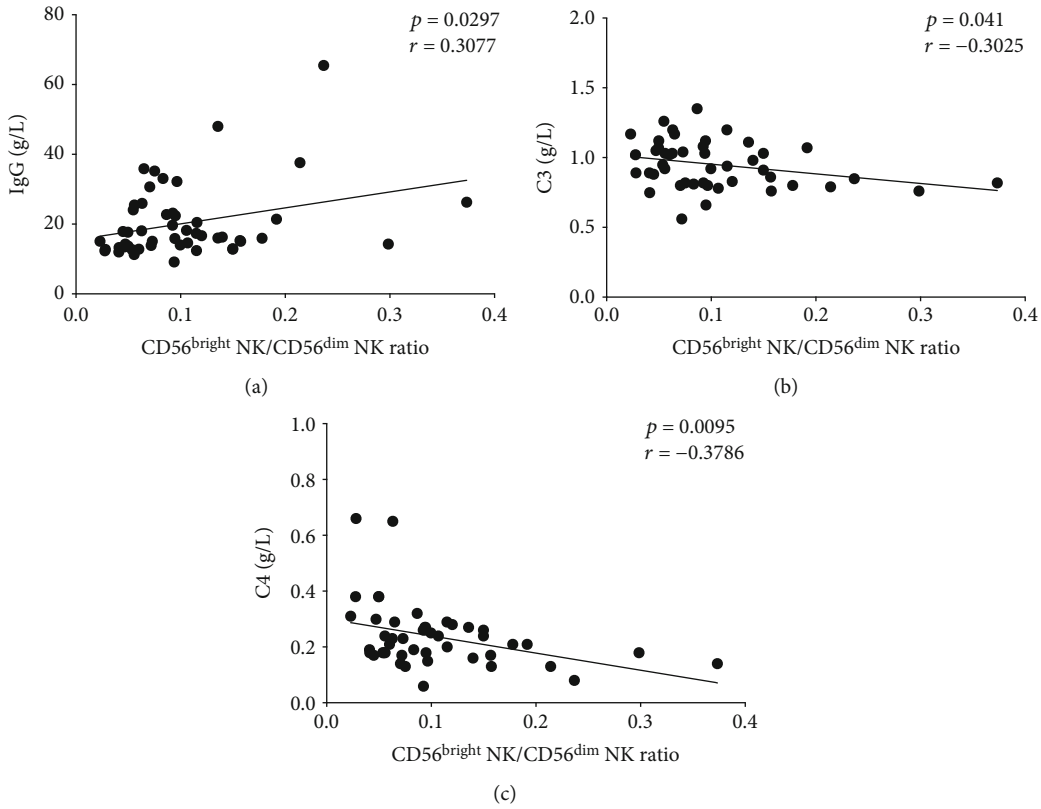


Figure 3: The correlation between $CD56^{bright}$ NK/$CD56^{dim}$ NK ratio and disease activity. (a) The correlation analysis between the $CD56^{bright}$ NK/$CD56^{dim}$ NK ratio and the serum IgG level or (b) complement C3 level or (c) complement C4 value in pSS patients.

ESSDAI. Interestingly, we found this ratio was positively correlated with the serum IgG level and negatively associated with the complement C3 and C4 levels. Thus, our data are the in-depth study of the previous work about CD56 NK cell subsets in pSS.

In consistent with the previous report, our data showed that the proportion and absolute number of circulating $CD3^{-}CD56^{+}$ NK cells were significantly reduced in pSS patients versus controls [3, 29, 30]. The depletion of peripheral blood NK cells in pSS patients may be due to an increased homing of the cytotoxic cells to exocrine glands, which initiate and maintain tissue inflammation through the production of Th1 cytokines and cytotoxic mediators. In general, $CD56^{bright}$ NK cells are mainly responsible for cytokine production, whereas $CD56^{dim}$ NK cells are mostly cytolytic. However, recent studies have revealed $CD56^{dim}$ NK cells to be a major source of cytokine production [31, 32]. Our data showed that circulating $CD56^{dim}$ NK cell subsets exhibited a greater loss in pSS patients, maybe resulting in a more homing to the exocrine glands to promote the immune injury, which needs to be further confirmed.

There exist some limitations of this study. It is commonly recognized that $CD56^{+}$ NK cell subsets in the salivary gland of pSS patients can be more suitable to reflect the situation in the gland. In this study, we mainly focused on the circulating $CD56^{+}$ NK cell subsets. It is difficult to distinguish the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets by immunohistochemistry staining; also, the biopsy gland tissues of pSS patients are not enough to analyze with flow cytometry. In addition, the number of SLE patients was relatively small. We only included newly diagnosed SLE patients as controls, which limited the number of included patients.

In summary, our results suggest that the ratio of peripheral blood $CD56^{bright}$ NK to $CD56^{dim}$ NK has the potential to identify the pSS patients and was associated with serum IgG levels and complement C3 and C4 consumption. Further studies are required to better understand the crucial role of CD56 NK cell subsets in the pathogenesis of pSS in disease-affected tissues.

Data Availability

The data that support the findings of this study are available. If it is necessary, we will provide it at all.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Bingxia Ming and Tong Wu contributed equally to this work.

Acknowledgments

This work was supported by grants from the National Natural Scientific Foundation of China (No. 81771754 and No. 81901586) and the Tongji Hospital Clinical Research Flagship Program (No. 2019CR206).

Supplementary Materials

Figure 1: comparison of function of peripheral NK cells and CD56 NK cell subsets between pSS patients and HC. (A) The percentage of CD107a$^+$ cells in NK cells or CD56 NK cell subsets. Cells were collected from HCs ($n = 8$) and pSS patients ($n = 15$). (B) The percentage of intracellular expression of IFN-γ gated from NK cells or CD56 NK cell subsets. Cells were collected from HCs ($n = 10$) and pSS patients ($n = 18$). HC = healthy control; pSS = primary Sjögren's syndrome. Figure 2: the correlation between CD56^{bright} NK/CD56^{dim} NK ratio and ESSDAI in pSS patients. Definitions of abbreviations: ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index. Figure 3: the change of peripheral NK cells and CD56 NK cell subsets in pSS patients ($n = 12$) before and after treatment. (A) The absolute number of peripheral CD56^{dim} NK cell subsets, (B) the absolute number of peripheral CD56^{bright} NK cell subsets, (C) the ratio of CD56^{bright} NK/CD56^{dim} NK, (D) ESSDAI, and (E) Serum serum IgG level before and after treatment. *(Supplementary materials)*

References

[1] B. Qin, J. Wang, Z. Yang et al., "Epidemiology of primary Sjögren's syndrome: a systematic review and meta-analysis," *Annals of the Rheumatic Diseases*, vol. 74, no. 11, pp. 1983–1989, 2015.

[2] G. Nocturne and X. Mariette, "Advances in understanding the pathogenesis of primary Sjögren's syndrome," *Nature Reviews Rheumatology*, vol. 9, no. 9, pp. 544–556, 2013.

[3] S. Rusakiewicz, G. Nocturne, T. Lazure et al., "NCR3/NKp30 contributes to pathogenesis in primary Sjogren's syndrome," *Science Translational Medicine*, vol. 5, no. 195, article 195ra96, 2013.

[4] C. H. Shiboski, S. C. Shiboski, R. Seror et al., "2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 76, no. 1, pp. 9–16, 2016.

[5] A. Ambrosi and M. Wahren-Herlenius, "Update on the immunobiology of Sjögren's syndrome," *Current Opinion in Rheumatology*, vol. 27, no. 5, pp. 468–475, 2015.

[6] A. Hansen, P. E. Lipsky, and T. Dorner, "B cells in Sjögren's syndrome: indications for disturbed selection and differentiation in ectopic lymphoid tissue," *Arthritis Research & Therapy*, vol. 9, no. 4, p. 218, 2007.

[7] C. P. Mavragani, "Mechanisms and new strategies for primary Sjögren's syndrome," *Annual Review of Medicine*, vol. 68, no. 1, pp. 331–343, 2017.

[8] J. Luo, B. Ming, C. Zhang et al., "IL-2 inhibition of Th17 generation rather than induction of Treg cells is impaired in primary Sjögren's syndrome patients," *Frontiers in Immunology*, vol. 9, p. 1755, 2018.

[9] M. A. Cooper, T. A. Fehniger, and M. A. Caligiuri, "The biology of human natural killer-cell subsets," *Trends in Immunology*, vol. 22, no. 11, pp. 633–640, 2001.

[10] A. Awada, C. Nicaise, S. Ena et al., "Potential involvement of the IL-33-ST2 axis in the pathogenesis of primary Sjogren's syndrome," *Annals of the Rheumatic Diseases*, vol. 73, no. 6, pp. 1259–1263, 2014.

[11] U. Båve, G. Nordmark, T. Lövgren et al., "Activation of the type I interferon system in primary Sjögren's syndrome: a possible etiopathogenic mechanism," *Arthritis and Rheumatism*, vol. 52, no. 4, pp. 1185–1195, 2005.

[12] T. O. R. Hjelmervik, K. Petersen, I. Jonassen, R. Jonsson, and A. I. Bolstad, "Gene expression profiling of minor salivary glands clearly distinguishes primary Sjögren's syndrome patients from healthy control subjects," *Arthritis and Rheumatism*, vol. 52, no. 5, pp. 1534–1544, 2005.

[13] J. C. Hall, L. Casciola-Rosen, A. E. Berger et al., "Precise probes of type II interferon activity define the origin of interferon signatures in target tissues in rheumatic diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 43, pp. 17609–17614, 2012.

[14] J.-E. Gottenberg, N. Cagnard, C. Lucchesi et al., "Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 8, pp. 2770–2775, 2006.

[15] S. Pesce, L. Moretta, A. Moretta, and E. Marcenaro, "Human NK cell subsets redistribution in pathological conditions: a role for CCR7 receptor," *Frontiers in Immunology*, vol. 7, 2016.

[16] L. L. Lanier and J. H. Phillips, "Ontogeny of natural killer cells," *Nature*, vol. 319, no. 6051, pp. 269-270, 1986.

[17] M. Gao, Y. Yang, D. Li et al., "CD27 natural killer cell subsets play different roles during the pre-onset stage of experimental autoimmune encephalomyelitis," *Innate Immunity*, vol. 22, no. 6, pp. 395–404, 2016.

[18] A. Poli, T. Michel, M. Theresine, E. Andres, F. Hentges, and J. Zimmer, "CD56bright natural killer (NK) cells: an important NK cell subset," *Immunology*, vol. 126, no. 4, pp. 458–465, 2009.

[19] M. S. Hasan, P. L. Ryan, L. A. Bergmeier, and F. Fortune, "Circulating NK cells and their subsets in Behçet's disease," *Clinical and Experimental Immunology*, vol. 188, no. 2, pp. 311–322, 2017.

[20] J. E. Melsen, G. Lugthart, A. C. Lankester, and M. W. Schilham, "Human circulating and tissue-resident CD56bright natural killer cell populations," *Frontiers in Immunology*, vol. 7, 2016.

[21] R. Davies, D. Hammenfors, B. Bergum et al., "Patients with primary Sjögren's syndrome have alterations in absolute quantities of specific peripheral leucocyte populations," *Scandinavian Journal of Immunology*, vol. 86, no. 6, pp. 491–502, 2017.

[22] F. Mackay, S. A. Woodcock, P. Lawton et al., "Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations," *The Journal of Experimental Medicine*, vol. 190, no. 11, pp. 1697–1710, 1999.

[23] G. W. Nelson, M. P. Martin, D. Gladman, J. Wade, J. Trowsdale, and M. Carrington, "Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/-susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis," *Journal of Immunology*, vol. 173, no. 7, pp. 4273–4276, 2004.

[24] F. Segerberg, C. Lundtoft, S. Reid et al., "Autoantibodies to killer cell immunoglobulin-like receptors in patients with systemic lupus erythematosus induce natural killer cell hyporesponsiveness," *Frontiers in Immunology*, vol. 10, p. 2164, 2019.

[25] K. Takahashi, "The regulatory role of natural killer cells in multiple sclerosis," *Brain*, vol. 127, no. 9, pp. 1917–1927, 2004.

[26] G. Ruacho, M. Kvarnström, A. Zickert et al., "Sjögren syndrome in systemic lupus Erythematosus: a subset characterized by a systemic inflammatory state," *The Journal of Rheumatology*, vol. 47, no. 6, pp. 865–875, 2020.

[27] A. V. Goules, E. K. Kapsogeorgou, and A. G. Tzioufas, "Insight into pathogenesis of Sjögren's syndrome: dissection on autoimmune infiltrates and epithelial cells," *Clinical Immunology*, vol. 182, pp. 30–40, 2017.

[28] M. I. Christodoulou, E. K. Kapsogeorgou, and H. M. Moutsopoulos, "Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome," *Journal of Autoimmunity*, vol. 34, no. 4, pp. 400–407, 2010.

[29] Y. Izumi, H. Ida, M. Huang et al., "Characterization of peripheral natural killer cells in primary Sjögren's syndrome: impaired NK cell activity and low NK cell number," *The Journal of Laboratory and Clinical Medicine*, vol. 147, no. 5, pp. 242–249, 2006.

[30] G. Sudzius, D. Mieliauskaite, A. Siaurys et al., "Distribution of Peripheral Lymphocyte Populations in Primary Sjögren's Syndrome Patients," *Journal of Immunology Research*, vol. 2015, Article ID 854706, 10 pages, 2015.

[31] K. Juelke, M. Killig, M. Luetke-Eversloh et al., "CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells," *Blood*, vol. 116, no. 8, pp. 1299–1307, 2010.

[32] L. Moretta, "Dissecting CD56dim human NK cells," *Blood*, vol. 116, no. 19, pp. 3689–3691, 2010.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 2943848, 7 pages
https://doi.org/10.1155/2020/2943848

Clinical Study

Hippocampal Atrophy in Systemic Lupus Erythematosus Patients without Major Neuropsychiatric Manifestations

Shuang Liu,[1,2] Yuqi Cheng,[2,3] Yueyin Zhao,[1] Aiyun Lai,[1] Zhaoping Lv,[1] Zhongqi Xie,[1] Bibhuti Upreti,[1] Xiangyu Wang,[1] Xiufeng Xu,[3] Chunrong Luo,[4] Hongjun Yu,[4] Baoci Shan,[5] Lin Xu,[6] and Jian Xu[1,2]

[1]*Department of Rheumatology and Immunology, First Affiliated Hospital of Kunming Medical University, Kunming 650032, China*
[2]*Yunnan Key Laboratory of Laboratory Medicine, Kunming 650032, China*
[3]*Department of Psychiatry, First Affiliated Hospital of Kunming Medical University, Kunming 650032, China*
[4]*Magnetic Resonance Imaging Center, The First Hospital of Kunming, Kunming 650011, China*
[5]*Key Laboratory of Nuclear Analysis, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing 100049, China*
[6]*Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China*

Correspondence should be addressed to Jian Xu; casxujian@163.com

Received 13 March 2020; Accepted 21 May 2020; Published 12 June 2020

Academic Editor: Lihua Duan

This study was conducted to explore hippocampal structural changes and their possible associations with clinical characteristics, emotional status, and treatment regimens in patients with systemic lupus erythematosus (SLE) without major neuropsychiatric manifestations (non-NPSLE). Eighty-five non-NPSLE patients with normal conventional magnetic resonance imaging (MRI) and seventy-seven matched healthy control (HC) subjects were recruited. All participants underwent the standard high-resolution volumetric MRI. The bilateral hippocampal volume (HIPV) and hippocampal density (HIPD) were calculated, respectively, for each participant. We found that the bilateral HIPV and HIPD of the SLE patient group were significantly less than those of the HC group. The bilateral HIPV of female patients were significantly less than those of male patients. The SLE disease activity index (SLEDAI) was negatively correlated with the bilateral HIPV and the right HIPD. Urine protein quantity was negatively correlated with the bilateral HIPV and HIPD. Hydroxychloroquine (HCQ) showed a protective effect on right HIPV. In conclusion, we found that the early hippocampal atrophy could occur before obvious neuropsychiatric manifestations and might be associated with SLE disease activity and organ damages. Early detection and intervention of hippocampal damage might prevent the progression to NPSLE. More studies are needed to fully understand the underlying mechanisms of hippocampal atrophy in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with multiorgan involvement. It is characterized by high titers of various serum antibodies targeting nuclear or cytoplasmic antigens. Glucocorticoids (GC) and immunosuppressive agents (ISA) are used to help patients reach the target of remission or low disease activity. Hydroxychloroquine (HCQ), cyclophosphamide (CTX), and Mycophenolate Mofetil (MMF) are widely used ISA (1). The central nervous system (CNS) is commonly involved in SLE (2, 3). Brain atrophy has been detected in SLE patients using several neuroimaging techniques. It is often associated with clinical manifestations in SLE patients and sometimes even in patients without obvious CNS signs and symptoms (4, 5). The hippocampus is located in the temporal lobe of the brain and plays an important role in learning and memory processes. Hippocampal atrophy was found in SLE patients

and was related to cognitive dysfunction, disease duration, and history of CNS manifestations (6).

Magnetic resonance imaging (MRI) is one of the most commonly used techniques to evaluate brain abnormalities including brain atrophy. Many patients with mild cognitive impairment have normal conventional MRI findings because conventional MRI is nonspecific or not sensitive enough for delicate structures like hippocampus (4, 6). SLE patients without major neuropsychiatric manifestations are usually considered non-NPSLE patients. In our previous study, we have found that the white matter volume (WMV) of the non-NPSLE patient group was significantly less than that of healthy control (HC) group and that ISA treatment might have a protective effect on WMV (7). Furthermore, in another study, we also found that specific autoantibodies, such as anti-cardiolipin (aCL) antibodies, might contribute to the reduction of grey matter density (GMD) and white matter density (WMD) in non-NPSLE patients. ISA treatment also showed effects in preventing the reduction of GMD and WMD (8). It has been reported that abnormal hippocampal structural changes could be found in non-NPSLE patients with normal conventional brain MRI (9, 10). These results prompted our interest in exploring hippocampal structural changes in non-NPSLE patients by using volumetric MRI.

The underlying mechanisms of hippocampal involvement in SLE patients remain unclear. Various autoantibodies including aCL antibodies and anti-NR2 subtype of the N-methyl-D-aspartate receptor (NMDAR) antibodies were considered to play important roles in the pathogenesis of neuropsychiatric SLE (NPSLE) (8, 11, 12). However, few studies were focused on the relationship between autoantibodies and hippocampal atrophy.

Thus, we conducted this study to explore hippocampal structural changes in non-NPSLE patients and their possible associations with clinical characteristics including specific autoantibodies, disease activity, and emotional status as well as treatment regimens.

2. Material and Methods

2.1. Subjects. SLE patients were recruited from the inpatient and outpatient facilities of Department of Rheumatology and Immunology of First Affiliated Hospital of Kunming Medical University, a member of the Chinese SLE Treatment and Research Group (CSTAR), from September of 2012 to September of 2014. Each participant went through a standardized protocol and was evaluated by the same investigator throughout the study. All participants had received complete and detailed description of the study and had given written informed consent before enrollment into the study. This research protocol was approved by the Institutional Review Board of Kunming Medical University, Yunnan Province, P. R. China (ClinicalTrials.gov: NCT00703742).

The inclusion criteria included (1) patients diagnosed as SLE according to the 1997 revised American College of Rheumatology (ACR) criteria for the classification of SLE (13), (2) subjects between the ages of 18 and 60, and (3) subjects willing to participate in this study and give written informed consent.

The exclusion criteria included (1) patients fulfilling the ACR diagnostic criteria for rheumatoid arthritis, systemic sclerosis, Sjögren's syndrome (primary or secondary), or other connective tissue diseases and drug-induced SLE; (2) patients with neurological disorders that would affect the brain structure (e.g., history of head trauma, Parkinson's disease, or seizures); (3) patients with major psychiatric manifestations, such as obvious disorganized behaviors and disturbances of consciousness; (4) patients with history of substance abuse; (5) patients who were pregnant or suspected to be pregnant; (6) patients with contraindications to MRI, such as claustrophobia or having cardiac pacemakers; (7) patients with serious clinical conditions that could cause cerebral atrophy, such as history of hypertension, diabetes mellitus, and renal insufficiency; and (8) patients with brain structural abnormalities identified by conventional T1 and T2 weighted MRI.

All 85 SLE patients recruited had undergone full sets of laboratory tests and MRI scans and filled up general questionnaires. A rheumatologist and a neurologist performed complete physical examination including neurological examination on all SLE patients and 77 HC subjects to exclude major disorders. Additionally, psychiatric symptoms were screened by a psychiatrist using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders-IV Non-Patient Version (SCID-NP). All participants were Chinese Han people and were right-handed.

2.2. Clinical Characteristics of SLE Patients. We recorded the gender, age, and disease duration for each patient. The disease duration was defined as the period from the appearance of initial signs and symptoms of SLE to the day of MRI acquisition. All clinical manifestations and laboratory test results were systematically recorded. Disease activity was measured using SLE disease activity index (SLEDAI). Disease was considered to be active when the SLEDAI score was higher than nine (14, 15). All participants were right-handed as assessed by the Edinburgh Handedness Inventory (16). All clinical data were collected on the day of MRI scans.

The cumulative dosages of GC and ISA were documented through careful interviews and calculated by adding up all the daily dosages. The dosages of oral and intravenous GC were all converted to equivalent dosages of prednisone.

2.3. Autoantibodies. Each patient was tested for a set of antibodies, including antinuclear antibody (ANA), anti-double-stranded deoxyribonucleic acid (dsDNA), anti-SSA/Ro 52 kD, anti-SSA/Ro 60 kD, anti-SSB/La, anti-Sm, anti-histone, anti-ribosomal P0, anti-nucleosome, anti-U1 ribonucleoprotein (RNP), and aCL antibodies. ANA was tested with indirect immunofluorescence using Hep-2 cells as substrates (SCIMEDX Corporation, New Jersey, USA); ANA spectrum antibodies were tested with the line immunoassay method using IMTEC kit (IMTEC, Berlin, Germany); aCL antibodies were tested with conventional ELISA (Aesku Diagnostics, Wendelsheim, Germany). All blood samples were drawn on the day of MRI acquisitions.

Table 1: Demographic and clinical characteristics of SLE and HC groups.

	SLE ($n = 85$)	HC ($n = 77$)	t	p value
Age (year, mean ± SD)	29.28 ± 7.02	30.81 ± 7.44	-1.340	0.182
Female/male	71/14	57/20	2.200 (χ^2)	0.138
Disease duration (m, mean ± SD)	19.90 ± 28.90	NA		
SLEDAI (mean ± SD)	9.53 ± 6.13	NA		
Total GC (g, mean ± SD)	9.46 ± 13.49 ($n = 67$)	NA		
Total CTX (g, mean ± SD)	4.15 ± 3.41 ($n = 22$)	NA		
Total HCQ (g, mean ± SD)	55.06 ± 95.42 ($n = 38$)	NA		

SLE: systemic lupus erythematosus; HC: healthy control; SD: standard deviation; NA: not applicable; SLEDAI: SLE disease activity index; GC: glucocorticoid; CTX: cyclophosphamide; HCQ: hydroxychloroquine.

2.4. Image Acquisition. Image acquisitions were performed by an experienced neuroradiologist. MRI sequences were performed on all subjects using a 1.5 T clinical MRI scanner equipped with a birdcage head coil. It was manufactured by General Electric (GE) Company (Twin speed, Milwaukee, WI, USA). Supportive foam pads were used to minimize head movement. A rapid sagittal localizer scan was acquired to ensure alignment. Conventional T1 and T2 MRI scans were taken to exclude obvious structural abnormalities. A set of three-dimensional volumetric structural MRI scans was performed on each subject using a fast spoiled gradient echo sequence (FSPGR) with the following parameters: repetition time (TR)/echo time (TE) = 10.5/2 ms, matrix size = 256 × 256, thickness = 1.8 mm with no interslice gap, field of view = 240 mm, flip angle = 15°, and resolution = $0.94 \times 0.94 \times 0.9\,\text{mm}^3$. A total of 172 continuous slices of whole brain data were acquired in axial planes parallel to the anterior commissure-posterior commissure line.

2.5. Data Preprocessing and VBM Statistical Analysis. Digital Imaging and Communications in Medicine (DICOM) image data were processed with MRIcro software (version 1.40, Chris Rorden's Neuropsychology Laboratory, University of South Carolina, Columbia, SC, USA; http://www.mricro.com). All data were analyzed by using statistical parametric mapping (SPM) 5 (Wellcome Department of Cognitive Neurology, Institute of Neurology, London, UK; http://www.fil.ion.ucl.ac.uk/) and voxel-based morphometry (VBM) 5 (Department of Neurology, Department of Psychiatry, Friedrich Schiller University Jena, Thuringia, Germany; http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/) software based on Matlab 7.1 (The MathWorks, Inc., Natick, MA, USA). Each individual image was normalized and transformed into the standardized Montreal Neurological Institute (MNI) template, then resampled in 2 × 2 × 2 mm scale. Normalized images were then segmented into grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF). Modulated GM and WM images were separately smoothened to remove noise using a filter with full width at half maximum (FWHM) of 8 mm.

2.6. Analysis of Hippocampal Volume (HIPV) and Hippocampal Density (HIPD). At first, we used the standard GM and WM templates implanted in SPM 5 as the whole brain GM and WM masks to get the total brain volume. Then, we used WFU PickAtlas software (NeuroImaging Tools and Resources Collaboratory, Radiology Informatics and Imaging Laboratory, Department of Radiology, Wake Forest University Health Sciences Medical Center, NC, USA; http://www.nitrc.org/projects/wfu_pickatlas) to get the hippocampal mask. Finally, we retrieved HIPV and HIPD data. Statistical analysis was conducted with SPSS 20.0 (IBM Inc., Armonk, NY, USA). Covariance analysis was performed to analyze the differences in HIPV/HIPD between SLE and HC groups as well as different subgroups of SLE patients. Age and total brain volume were covariates. We used the Bonferroni method for HIPV/HIPD correction in multiple comparisons. Partial correlation analysis, controlling for age and total brain volume, was used to explore possible correlations between disease characteristics and HIPV/HIPD. The results were believed to be statistically significant when $p < 0.05$. All statistical tests were two-sided.

3. Results

3.1. Demographic Data in SLE and HC Groups. In this study, 85 SLE and 77 HC subjects were recruited. The mean age of SLE patients was 29.28 years (standard deviation (SD) = 7.02, range 18-48) and the mean age of HC subjects was 30.81 years (SD = 7.44, range 18-50). There were no significant differences in age or gender between these two groups. The disease duration in SLE patients ranged from 0.25 to 204 months (mean = 19.90 months, SD = 28.90). The mean SLEDAI was 9.53 (SD = 6.13). Detailed data are showed in Table 1.

Among the 85 SLE patients, 40 (47.1%) patients were positive for anti-Sm antibody, 55 (64.7%) were positive for anti-dsDNA antibody, 45 (52.9%) were positive for anti-SSA/Ro 52 kD antibody, 56 (65.9%) were positive for anti-SSA/Ro 60 kD antibody, 30 (35.3%) were positive for anti-SSB/La antibody, and 10 (11.8%) were positive for aCL antibody.

3.2. HIPV/HIPD Differences between SLE and HC Groups. The bilateral HIPV and HIPD of SLE group were significantly less than those of the HC group (see Figure 1(a), Table 2). The bilateral HIPV of male patients were significantly greater than those of female patients. However, the right HIPD of male patients was significantly less than that

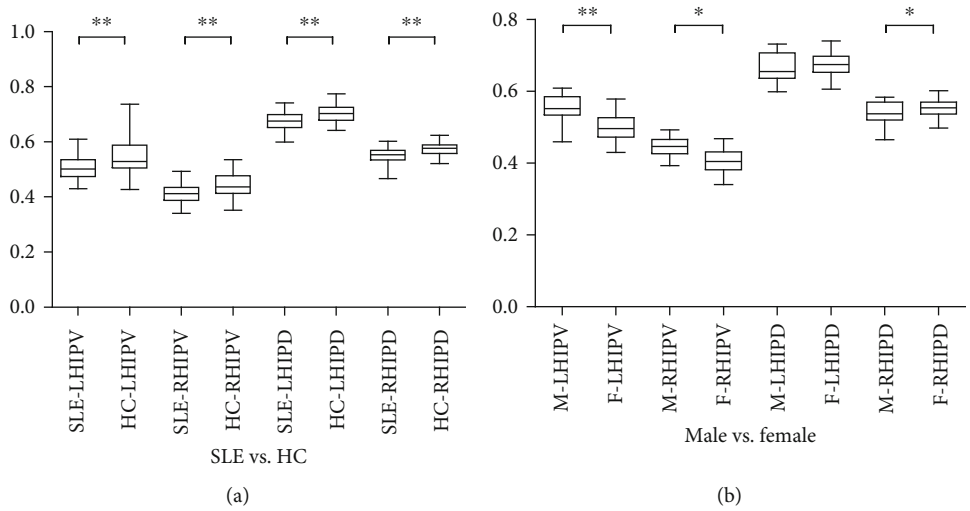


Figure 1: Comparisons of hippocampal volume and density in different groups. (a). Comparisons of hippocampal volume and density between SLE patients and HC group; (b). Comparisons of hippocampal volume and density between male and female SLE patients. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 2: Hippocampal volume and density in SLE and HC groups.

	SLE (n = 85)		HC (n = 77)		p value	Adjusted p value
	Mean	SD	Mean	SD		
LHIPV	0.5079	0.0405	0.5463	0.0555	0.001^{**}	0.001^{**}
RHIPV	0.4132	0.0320	0.4427	0.0406	0.001^{**}	0.001^{**}
LHIPD	0.6756	0.0305	0.7039	0.0297	$<0.001^{**}$	—
RHIPD	0.5500	0.0237	0.5730	0.0229	$<0.001^{**}$	$<0.001^{**}$

SLE: systemic lupus erythematosus; HC: healthy control; SD: standard deviation; L: left; HIPV: hippocampal volume; R: right; HIPD: hippocampal density. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 3: Gender differences of hippocampal volume and density in SLE patients.

	Male (n = 14)		Female (n = 71)		p value
	Mean	SD	Mean	SD	
LHIPV	0.5515	0.0399	0.4993	0.0349	0.005^{**}
RHIPV	0.4458	0.0284	0.4068	0.0287	0.021^{*}
LHIPD	0.6657	0.0422	0.6775	0.0284	0.118
RHIPD	0.5379	0.0322	0.5524	0.0211	0.047^{*}

SLE: systemic lupus erythematosus; SD: standard deviation; L: left; HIPV: hippocampal volume; R: right; HIPD: hippocampal density. $^{*}p < 0.05$, $^{**}p < 0.01$.

of female patients, while the left HIPD was comparable between different genders (see Figure 1(b), Table 3).

Due to the gender differences, we reanalyzed the bilateral HIPV and right HIPD in the SLE and HC groups while controlling for gender as a covariate. The adjusted p values are shown in Table 2.

3.3. Associations between HIPV/HIPD and Clinical Characteristics. All the statistical analyses involving the bilateral HIPV and the right HIPD were done while controlling for gender. We divided patients into two groups: a positive aCL antibody group and a negative aCL antibody group. Bilateral HIPV/HIPD in these two groups showed no significant differences. Similarly, we divided the patients into two groups according to the results of the rest of the antibodies tested and we found that each antibody positivity/negativity pair had no significant differences in their HIPV/HIPD. Patients with active disease (SLEDAI > 9) showed less right HIPD than those with low disease activity or in remission (0.5444 ± 0.0256 vs. 0.5544 ± 0.0214; $p = 0.032$). SLEDAI scores showed negative correlations with bilateral HIPV and right HIPD ($r = -0.239$, $p = 0.031$; $r = -0.245$, $p = 0.027$; $r = -0.221$, $p = 0.046$, respectively). The urine protein quantities were negatively correlated with bilateral HIPV and HIPD ($r = -0.300$, $p = 0.006$; $r = -0.255$, $p = 0.021$; $r = -0.272$, $p = 0.011$; $r = -0.247$, $p = 0.025$, respectively). The total HCQ dose showed a positive correlation with right HIPV ($r = 0.254$, $p = 0.021$). There were no significant correlations between HIPV/HIPD and other factors including disease duration, total GC, total CTX, HAMD, and HAMA scores. Detailed data are showed in Table 4.

4. Discussion

In this study, we found a significant reduction in the hippocampal volume and density in non-NPSLE patients when compared to those of the HC group. Female patients showed greater reductions in the hippocampal volume. Hippocampal atrophy was associated with disease activity and urine protein. HCQ showed a protective effect on hippocampal volume.

Although MRI is considered to be a good method to evaluate CNS damage in SLE, conventional MRI findings are often nonspecific or unable to detect any changes in patients with or without NPSLE (17). Hippocampus is crucial in learning and memory processes and plays a role in the

Table 4: Correlations between HIPV/HIPD and clinical characteristics.

	LHIPV		RHIPV		LHIPD		RHIPD	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Disease duration (m)	-0.193	0.082	-0.115	0.302	-0.141	0.192	-0.136	0.222
Urine protein (g/d)	-0.300	0.006**	-0.255	0.021*	-0.272	0.011*	-0.247	0.025*
SLEDAI	-0.239	0.031*	-0.245	0.027*	-0.183	0.090	-0.221	0.046*
Total GC (g)	-0.215	0.053	-0.127	0.256	-0.204	0.058	-0.148	0.183
Total HCQ (g)	0.111	0.320	0.254	0.021*	0.024	0.825	0.069	0.537
Total CTX (g)	-0.047	0.674	-0.083	0.457	-0.052	0.635	-0.035	0.755
HAMD	0.004	0.969	0.010	0.929	-0.028	0.799	-0.005	0.965
HAMA	-0.022	0.844	0.056	0.616	-0.035	0.745	0.045	0.688

L: left; HIPV: hippocampal volume; R: right; HIPD: hippocampal density; SLICC: Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for Systemic Lupus Erythematosus; SLEDAI: systemic lupus erythematosus disease activity index; GC: glucocorticoid; HCQ: hydroxychloroquine; CTX: cyclophosphamide. $^{*}p < 0.05$, $^{**}p < 0.01$.

regulation of emotional status (18). A temporal progression of hippocampal volume reduction has been reported in SLE patients with cognitive dysfunction (6). Patients in our study had no major neuropsychiatric manifestations or abnormal conventional MRI findings; i.e., they were non-NPSLE patients. Therefore, the hippocampal atrophy implied that brain damage could occur even before the appearance of obvious neuropsychiatric signs and symptoms. Previous studies have also shown that abnormal structural changes, such as hippocampal atrophy, could occur in early phases and affect cognitive function in SLE patients (9, 19, 20). Our study used a quantitative volumetric MRI technique to measure the hippocampal volume and found hippocampal atrophy in non-NPSLE patients.

Gender differences in hippocampal density and volume were found in our study. Bilateral HIPV of female patients were significantly less than those of male patients. It was consistent with another study of our team, in which we found that female SLE patients had significantly lower whole brain grey matter volume (GMV) and WMV than male patients (21). We hypothesized that these findings might be related to the higher estrogen levels in females. Estrogen was considered to be one cause of the higher prevalence of SLE in females. Some researchers believed that gender differences could be found in almost all brain activities and that estrogen could regulate the hippocampus associated learning and memory processes differently in different genders (22). However, when compared with male patients, we also found a greater right HIPD in female patients. When processed by the VBM method, the volume data were modulated and might be more reliable than density data. The higher right HIPD values in females with a p value of 0.047 might not be as meaningful as the HIPV differences. Another possible explanation for the variation could be a different aspect of gender differences, the compensation mechanism in females, which has also been mentioned previously in a study (23). More studies are needed to confirm the gender differences and reveal the underlying mechanisms.

The exact roles of autoantibodies in the pathogenesis of brain damage in SLE remain unclear. However, several studies suggest that because blood brain barrier (BBB) lesions could bring high titer auto antibodies in contact with myeloid or glial cells and cause their activation; they could induce production of local cytokines, such as IL-6, which was found elevated in CSF of SLE patients (24–29). Also, some researchers reported that autoantibodies could affect certain brain regions due to BBB lesions and cause organ damages through different pathways (24). However, we failed to find the associations between autoantibodies and hippocampal atrophy. Further investigations are needed to know whether autoantibodies play any role in the pathogenesis of hippocampal atrophy.

On the other hand, we found negative correlations between the HIPV/HIPD and urine protein quantities as well as SLEDAI scores. It implied that early hippocampal atrophy might be associated with SLE disease activity and other organ damages. The associations between early brain atrophy and SLE disease activity were reported by our team earlier (7, 8). Appenzeller et al. reported that SLE patients with white matter lesions had hippocampal atrophy more frequently than patients without it. Furthermore, the hippocampal loss in MRI might become progressive over time in patients with long disease duration (6).

Additionally, we found that HCQ might prevent HIPV reduction. This is consistent with our previous studies which showed that ISA had protective effects on brain atrophy in SLE patients (7, 8, 21). In our studies, ISA included CTX and HCQ. CTX is the most common and efficacious immunosuppressant used for the treatment of lupus. Leung et al. reported that CTX could improve brain demyelinating lesions in SLE (30). Ballok et al. reported that the hippocampus of a NPSLE patient showed decreased neuronal density and suggested that autoimmunity might contribute to the hippocampal damage. They then observed that hippocampal damage could be improved by CTX treatment in Murphy Roths Large-lymphoproliferation (MRL-lpr) mice (31). As an antimalarial drug, HCQ shows good immunomodulation. It could reduce damage accrual, improve survival rate, prevent thrombotic events, improve dyslipidemia, and prevent SLE flares (32, 33). González et al. reported that HCQ was associated with a longer time to neuropsychiatric damage occurrence, which might reveal the protective effect on early brain damage in SLE (34). However, no other study has shown a similar protective effect of HCQ. Also, the exact

mechanism of this effect remains unclear. More studies, especially prospective studies and animal experiments to explore it further are warranted.

5. Limitations

There are still some flaws in our study. One is that we only used the VBM method to calculate the hippocampal volume even though there were other available methods such as manual tracing and FreeSurfer segmentation. Grimm et al. reported that VBM and FreeSurfer were comparable in calculating the hippocampal volume (35). Wenger et al. reported that FreeSurfer was more reliable in assessing the hippocampal volume in young adults than older ones (36). In our study, we considered the VBM method to be feasible to calculate the hippocampal volume.

Our study shows that hippocampal atrophy detected by volumetric MRI could occur before obvious neurological manifestations in SLE and is one of the primary deficits in SLE. It might be associated with SLE disease activity and other organ damages, especially proteinuria. HCQ might have a protective effect. Thus, early detection and intervention of hippocampal damage might prevent the progression to NPSLE. Further studies are still required to explore the underlying mechanisms of hippocampal involvement in SLE.

Data Availability

All datasets for this study can be found in the Figshare. Please see the 10.6084/m9.figshare.12017730.v3 for details.

Disclosure

An introduction of this manuscript was presented as a poster presentation in Abstracts 000000of IUIS 2019 Beijing—17th International Congress of Immunology (II), 19 October–23 October 2019, Beijing, China.

Conflicts of Interest

All authors have no conflict of interest to declare.

Authors' Contributions

Shuang Liu and Yuqi Cheng contributed equally to this work.

Acknowledgments

We thank all the volunteers who participated in this study. We thank Dr. Daying Feng of Department of Rheumatology and Immunology of First Affiliated Hospital of Kunming Medical University for recruiting the volunteers. This work is supported by grants from National Natural Science Foundation of China (81160379, 81460256, 81560233, 81501406, and 81760296), the Funding of Yunnan Provincial Health Science and Technology Plan (2014NS171, 2016NS026, 2017NS051, 2018NS0133, and 2018NS0134), Yunnan Provincial Fund for High Level Reserve Talents in Health Science (H-2017068), The Hundred-Talent Program of Kunming Medical University (60117190457), Innovative Research Team of Kunming Medical University (CXTD201613), Yunnan Provincial Fund for Preparatory Young Leaders in Academia and Technology (2015HB071), the Funding of Ministry of Science and Technology of Yunnan Province (2014HC018 and 2018ZF016), the Funding of Yunnan Provincial Department of Education, the Funding of Health Department of Yunnan Province, Yunnan Applied Basic Research Projects-Union Foundation (2017FE467, 2017FE467(-138)), The Youth Talent of Ten Thousand Scientists Program of Yunnan Province (YNWR-MY-2018-040, YNWR-QNBJ-2018-152), the Funding of Ministry of Science and Technology of China (2017YFC0907600), Major science and technology projects of Yunnan Clinical Medical Center for Skin Immune Diseases (ZX2019-03-02), and Yunnan Province Clinical Research Center for Skin Immune Diseases (2019ZF012).

References

[1] R. F. van Vollenhoven, M. Mosca, G. Bertsias et al., "Treat-to-target in systemic lupus erythematosus: recommendations from an international task force," *Annals of the Rheumatic Diseases*, vol. 73, no. 6, pp. 958–967, 2014.

[2] D. C. Adelman, E. Saltiel, and J. R. Klinenberg, "The neuropsychiatric manifestations of systemic lupus erythematosus: an overview," *Seminars in Arthritis and Rheumatism*, vol. 15, no. 3, pp. 185–199, 1986.

[3] G. Sanna, M. Piga, J. W. Terryberry et al., "Central nervous system involvement in systemic lupus erythematosus: cerebral imaging and serological profile in patients with and without overt neuropsychiatric manifestations," *Lupus*, vol. 9, no. 8, pp. 573–583, 2016.

[4] T. W. J. Huizinga, S. C. A. Steens, and M. A. van Buchem, "Imaging modalities in central nervous system systemic lupus erythematosus," *Current Opinion in Rheumatology*, vol. 13, no. 5, pp. 383–388, 2001.

[5] S. Appenzeller, G. B. Pike, and A. E. Clarke, "Magnetic resonance imaging in the evaluation of central nervous system manifestations in systemic lupus erythematosus," *Clinical Reviews in Allergy and Immunology*, vol. 34, no. 3, pp. 361–366, 2008.

[6] S. Appenzeller, A. D. Carnevalle, L. M. Li, L. T. L. Costallat, and F. Cendes, "Hippocampal atrophy in systemic lupus erythematosus," *Annals of the Rheumatic Diseases*, vol. 65, no. 12, pp. 1585–1589, 2006.

[7] J. Xu, Y. Cheng, P. Chai et al., "White-matter volume reduction and the protective effect of immunosuppressive therapy in systemic lupus erythematosus patients with normal appearance by conventional magnetic resonance imaging," *The Journal of Rheumatology*, vol. 37, no. 5, pp. 974–986, 2010.

[8] J. Xu, Y. Cheng, A. Lai et al., "Autoantibodies affect brain density reduction in nonneuropsychiatric systemic lupus erythematosus patients," *Journal of Immunology Research*, vol. 2015, 920711 pages, 2015.

[9] E. Kozora, M. S. Brown, C. M. Filley et al., "Memory impairment associated with neurometabolic abnormalities of the hippocampus in patients with non-neuropsychiatric systemic lupus erythematosus," *Lupus*, vol. 20, no. 6, pp. 598–606, 2010.

[10] I. Shapira-Lichter, E. Vakil, I. Litinsky et al., "Learning and memory-related brain activity dynamics are altered in

systemic lupus erythematosus: a functional magnetic resonance imaging study," *Lupus*, vol. 22, no. 6, pp. 562–573, 2013.

[11] M. B. Lauvsnes, M. K. Beyer, J. T. Kvaløy et al., "Association of hippocampal atrophy with cerebrospinal fluid antibodies against the NR2 subtype of the N-methyl-D-aspartate receptor in patients with systemic lupus erythematosus and patients with primary Sjögren's syndrome," *Arthritis & Rhematology*, vol. 66, no. 12, pp. 3387–3394, 2014.

[12] J. Zhang, A. M. Jacobi, T. Wang, R. Berlin, B. T. Volpe, and B. Diamond, "Polyreactive autoantibodies in systemic lupus erythematosus have pathogenic potential," *Journal of Autoimmunity*, vol. 33, no. 3-4, pp. 270–274, 2009.

[13] M. C. Hochberg, "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 40, no. 9, p. 1725, 1997.

[14] C. Bombardier, D. D. Gladman, M. B. Urowitz et al., "Derivation of the SLEDAI. A disease activity index for lupus patients," *Arthritis and Rheumatism*, vol. 35, no. 6, pp. 630–640, 1992.

[15] D. D. Gladman, D. Ibañez, and M. B. Urowitz, "Systemic lupus erythematosus disease activity index 2000," *The Journal of Rheumatology*, vol. 29, no. 2, pp. 288–291, 2002.

[16] R. C. Oldfield, "The assessment and analysis of handedness: the Edinburgh inventory," *Neuropsychologia*, vol. 9, no. 1, pp. 97–113, 1971.

[17] R. L. Brey, "Neuropsychiatric lupus: clinical and imaging aspects," *Bulletin of the NYU Hospital for Joint Diseases*, vol. 65, no. 3, pp. 194–199, 2007.

[18] T. Bartsch and P. Wulff, "The hippocampus in aging and disease: from plasticity to vulnerability," *Neuroscience*, vol. 309, pp. 1–16, 2015.

[19] H. X. Gao, S. R. Campbell, M. H. Cui et al., "Depression is an early disease manifestation in lupus-prone MRL/lpr mice," *Journal of Neuroimmunology*, vol. 207, no. 1-2, pp. 45–56, 2009.

[20] E. Kozora, D. B. Arciniegas, C. M. Filley et al., "Cognition, MRS neurometabolites, and MRI volumetrics in non-neuropsychiatric systemic lupus erythematosus: preliminary data," *Cognitive and Behavioral Neurology*, vol. 18, no. 3, pp. 159–162, 2005.

[21] S. Liu, Y. Cheng, Y. Zhao et al., "Clinical factors associated with brain volume reduction in systemic lupus erythematosus patients without major neuropsychiatric manifestations," *Frontiers in Psychiatry*, vol. 9, p. 8, 2018.

[22] G. E. Gillies and S. McArthur, "Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines," *Pharmacological Reviews*, vol. 62, no. 2, pp. 155–198, 2010.

[23] T. Ren, R. C. M. Ho, and A. Mak, "Dysfunctional cortico-basal ganglia-thalamic circuit and altered hippocampal-amygdala activity on cognitive set-shifting in non-neuropsychiatric systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 64, no. 12, pp. 4048–4059, 2012.

[24] B. Diamond and B. T. Volpe, "A model for lupus brain disease," *Immunological Reviews*, vol. 248, no. 1, pp. 56–67, 2012.

[25] N. J. Abbott, L. L. F. Mendonça, and D. E. M. Dolman, "The blood-brain barrier in systemic lupus erythematosus," *Lupus*, vol. 12, no. 12, pp. 908–915, 2016.

[26] C. Kowal, L. A. DeGiorgio, T. Nakaoka et al., "Cognition and Immunity: Antibody Impairs Memory," *Immunity*, vol. 21, no. 2, pp. 179–188, 2004.

[27] E. Trysberg, H. Carlsten, and A. Tarkowski, "Intrathecal cytokines in systemic lupus erythematosus with central nervous system involvement," *Lupus*, vol. 9, no. 7, pp. 498–503, 2016.

[28] K. Baraczka, K. Nékám, T. Pozsonyi, I. Szüts, and G. Ormos, "Investigation of cytokine (tumor necrosis factor-alpha, interleukin-6, interleukin-10) concentrations in the cerebrospinal fluid of female patients with multiple sclerosis and systemic lupus erythematosus," *European Journal of Neurology*, vol. 11, no. 1, pp. 37–42, 2004.

[29] M. Bravo-Zehnder, E. M. Toledo, F. Segovia-Miranda et al., "Anti-ribosomal P protein autoantibodies from patients with neuropsychiatric lupus impair memory in mice," *Arthritis & Rhematology*, vol. 67, no. 1, pp. 204–214, 2015.

[30] F. K. Leung and P. R. Fortin, "Intravenous cyclophosphamide and high dose corticosteroids improve MRI lesions in demyelinating syndrome in systemic lupus erythematosus," *The Journal of Rheumatology*, vol. 30, no. 8, pp. 1871–1873, 2003.

[31] D. A. Ballok, J. Woulfe, M. Sur, M. Cyr, and B. Sakic, "Hippocampal damage in mouse and human forms of systemic autoimmune disease," *Hippocampus*, vol. 14, no. 5, pp. 649–661, 2004.

[32] G. Ruiz-Irastorza, M. Ramos-Casals, P. Brito-Zeron, and M. A. Khamashta, "Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review," *Annals of the Rheumatic Diseases*, vol. 69, no. 1, pp. 20–28, 2009.

[33] N. Costedoat-Chalumeau, B. Dunogué, N. Morel, V. le Guern, and G. Guettrot-Imbert, "Hydroxychloroquine: a multifaceted treatment in lupus," *Presse Médicale*, vol. 43, no. 6, pp. e167–e180, 2014.

[34] L. A. González, G. J. Pons-Estel, J. Zhang et al., "Time to neuropsychiatric damage occurrence in LUMINA (LXVI): a multi-ethnic lupus cohort," *Lupus*, vol. 18, no. 9, pp. 822–830, 2009.

[35] O. Grimm, S. Pohlack, R. Cacciaglia et al., "Amygdalar and hippocampal volume: a comparison between manual segmentation, Freesurfer and VBM," *Journal of Neuroscience Methods*, vol. 253, pp. 254–261, 2015.

[36] E. Wenger, J. Mårtensson, H. Noack et al., "Comparing manual and automatic segmentation of hippocampal volumes: reliability and validity issues in younger and older brains," *Human Brain Mapping*, vol. 35, no. 8, pp. 4236–4248, 2014.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 7352637, 9 pages
https://doi.org/10.1155/2020/7352637

Research Article

The Impact of Psoriasis and Metabolic Syndrome on the Systemic Inflammation and Oxidative Damage to Nucleic Acids

Drahomira Holmannova,[1] Lenka Borska,[2] Ctirad Andrys,[3] Pavel Borsky,[1,2] Jan Kremlacek,[2] Kvetoslava Hamakova,[4] Vit Rehacek,[5] Andrea Malkova,[1] Tereza Svadlakova,[1,3] Vladimir Palicka,[6] Jan Krejsek,[3] and Zdenek Fiala[1]

[1]*Institute of Hygiene and Preventive Medicine, Faculty of Medicine in Hradec Kralove, Charles University, Czech Republic*
[2]*Institute of Pathological Physiology, Faculty of Medicine in Hradec Kralove, Charles University, Czech Republic*
[3]*Institute of Clinical Immunology and Allergology, University Hospital and Faculty of Medicine in Hradec Kralove, Charles University, Czech Republic*
[4]*Clinic of Dermatology and Venereology, University Hospital Hradec Kralove, Czech Republic*
[5]*Transfusion Center, University Hospital, Hradec Kralove 500 03, Czech Republic*
[6]*Institute of Clinical Biochemistry and Diagnostics, University Hospital Hradec Kralove and Faculty of Medicine in Hradec Kralove, Charles University, Czech Republic*

Correspondence should be addressed to Pavel Borsky; borskyp@lfhk.cuni.cz

Received 16 February 2020; Revised 14 April 2020; Accepted 27 April 2020; Published 20 May 2020

Academic Editor: Lihua Duan

Background. Psoriasis is a chronic systemic inflammatory disease associated with a wide range of comorbidities, including metabolic syndrome (MetS). Serum calprotectin, ANGPTL8, and oxidative damage to nucleic acids might be associated with both diseases. The presented study describes the influence of psoriasis and MetS on the serum levels of markers of systemic inflammation (calprotectin and ANGPTL8) and markers of oxidative damage to nucleic acids. The applicability of serum levels of calprotectin and ANGPTL8 for monitoring of the activity of psoriasis (diagnostic markers) is also evaluated. *Methods*. Clinical examination (PASI score, MetS), enzyme-linked immunosorbent assay (ELISA), and Enzyme Immunoassay (EIA). Serum calprotectin, ANGPTL8, 8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanosine, and 8-hydroxyguanine. *Results and Conclusions*. The psoriasis significantly increased the serum level of calprotectin and the serum level of oxidative damage to nucleic acids, however not the serum level of ANGPTL8. The presence of MetS did not significantly affect the serum levels of calprotectin, ANGPTL8, and oxidative damage to nucleic acids in either psoriasis patients or controls. It seems that the serum level of calprotectin (but not the serum level of ANGPTL8) could be used as a biomarker for monitoring the activity of psoriasis.

1. Introduction

Inflammation is a precisely controlled, well-regulated response of the immune system to diverse stimuli, which provides protection and defense against both external and internal danger (such as pathogens, toxins, chemicals, cancer, and damaged cells) and promotes regeneration of damaged tissue. A wide range of factors can disrupt immune system functions (immune system homeostasis, self-tolerance, inhibitory/anti-inflammatory mechanisms, termination of inflammation, proresolving, and regenerative processes) and thus transform initially healing process into a destructive immune system response.

Psoriasis, metabolic syndrome (MetS), and oxidative stress are accompanied by low-grade chronic inflammation [1, 2]. Psoriasis is a chronic systemic genetically linked inflammatory (immune-mediated) hyperproliferative condition with a diverse array of clinical manifestations [3, 4]. Psoriasis preferentially affects the skin and also the nails, joints, mucous membranes, etc. The pathogenesis of psoriasis is still

not fully understood. Multiple factors are known to be implicated into the pathogenesis of psoriasis: genetic predisposition (polymorphisms in genes controlling different immunological signaling pathways and processes), abnormal individual reactivity, epigenetic factors, oxidative stress, or skin microbiome alteration [5–9]. These factors alter skin barrier functions; cause defects in differentiation and proliferation of keratinocytes; increase the skin infiltration by immune cells (T cells, macrophages, neutrophils, and dendritic cells); intensify the production of IL-23, IL-22, IL-17A, TNFα, INFγ, and IL-12; and drive the assembly of inflammasomes, and thus the inflammation is triggered and maintained [10, 11].

Oxidative stress, reactive oxygen and nitrogen species, is a key player in the pathogenesis of many human diseases. A wide range of environmental factors (toxins, chemicals, and UV radiation) that interact with the skin and inflammatory reactions in the organisms enhance oxidative stress which then damages cells, their membranes and DNA/RNA, and leads to the release of DAMPs (damage/danger-associated molecular patterns) which include alarmins such as IL1α, HMGB1, and calprotectin, as well as boosts the immune-inflammatory response. Studies have shown that the treatment with antioxidant ameliorates symptoms of inflammatory diseases and reduces their progression (e.g., psoriasis, rheumatoid arthritis, and neurodegenerative or cardiovascular diseases) and decreases the level of calprotectin [12–14].

Calprotectin is a heterocomplex of two calcium-binding molecules S100A8 and S100A9. These alarmins belong to the S100 protein family and are released in heterodimeric form from immune cells in the response to the environmental triggers, oxidative stress, cellular damage, bacterial infections, and inflammation. The main source of calprotectin is neutrophils, monocytes, and macrophages [15]. Calprotectin can serve as a noninvasive biomarker for inflammatory diseases (for example, IBD, rheumatoid arthritis, infections, cardiovascular diseases, MetS, and bronchiolitis obliterans). It can help diagnose inflammation and predict relapse in patients in clinical remission [16–19].

There is also a link between inflammation and MetS and thus psoriasis as well. The MetS is associated with overweight or obesity. Adipose tissue is an active endocrine organ which produces adipokines and proinflammatory cytokines that attract immune system cells, mainly monocytes, and support differentiation of infiltrating macrophages into M1 proinflammatory subset; therefore, the presence of the MetS might negatively regulate the course psoriasis and vice versa [20–23]. The MetS, as well as dyslipidemia, insulin resistance, and fatty liver disease, is characterized by an increased expression of angiopoietin-like 8 (ANGPTL8; betatrophin). ANGPTL8 is a liver and adipose tissue-specific glycoprotein with ability to regulate both glucose and lipid metabolism by inhibition of lipoprotein lipase. ANGPTL8 is rhythmically expressed, but the level of expression depends on stimuli such as insulin and calorie intake [24–26].

The study describes the influence of psoriasis and MetS on the serum indicators of systemic inflammation (calprotectin and ANGPTL8) and on the serum indicators of oxidative damage to nucleic acids. The paper also evaluates the applicability of serum levels of calprotectin and ANGPTL8 for monitoring of the activity of psoriasis (diagnostic markers).

2. Methods

2.1. Study Groups. The experimental group consisted of 44 patients with psoriasis. Patients were examined at the Clinic of Dermal and Venereal Disease, Charles University Hospital in Hradec Kralove. The control group included 80 healthy blood donors from the Department of Transfusion Medicine, Charles University Hospital in Hradec Kralove. All subjects signed the informed consent before participating in the study. The persons with any inflammatory diseases, pregnancy, and those using nonsteroidal or anti-inflammatory medications were excluded. Patients had no treatment of psoriasis in three months before the enrollment of the study.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Faculty Hospital in Hradec Kralove, Czech Republic (project identification code PROGRES Q40-09 and Q40-10, reference number 201705 I83P, date 2 May 2017).

2.2. Disease Status Determination: PASI Score. The severity of the disease was assessed using a standardized clinical evaluation—Psoriasis Area Severity Index (PASI), which is calculated based on erythema, desquamation, and skin infiltration [27].

2.3. Metabolic Syndrome (MetS). We followed the methods of Borska et al. We evaluated the presence of MetS in observed subjects according to the criteria of the National Cholesterol Education Program Adult Treatment Panel (NCE/ATPIII). The diagnosis of MetS was declared when three of the five listed criteria were present: (1) increased waist circumference or abdominal obesity (≥102 cm for men; ≥88 cm for women), (2) glucose intolerance (fasting glucose ≥ 5.6 mmol/L or known treatment of diabetes), (3) elevated level of triglycerides (TAG) ≥ 1.7 mmol/L, (4) reduced level of high-density lipoproteins (HDL cholesterol), and (5) elevated blood pressure (>130/85 mmHg) [28, 29].

2.4. Blood Sample Collection. Peripheral blood samples were collected from the cubital vein in both groups by BD Vacutainer sampling tubes. Blood serum was isolated by centrifugation, and the samples were stored at −70°C. Repeated thawing and freezing were avoided.

2.5. Analysis of Calprotectin. Concentrations of calprotectin were analyzed in the serum samples with enzyme-linked immunosorbent assay (ELISA) using the Human S100A8/A9 (calprotectin) ELISA kit (BioVendor, Czech Republic) according to the manufacturer's instructions. Samples were 200-fold diluted. The sensitivity of the kit was 0.22 ng/mL. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Analysis of ANGPTL8. Serum levels of ANGPTL8 were determined by using the ELISA kit (enzyme-linked immunosorbent assay kit) for ANGPTL8 (Cloud-Clone

Corp., TX, USA) according to the manufacturer's instructions. Detection limit of the kit was 0.056 ng/mL. Samples were 30-fold diluted. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Analysis of Oxidative Damage to Nucleic Acids. The EIA Kit (Enzyme Immunoassay, Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure the level of oxidative damage to nucleic acids (DNA/RNA damage). The damage was shown as the sum of three oxidized guanine species in serum: 8-hydroxy-2′-deoxyguanosine released from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from DNA or RNA. We measured the level of DNA/RNA damage in pg of all guanine species per mL of serum. The detection limit was 33 pg/mL of serum. We followed the methods of Borska et al. [28].

2.8. Statistical Analysis. The data were statistically processed by the R software version 3.6.1 "nortest," "compute.es," and "ggplot2." Based on normality distribution evaluation (the Anderson–Darling test), parametric or nonparametric tests were used. The relationship between parameters was evaluated either by Pearson's or by Spearman's correlation test. Differences among groups were assessed using Student's t or Wilcoxon rank-sum test. The null hypothesis was rejected when the probability level (p) reached below 0.05 (the alpha level) [30–32].

3. Results

3.1. Study Groups. A total of 124 subjects were examined: 44 patients with psoriasis (22 patients without metabolic syndrome = patients No-MetS and 20 patients with metabolic syndrome = patients MetS) and 80 healthy controls (44 controls without metabolic syndrome = controls No-MetS and 36 controls with metabolic syndrome = controls MetS). The age distribution did not differ significantly between patients and controls (average age: 45.4 vs. 43.6; min–max age: 18–84 vs. 18–80; Figure 1).

We found that PASI scores of patients MetS were 17.4/19.3 (median/mean) and patients No-MetS 19.7/23.6 (Figure 2). No significant difference in the PASI score was observed between patients MetS and patients No-MetS.

3.2. Analysis of Calprotectin. The serum level of calprotectin was significantly elevated in patients compared to the controls regardless of the presence of MetS ($p < 0.001$; Table 1, Figures 3 and 4). In both, the patient and control groups, the presence of MetS resulted in an insignificant increase in calprotectin levels (Table 2, Figure 4).

3.3. Analysis of ANGPTL8. No significant differences in the serum level of ANGPTL8 were observed between patients and controls, even among subgroups (Table 1). Neither in the patient group nor in the control group has the presence of MetS significantly influence the ANGPTL8 level compared to those without MetS (Table 2).

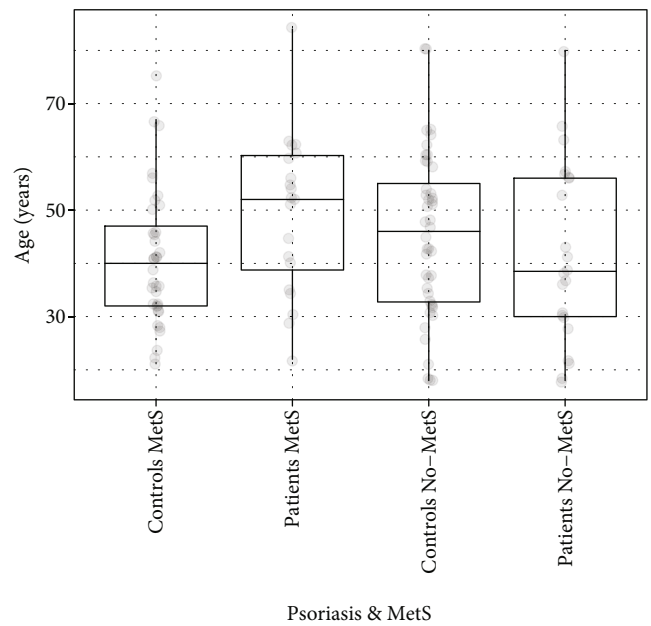


Figure 1: Age characteristics of monitored groups. Legend: data are graphically displayed as box blots (the minimum value is represented by points below the box, 1st line (bottom) of the box represents first quartile (Q1), 2nd (middle of the box) median, 3rd (top of the box) third quartile (3Q), points above the box represent the maximum value).

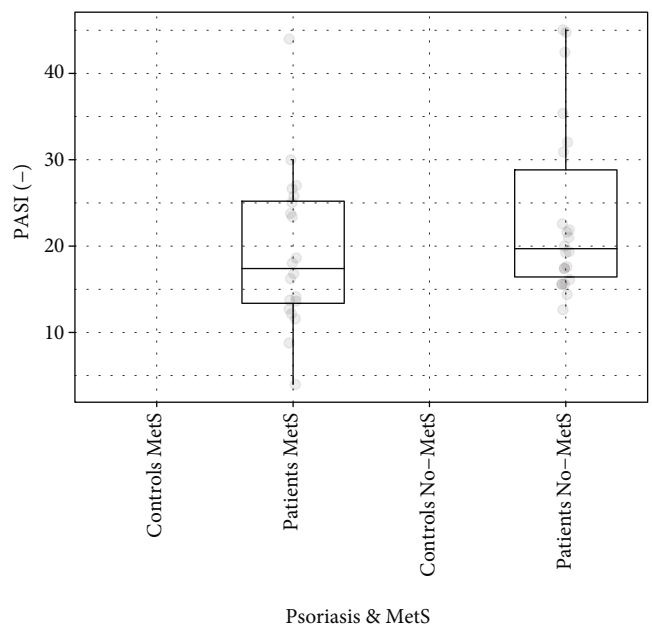


Figure 2: The PASI score in patients with and without MetS.

3.4. Analysis of Oxidative Damage to Nucleic Acids. The serum level of the oxidative damage to nucleic acids was significantly elevated in patients compared to controls regardless of the presence of MetS ($p < 0.001$; Table 1, Figures 5 and 6). Neither in the patient group nor in the control group has the presence of MetS significantly influence the oxidative

Table 1: The levels of calprotectin, ANGPTL8, and oxidative damage to nucleic acids in controls and patients.

Parameter	Median (Q1–Q3)	Significance of the differences
Calprotectin (pg/mL)		
Controls	1943.0 (1260.0–2488.0)	$p < 0.001$
Patients	5231.0 (4159.0–8041.0)	
ANGPTL8 (ng/mL)		
Controls	11.97 (9.63–15.10)	NS
Patients	13.80 (10.83–14.72)	
Oxidative damage to nucleic acids (pg/mL)		
Controls	1730.0 (981.0–2643.4)	$p < 0.001$
Patients	3269.3 (2004.4–4505.9)	

Legend: Q1: first quartile; Q3: third quartile.

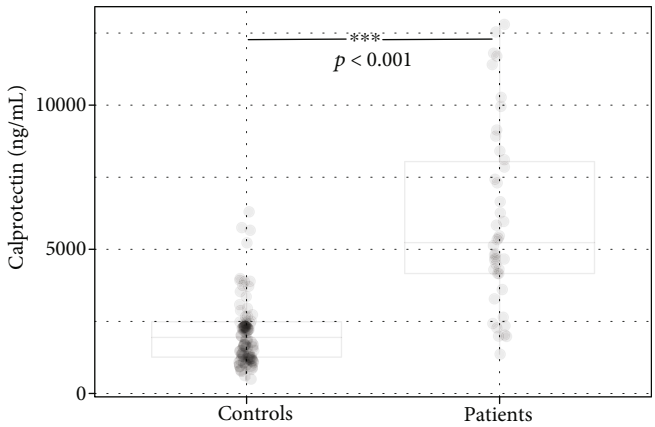


Figure 3: The levels of calprotectin in controls and patients.

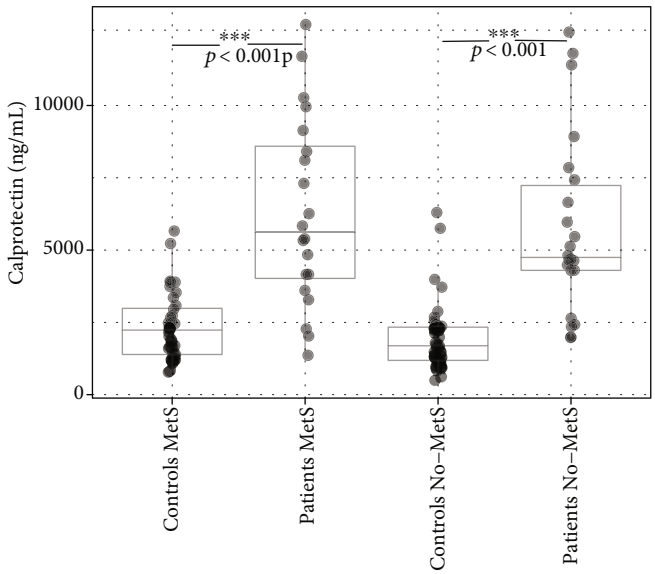


Figure 4: The levels of calprotectin in controls and patients with and without MetS.

damage to nucleic acids compared to those without MetS (Table 2, Figure 6).

3.5. Relationships among Measured Parameters. The relations between the age, PASI score, ANGPTL8, oxidative damage to nucleic acids, and calprotectin were investigated in patients. We found negative correlation between the age and PASI score ($r = -0.40$; $p < 0.01$) and positive correlation between the PASI score and oxidative damage to nucleic acids (0.44; $p < 0.01$) (Figure 7).

4. Discussion

Psoriasis is a chronic systemic multifactorial inflammatory disease with genetic predisposition which is accompanied with the activation of the immune system cells, resident skin cells, increased production of cytokines, chemokines, and other soluble substrates; some of them could serve as a screening indicators for psoriasis (for diagnostics, prognostics, or monitoring the treatment efficacy), for oxidative stress, and for MetS.

Previous studies have shown that serum levels of calprotectin and oxidative damage to nucleic acids are increased in patients with psoriasis. Also, in our study, the levels of calprotectin were significantly higher in patients with psoriasis compared to control. The presence of MetS led to an increase in calprotectin levels in patients and controls; however, this increase did not reach the level of statistical significance. Other authors obtained similar results as we did [33–35].

Calprotectin can be considered as a promising biomarker of inflammation, psoriasis, and its complication, such as arthritis. Zaki et al. as well as D'Amico et al. confirmed that biological therapy decreased both inflammation and the level of calprotectin [34, 35]. Zaki et al., Hamza et al., and Greco et al. documented that the levels of calprotectin significantly correlated with the PASI score; however, in our study, the correlation between PASI score and calprotectin was not statistically significant [33, 34].

As we mentioned previously, the level of calprotectin was increased in our patients with psoriasis, especially in patients with metabolic syndrome. Unsurprisingly, the same pattern of calprotectin secretion was documented in healthy controls; increased levels were in control with metabolic syndrome. Psoriasis is undoubtedly associated with inflammation and metabolic syndrome which is also inflammatory condition; thus, both psoriasis and metabolic syndrome might be accompanied by the elevated level of calprotectin. Pedersen et al. also proved that patients with diabetes mellitus type II with clinical complication such as cardiovascular disease and obesity had higher concentration of plasma calprotectin than the general population; they also described a positive correlation between the level of calprotectin BMI, triglycerides, hs-CRP, insulin, and negative correlation with HDL cholesterol, etc. [18]. Metabolic syndrome includes, besides other disorders, obesity and atherosclerosis. Obesity has been shown to promote expansion of Th17 cells and IL-17 production in adipose tissue; atherosclerosis is defined as a chronic inflammatory process with endothelial dysfunction, deposits of lipids, and macrophages in the arterial walls; moreover, it

Table 2: The levels of calprotectin, ANGPTL8, and oxidative damage to nucleic acids in patients and controls with and without MetS.

Parameter	Q1	Median	Q3	Geometric mean	Min	Max	Significance of the differences
Calprotectin (pg/mL)							
Patients No-MetS	4298.0	4746.0	7233.8	5746.0	1976.0	12538.0	NS
Patients MetS	4020.3	5612.5	8590.8	6310.3	1360.0	12800.0	
Controls No-MetS	1185.8	1690.5	2334.0	1964.5	497.0	6299.0	NS
Controls MetS	1389.3	2232.5	2984.5	2358.7	779.0	5659.0	
ANGPTL8 (ng/mL)							
Patients No-MetS	10.78	13.80	19.00	14.70	5.78	25.50	NS
Patients MetS	10.98	13.92	19.98	14.74	4.60	24.30	
Controls No-MetS	10.02	12.20	15.09	12.63	2.30	25.40	NS
Controls MetS	9.47	11.75	15.60	12.98	2.10	32.80	
Oxidative damage to nucleic acids (pg/mL)							
Patients No-MetS	2004	2931	4506	3415	700	8150	NS
Patients MetS	2270	3517	4523	3651	500	13050	
Controls No-MetS	1250	1805	3612	2665	191	16100	NS
Controls MetS	786	1465	2339	1897	128	7800	

Legend: Q1: first quartile; Q3: third quartile.

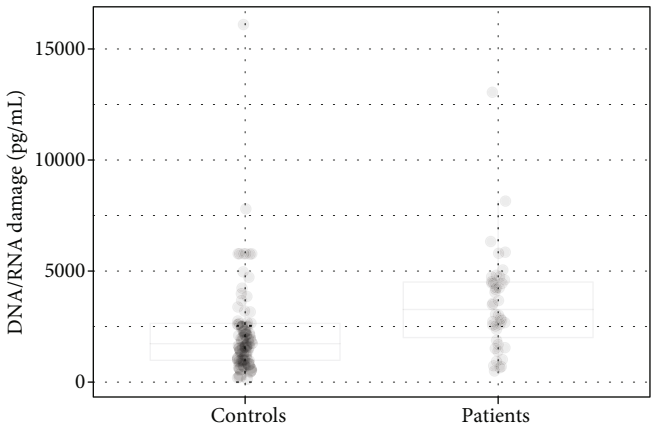


Figure 5: The levels of oxidative damage to nucleic acids in controls and patients.

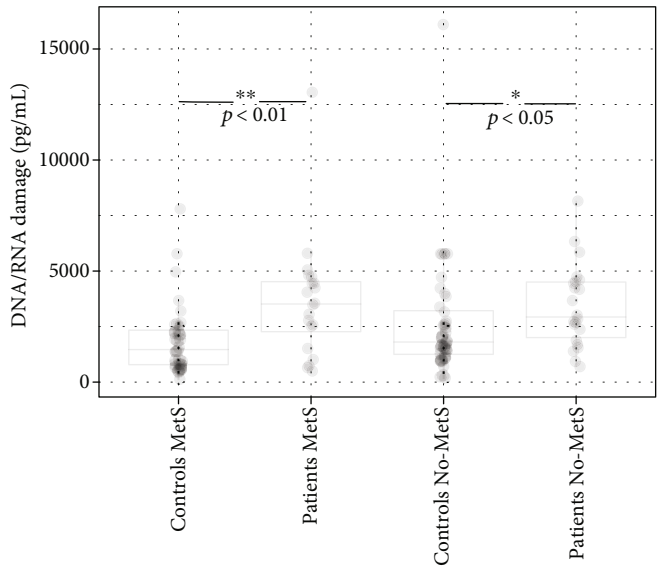


Figure 6: The levels of oxidative damage to nucleic acids in controls and patients with and without MetS.

has been reported that Th17 and IL-17A are involved in the progression of atherosclerosis. Th17 and IL-17 are important players in pathogenesis of psoriasis, one of the many proinflammatory signals linking atherosclerosis (obesity and metabolic syndrome) and psoriasis [36, 37].

Pirowska et al. assessed the levels of proinflammatory cytokines IL-23, IL-17, etc. in serum of patients with psoriasis and psoriatic arthritis in order to establish the correlation between cytokines and PASI score and risk of obesity and metabolic syndrome. Psoriatic patients with metabolic syndrome had a higher level of serum IL-17 and IL-23 than patients without metabolic syndrome [38]. But we have to take into consideration that there are other factors involved in the pathogenesis of psoriasis and metabolic syndrome. These processes are much more complex and influenced by many endogenous and exogenous factors, e.g., oxidative stress. Oxidative stress is a condition that refers to an imbalance between the production of oxygen/nitrogen reactive species and the ability of an organism to detoxify them. Inflammation and oxidative stress are closely linked and implicated in many chronic diseases.

Psoriasis and metabolic syndrome, as systemic inflammatory diseases, are accompanied with higher production of ROS that react with cellular biomolecules including DNA and RNA. Other studies have proven that levels of oxidative damage to nucleic acids are increased in patients with psoriasis [12, 14, 39–42]. Kaur et al. documented that activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase enzymes, is reduced in patients with psoriasis [43]. Our study confirmed the results of previous studies. The level of oxidative damage to nucleic acids was significantly increased in patients with psoriasis

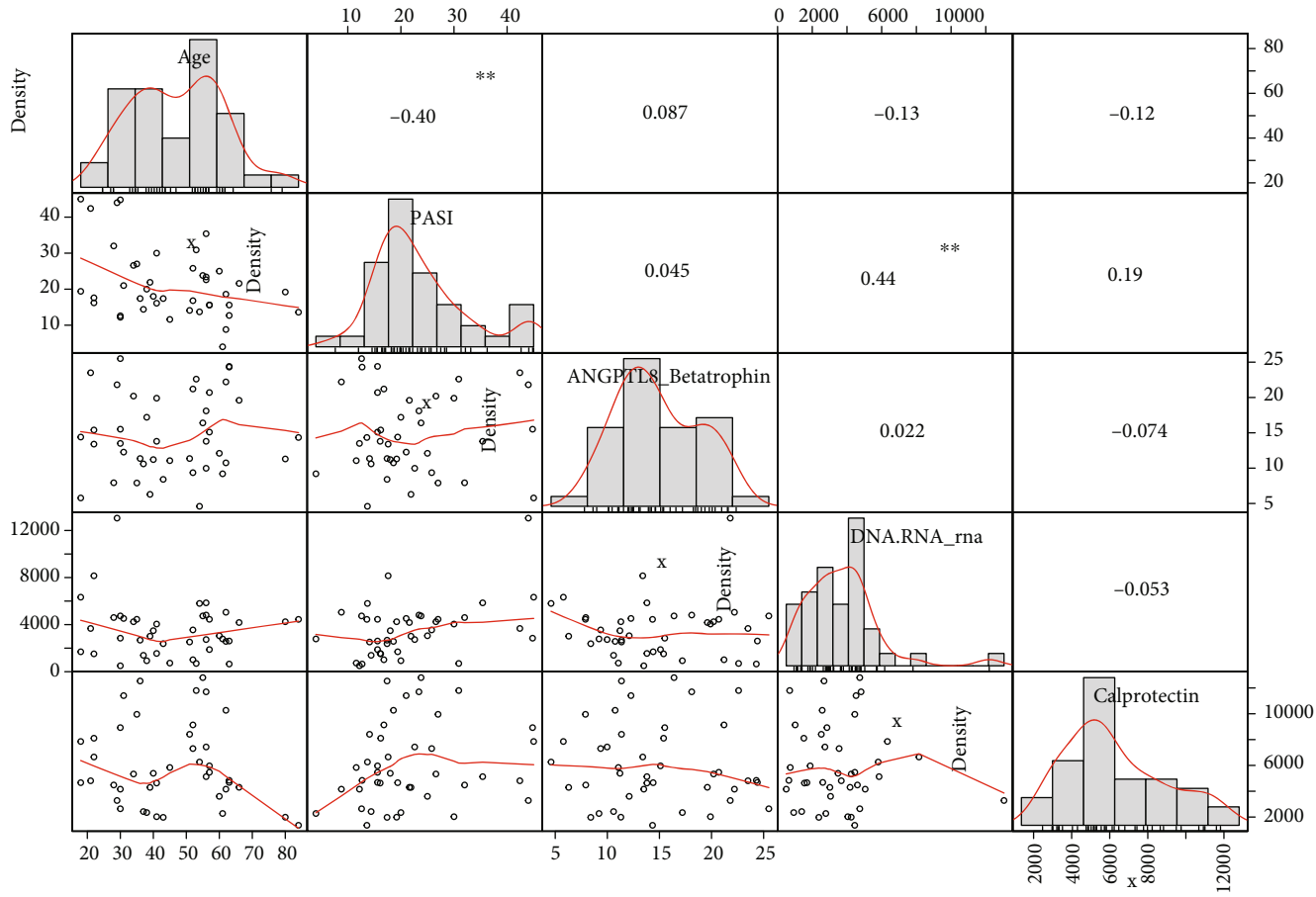


Figure 7: Relationships among measured parameters (Spearman rank correlation).

compared to healthy controls. Subsequent analysis revealed that levels of oxidative damage to nucleic acids did not significantly differ among subjects with and without metabolic syndrome. The levels of the damage were insignificantly higher in patients with metabolic syndrome, but insignificantly lower in controls with metabolic syndrome. We do not have yet an objective explanation for the latter phenomenon. The level of oxidative damage to nucleic acids also positively correlated with the PASI score; negative correlation was documented between the PASI score and age of patients enrolled in the study.

There are many molecules that modulate inflammatory processes, and their expression is modulated by acute or chronic inflammation. One of them is ANGPTL8, a glycoprotein involved in inflammatory response, fatty acid, and saccharide metabolism. The expression of ANGPTL8 is driven by inflammatory environment, feeding, glucose, and insulin level. Its levels are elevated in patients with dyslipidemia, cardiovascular diseases, diabetes mellitus, SIRS, hepatosteatosis, hypothyroidism, diabetic retinopathy, pregnancy, PCOS (especially in patients with metabolic syndrome), and nephropathy associated with diabetes mellitus. ANGPTL8 levels negatively correlates with HDL cholesterol and LPL and positively with TCG and VEGF [44–53]. The opposite observations have been presented by Gómez-Ambrosi et al. and Wang et al. [54, 55]. Their results provided evidence that the level of ANGPTL 8 is decreased in patients with obesity and diabetes mellitus type II. These conflicting results might be explained with ANGPTL8 complexity, its involvement in a wide range of processes, or methods used for ANGPTL8 detection. Some commercial kits detect only a full-length form, while some measured both full-length and cleaved C and N-terminus of ANGPTL8. Both Gómez-Ambrosi et al. and Wang et al. used the same kit for ANGPTL8 detection.

Factors that decrease the level of ANGPTL8 includes hyperinsulinemia, TNFα, miRNA 143-3p from hepatocytes, and miRNA 221-3p which expression is promoted by activated macrophages in adipose cells, glucocorticoids during fasting, etc. [51, 56–58]. The level of ANGPTL is also lower in patients with Graves' disease [59]. The study conducted by Zhang et al. proved that ANGPTL8 negatively regulates NF-κB activation. ANGPTL8 binds p62, as a coreceptor, and IKKγ, thereby forms a functional complex, and facilitates selective autophagic IKKγ degradation. ANGPTL 8 might suppress acute inflammatory response; it is a negative feedback regulator controlling inflammation [60]. The opposite results were described by Liao et al. The grade of intervertebral disc degeneration which is associated with inflammation positively correlated with mRNA ANGPTL8. The expression of ANGPLT8 was upregulated by TNFα and overexpression of ANGPTL8 significantly increased the expression of MMP3, MMP9, and IL-6 [61].

Knowing these data, we assumed that the increase or decrease of levels of ANGPTL8 in psoriatic patients and all subjects with metabolic syndrome could occur. To date, the levels of ANGPTL8 were not measured in psoriatic patients with/without metabolic syndrome. Although psoriasis as well as metabolic syndrome is related to systemic inflammatory conditions, and metabolic syndrome is associated with a higher value of ANGPTL8 in some previous studies, we found only insignificant differences among groups of patients and controls (with a lower level in controls), among patients with and without metabolic syndrome and among controls with and without metabolic syndrome. It seems that the presence of metabolic syndrome did not influence the levels of ANGPTL8; on the other hand, the presence of psoriasis slightly altered the expression of ANGPTL8. So, there is a possibility that pathological reactions involved in the pathogenesis of psoriasis might influence the expression of ANGPTL8 more than those involved in metabolic syndrome.

We might only speculate why the level of ANGPTL8 did not increase, despite the presence of inflammation and metabolic syndrome, as we expected. There are still many unknown factors and uncovered mechanisms that might interfere with the production and functions of ANGPTL8 (factors inhibiting or altering the synthesis of ANGPTL8, the interactions of ANGPTL8 with other molecules, the role of microbiota which regulates lipid metabolism and the immune system functions, and the presence of truncated variants of ANGPTL8) [62]. As we mentioned previously, the study performed by Gómez-Ambrosi et al. and Wang et al. [54, 55] did not document the increase of the ANGPTL8 level. Similar results as those obtained by Gómez-Ambrosi et al. and Wang et al. were also presented by Guo et al. and Tuhan et al. [63, 64]. They reported that the level of ANGPTL8 negatively correlated with hyperglycaemia and insulin resistance, the conditions which are normally associated with metabolic syndrome, but not with overweight or obesity. Fu et al. also pointed the conflicted results regarding the level of ANGPTL8 in human studies. They revealed the positive correlation between the level of vitamin D and ANGPTL8, so the vitamin D deficiency was associated with a lower level of ANGPTL8 [65]. The prevalence of vitamin D deficiency is high in the population of the Czech Republic as the deficiency is presented in 30-60% of Czechs [66, 67]. We cannot omit the fact that the results (level of ANGPTL8) also depend on the kit used, as mentioned earlier.

Considering these facts (unknown factors, hyperglycaemia, high intensity of insulin resistance, vitamin D deficiency, etc.), it is impossible to come to any proper conclusion why the level of ANGPTL8 did not increase. This research has raised many questions in need of further investigation.

5. Conclusions

The results of the study show that psoriasis significantly increased the serum level of calprotectin (indicator of systemic inflammation) and the serum level of oxidative damage to nucleic acids, however not the serum level of ANGPTL8 (indicator of systemic inflammation). The presence of MetS did not significantly affect the serum levels of calprotectin, ANGPTL8, and oxidative damage to nucleic acids in either psoriasis patients or controls. It seems that the serum level of calprotectin (but not serum level of ANGPTL8) could be used as a biomarker for monitoring the activity of psoriasis (diagnostic marker).

Data Availability

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

Conflicts of Interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors acknowledge Mgr. Dana Knajflova for text proofreading and linguistics. The study was supported by Charles University, Faculty of Medicine in Hradec Kralove, the Czech Republic, by projects Q40-09, Q40-10, Q40-11, and SVV-260397/2017.

References

[1] M. M. J. van Greevenbroek, C. G. Schalkwijk, and C. D. A. Stehouwer, "Dysfunctional adipose tissue and low-grade inflammation in the management of the metabolic syndrome: current practices and future advances," *F1000Research*, vol. 5, article 2515, 2016.

[2] B. B. Davidovici, N. Sattar, P. C. Jörg et al., "Psoriasis and systemic inflammatory diseases: potential mechanistic links between skin disease and co-morbid conditions," *The Journal of Investigative Dermatology*, vol. 130, no. 7, pp. 1785–1796, 2010.

[3] A. Rendon and K. Schäkel, "Psoriasis pathogenesis and treatment," *International Journal of Molecular Sciences*, vol. 20, no. 6, p. 1475, 2019.

[4] N. J. Korman, "Management of psoriasis as a systemic disease: what is the evidence?," *British Journal of Dermatology*, vol. 182, 2020.

[5] A. Osmola-Mańkowska, E. Teresiak-Mikołajczak, M. Skrzypczak-Zielińska, and Z. Adamski, "Genetic polymorphism in psoriasis and its meaning for the treatment efficacy in the future," *Advances in Dermatology and Allergology*, vol. 35, no. 4, pp. 331–337, 2018.

[6] J. M. Whyte, J. J. Ellis, M. A. Brown, and T. J. Kenna, "Best practices in DNA methylation: lessons from inflammatory bowel disease, psoriasis and ankylosing spondylitis," *Arthritis Research & Therapy*, vol. 21, no. 1, p. 133, 2019.

[7] W.-M. Wang and H.-Z. Jin, "Skin microbiome: an actor in the pathogenesis of psoriasis," *Chinese Medical Journal*, vol. 131, no. 1, pp. 95–98, 2018.

[8] H. W. Chang, D. Yan, R. Singh et al., "Alteration of the cutaneous microbiome in psoriasis and potential role in Th17 polarization," *Microbiome*, vol. 6, no. 1, p. 154, 2018.

[9] N. D. Loft, L. Skov, M. K. Rasmussen et al., "Genetic polymorphisms associated with psoriasis and development of psoriatic arthritis in patients with psoriasis," *PLoS One*, vol. 13, no. 2, article e0192010, 2018.

[10] C. Albanesi, S. Madonna, P. Gisondi, and G. Girolomoni, "The interplay between keratinocytes and immune cells in the pathogenesis of psoriasis," *Frontiers in Immunology*, vol. 9, p. 1549, 2018.

[11] I. Lorthois, M. Simard, S. Morin, and R. Pouliot, "Infiltration of T cells into a three-dimensional psoriatic skin model mimics pathological key features," *International Journal of Molecular Sciences*, vol. 20, no. 7, p. 1670, 2019.

[12] D. P. Kadam, A. N. Suryakar, R. D. Ankush, C. Y. Kadam, and K. H. Deshpande, "Role of oxidative stress in various stages of psoriasis," *Indian Journal of Clinical Biochemistry*, vol. 25, no. 4, pp. 388–392, 2010.

[13] X. Lin and T. Huang, "Oxidative stress in psoriasis and potential therapeutic use of antioxidants," *Free Radical Research*, vol. 50, no. 6, pp. 585–595, 2016.

[14] V. Rani, G. Deep, R. K. Singh, K. Palle, and U. C. S. Yadav, "Oxidative stress and metabolic disorders: pathogenesis and therapeutic strategies," *Life Sciences*, vol. 148, pp. 183–193, 2016.

[15] I. Stríz and I. Trebichavský, "Calprotectin - a pleiotropic molecule in acute and chronic inflammation," *Physiological Research*, vol. 53, no. 3, pp. 245–253, 2004.

[16] F. Ometto, L. Friso, D. Astorri et al., "Calprotectin in rheumatic diseases," *Experimental Biology and Medicine*, vol. 242, no. 8, pp. 859–873, 2017.

[17] L. Pedersen, M. Nybo, M. K. Poulsen, J. E. Henriksen, J. Dahl, and L. M. Rasmussen, "Plasma calprotectin and its association with cardiovascular disease manifestations, obesity and the metabolic syndrome in type 2 diabetes mellitus patients," *BMC Cardiovascular Disorders*, vol. 14, no. 1, p. 196, 2014.

[18] C. Kerkhoff, A. Voss, T. E. Scholzen, M. M. Averill, K. S. Zänker, and K. E. Bornfeldt, "Novel insights into the role of S100A8/A9 in skin biology," *Experimental Dermatology*, vol. 21, no. 11, pp. 822–826, 2012.

[19] P.-S. Jerkic, F. Michel, A. Kochems, R. Schubert, M. Rosewich, and S. Zielen, "Calprotectin as new sensitive marker of neutrophilic inflammation in patients with bronchiolitis obliterans," *European Respiratory Journal*, vol. 52, article PA4649, 2018.

[20] S. B. Kaushik and M. G. Lebwohl, "Psoriasis: which therapy for which patient: psoriasis comorbidities and preferred systemic agents," *Journal of the American Academy of Dermatology*, vol. 80, no. 1, pp. 27–40, 2019.

[21] L. Russo and C. N. Lumeng, "Properties and functions of adipose tissue macrophages in obesity," *Immunology*, vol. 155, no. 4, pp. 407–417, 2018.

[22] L. Boutens, G. J. Hooiveld, S. Dhingra, R. A. Cramer, M. G. Netea, and R. Stienstra, "Unique metabolic activation of adipose tissue macrophages in obesity promotes inflammatory responses," *Diabetologia*, vol. 61, no. 4, pp. 942–953, 2018.

[23] F. Villarroya, R. Cereijo, A. Gavaldà-Navarro, J. Villarroya, and M. Giralt, "Inflammation of brown/beige adipose tissues in obesity and metabolic disease," *Journal of Internal Medicine*, vol. 284, no. 5, pp. 492–504, 2018.

[24] J. F. Haller, I. J. Mintah, L. M. Shihanian et al., "ANGPTL8 requires ANGPTL3 to inhibit lipoprotein lipase and plasma triglyceride clearance," *Journal of Lipid Research*, vol. 58, no. 6, pp. 1166–1173, 2017.

[25] M. Luo and D. Peng, "ANGPTL8: an important regulator in metabolic disorders," *Frontiers in Endocrinology*, vol. 9, p. 169, 2018.

[26] O. Kovrov, K. K. Kristensen, E. Larsson, M. Ploug, and G. Olivecrona, "On the mechanism of angiopoietin-like protein 8 for control of lipoprotein lipase activity," *Journal of Lipid Research*, vol. 60, no. 4, pp. 783–793, 2019.

[27] E. Salihbegovic, N. Hadzigrahic, and A. Cickusic, "Psoriasis and metabolic syndrome," *Medical Archives*, vol. 69, no. 2, pp. 85–87, 2015.

[28] L. Borska, J. Kremlacek, C. Andrys et al., "Systemic inflammation, oxidative damage to nucleic acids, and metabolic syndrome in the pathogenesis of psoriasis," *International Journal of Molecular Sciences*, vol. 18, no. 11, p. 2238, 2017.

[29] "Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III) final report," *Circulation*, vol. 106, no. 25, pp. 3143–3421, 2002.

[30] H. Wickham, *Ggplot 2: Elagant Graphics for Data Analysis*, Springer, 2016.

[31] A. C. Del Re, *Compute.es: compute effect sizes*, R Packag, 2013, http://cran.r-project.org/web/packages/compute.es.

[32] Gross Juergen LU, *Nortest: tests for normality*, 2015, https://cran.r-project.org/package=nortest.

[33] A. V. Greco and A. Gasbarrini, "Serum calprotectin correlates with risk and disease severity in psoriasis patients and the decrease of calprotectin predicts better response to tumor necrosis factor inhibitors," *European Review for Medical and Pharmacological Sciences*, vol. 14, no. 2, 2010.

[34] A. M. Zaki, M. A. Amer, N. M. A. Mohamed, and M. A. E. Abdelkhalik, "Evaluation of serum level of calprotectin in patients with psoriasis and its relation to the clinical severity of the disease," *The Egyptian Journal of Hospital Medicine*, vol. 76, no. 4, pp. 3919–3923, 2019.

[35] C. Hansson, C. Eriksson, and G.-M. Alenius, "S-calprotectin (S100A8/S100A9): a potential marker of inflammation in patients with psoriatic arthritis," *Journal of Immunology Research*, vol. 2014, Article ID 696415, 5 pages, 2014.

[36] S. Winer, G. Paltser, Y. Chan et al., "Obesity predisposes to Th17 bias," *European Journal of Immunology*, vol. 39, no. 9, pp. 2629–2635, 2009.

[37] S. Taleb, A. Tedgui, and Z. Mallat, "IL-17 and Th17 cells in Atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 35, no. 2, pp. 258–264, 2015.

[38] M. Pirowska, A. Obtułowicz, S. Lipko-Godlewska, A. Goździalska, K. Podolec, and A. Wojas-Pelc, "The level of proinflammatory cytokines: interleukins 12, 23, 17 and tumor necrosis factor α in patients with metabolic syndrome accompanying severe psoriasis and psoriatic arthritis," *Advances in Dermatology and Allergology*, vol. 35, no. 4, pp. 360–366, 2018.

[39] A. Karaman, H. Aydın, B. Geçkinli, A. Çetinkaya, and S. Karaman, "DNA damage is increased in lymphocytes of patients with metabolic syndrome," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 782, pp. 30–35, 2015.

[40] Q. Zhou, U. Mrowietz, and M. Rostami-Yazdi, "Oxidative stress in the pathogenesis of psoriasis," *Free Radical Biology & Medicine*, vol. 47, no. 7, pp. 891–905, 2009.

[41] K. Asha, A. Singal, S. B. Sharma, V. K. Arora, and A. Aggarwal, "Dyslipidaemia & oxidative stress in patients of psoriasis: emerging cardiovascular risk factors," *The Indian Journal of Medical Research*, vol. 146, no. 6, pp. 708–713, 2017.

[42] I. Shimizu, Y. Yoshida, M. Suda, and T. Minamino, "DNA damage response and metabolic disease," *Cell Metabolism*, vol. 20, no. 6, pp. 967–977, 2014.

[43] M. Kaur, S. Sharma, S. Kukreja, J. Kaur, and R. Bassi, "Study of oxidative stress in patients of psoriasis," *International Journal of Research in Dermatology*, vol. 2, no. 4, p. 95, 2016.

[44] M. Abu-Farha, J. Abubaker, I. Al-Khairi et al., "Circulating angiopoietin-like protein 8 (betatrophin) association with HsCRP and metabolic syndrome," *Cardiovascular Diabetology*, vol. 15, no. 1, 2016.

[45] Z. Fu, F. Berhane, A. Fite, B. Seyoum, A. B. Abou-Samra, and R. Zhang, "Elevated circulating lipasin/betatrophin in human type 2 diabetes and obesity," *Scientific Reports*, vol. 4, p. 5013, 2014.

[46] M. Yi, R.-P. Chen, R. Yang, X.-F. Guo, J.-C. Zhang, and H. Chen, "Betatrophin acts as a diagnostic biomarker in type 2 diabetes mellitus and is negatively associated with HDL-cholesterol," *International Journal of Endocrinology*, vol. 2015, Article ID 479157, 7 pages, 2015.

[47] Y.-H. Lee, S.-G. Lee, C. J. Lee et al., "Association between betatrophin/ANGPTL8 and non-alcoholic fatty liver disease: animal and human studies," *Scientific Reports*, vol. 6, p. 24013, 2016.

[48] M. Calan, O. Yilmaz, T. Kume et al., "Elevated circulating levels of betatrophin are associated with polycystic ovary syndrome," *Endocrine*, vol. 53, no. 1, pp. 271–279, 2016.

[49] L. K. Trebotic, P. Klimek, A. Thomas et al., "Circulating betatrophin is strongly increased in pregnancy and gestational diabetes mellitus," *PLoS One*, vol. 10, no. 9, article e0136701, 2015.

[50] C.-C. Chen, H. Susanto, W.-H. Chuang, T.-Y. Liu, and C.-H. Wang, "Higher serum betatrophin level in type 2 diabetes subjects is associated with urinary albumin excretion and renal function," *Cardiovascular Diabetology*, vol. 15, p. 3, 2016.

[51] P. A. Nidhina Haridas, J. Soronen, S. Sädevirta et al., "Regulation of angiopoietin-like proteins (ANGPTLs) 3 and 8 by insulin," *The Journal of Clinical Endocrinology and Metabolism*, vol. 100, no. 10, pp. E1299–E1307, 2015.

[52] L. Yang, R. Yin, Z. Wang, X. Wang, Y. Zhang, and D. Zhao, "Circulating Angptl3 and Angptl8 are increased in patients with hypothyroidism," *BioMed Research International*, vol. 2019, 3814689 pages, 2019.

[53] Q. Lu, L. Lu, W. Chen, and P. Lu, "Expression of angiopoietin-like protein 8 correlates with VEGF in patients with proliferative diabetic retinopathy," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 255, no. 8, pp. 1515–1523, 2017.

[54] J. Gómez-Ambrosi, E. Pascual, V. Catalán et al., "Circulating betatrophin concentrations are decreased in human obesity and type 2 diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 10, pp. E2004–E2009, 2014.

[55] H. Wang, Y. Lai, C. Han et al., "The effects of serum ANGPTL8/betatrophin on the risk of developing the metabolic syndrome – a prospective study," *Scientific Reports*, vol. 6, no. 1, p. 28431, 2016.

[56] J. K. DiStefano, "Angiopoietin-like 8 (ANGPTL8) expression is regulated by miR-143-3p in human hepatocytes," *Gene*, vol. 681, pp. 1–6, 2019.

[57] R. Mysore, F. J. Ortega, J. Latorre et al., "MicroRNA-221-3p regulates angiopoietin-like 8 (ANGPTL8) expression in adipocytes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 102, no. 11, pp. 4001–4012, 2017.

[58] F. Dang, R. Wu, P. Wang et al., "Fasting and feeding signals control the oscillatory expression of Angptl8 to modulate lipid metabolism," *Scientific Reports*, vol. 6, no. 1, p. 36926, 2016.

[59] H. Li, M. Xu, L. Zhao et al., "Decreased circulating levels of ANGPTL8 in Graves' disease patients," *Hormones*, vol. 18, no. 2, pp. 189–195, 2019.

[60] Y. Zhang, X. Guo, W. Yan et al., "ANGPTL8 negatively regulates NF-κB activation by facilitating selective autophagic degradation of IKKγ," *Nature Communications*, vol. 8, no. 1, p. 2164, 2017.

[61] Z. Liao, X. Wu, Y. Song et al., "Angiopoietin-like protein 8 expression and association with extracellular matrix metabolism and inflammation during intervertebral disc degeneration," *Journal of Cellular and Molecular Medicine*, vol. 23, no. 8, pp. 5737–5750, 2019.

[62] N. Akimoto, R. Wada, K. Iwakiri, and Z. Naito, "Histology and molecular biology studies on the expression and localization of angiopoietin-like protein 8 in human tissues," *Biomed Reports*, vol. 11, no. 5, pp. 215–221, 2019.

[63] K. F. Guo, J. X. Lu, H. Y. Yu et al., "Serum betatrophin concentrations are significantly increased in overweight but not in obese or type 2 diabetic individuals," *Obesity*, vol. 23, no. 4, pp. 793–797, 2015.

[64] H. Tuhan, A. Abacı, A. Anık et al., "Circulating betatrophin concentration is negatively correlated with insulin resistance in obese children and adolescents," *Diabetes Research and Clinical Practice*, vol. 114, pp. 37–42, 2016.

[65] J. Fu, C. Hou, L. Li et al., "Vitamin D modifies the associations between circulating betatrophin and cardiometabolic risk factors among youths at risk for metabolic syndrome," *Cardiovascular Diabetology*, vol. 15, no. 1, p. 142, 2016.

[66] P. Lips, K. D. Cashman, C. Lamberg-Allardt et al., "Current vitamin D status in European and Middle East countries and strategies to prevent vitamin D deficiency: a position statement of the European Calcified Tissue Society," *European Journal of Endocrinology*, vol. 180, no. 4, pp. P23–P54, 2019.

[67] O. Mayer Jr., J. Filipovský, J. Seidlerová et al., "The association between low 25-hydroxyvitamin D and increased aortic stiffness," *Journal of Human Hypertension*, vol. 26, no. 11, pp. 650–655, 2012.

Hindawi
Journal of Immunology Research
Volume 2020, Article ID 3891425, 7 pages
https://doi.org/10.1155/2020/3891425

Research Article

Higher Serum CCN3 Is Associated with Disease Activity and Inflammatory Markers in Rheumatoid Arthritis

Yingying Wei,[1,2,3] Linan Peng,[2] Yi Li,[1] Na Zhang,[1] Ke Shang,[1] Lihua Duan,[1] Jixin Zhong,[3] and Jie Chen[4]

[1]*Department of Rheumatology and Clinical Immunology, Jiangxi Provincial People's Hospital, Nanchang University, Nanchang, China*
[2]*School of Medicine, Xiamen University, Xiamen, China*
[3]*Department of Rheumatology and Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China*
[4]*Department of Scientific Research and Education, Jiangxi Provincial People's Hospital, Nanchang University, Nanchang, China*

Correspondence should be addressed to Lihua Duan; lh-duan@163.com, Jixin Zhong; zhongjixin620@163.com, and Jie Chen; jiechen86213@163.com

Received 25 February 2020; Accepted 30 March 2020; Published 9 May 2020

Guest Editor: Yan Yang

Nephroblastoma overexpressed protein (NOV/CCN3), the early discovered member of the CCN family, has recently been suggested to be involved in a number of inflammatory processes, including wound healing, alveolar epithelial cell inflammation, cancer metastasis, and macrophage foam cell formation. However, the role of CCN3 in rheumatoid arthritis (RA), a classic autoimmune and inflammatory disease, remains elusive. RA is a chronic systemic autoimmune disease that eventually leads to cartilage and bone destruction and joint dysfunction. In this study, we investigated the potential of serum CCN3 as a biomarker for RA. The serum levels of CCN3 were measured by ELISA. The clinical and laboratory parameters were collected from a clinical record system, and disease activity was determined by joint disease activity score 28 (DAS28). Our results showed that the serum levels of CCN3 were significantly increased in RA patients compared to healthy controls. Furthermore, the CCN3 level was positively correlated with DAS28 (CRP), DAS28 (ESR), and the level of anti-CCP Ab, an autoantibody highly specific for RA. Furthermore, CCN3 showed a positive correlation with inflammatory cytokine IL-6, while no significant correlation with TNF-α was observed. These data suggest that CCN3 plays an important role in the development of RA and might be a potential disease activity biomarker for RA.

1. Introduction

RA is a chronic and systemic autoimmune disease with pathological characteristics of sustained inflammatory synovitis, pannus formation, and abundant lymphocyte infiltration, followed by the destruction of joint cartilage and bone. A number of risk factors are involved in the development of RA, including infection, sex hormones, and genetic background [1, 2]. For example, it is reported that HLA-DRB1 mutation is associated with an increased risk of RA [3]. Recent studies have demonstrated that immune imbalance plays a key role in the pathogenesis of RA. TNF-α and IFN-γ, which are Th1-related cytokines, are considered as the key proinflammatory cytokines in the progression of human RA [4, 5]. In contrast, most studies have shown that Th2-type immune response alleviates Th1-mediated RA [6]. Recently, the imbalance of Th17 and regulatory T cell (Treg) has also been shown to play a critical role in autoimmune diseases [7], including human RA and collagen-induced arthritis [7, 8].

Nephroblastoma overexpressed protein (NOV/CCN3) is a member of the CCN family, which includes CYR61/CCN1, CTGF/CCN2, NOV/CCN3, WISP1/CCN4, WISP2/CCN5, and WISP3/CCN6 [9]. CCN3 gene was firstly identified as an integration site from nephroblastoma of newborn chicken infected with myeloblastoma-associated virus (MAV) [10]. Previous studies have demonstrated that CCN3 protein is

involved in multiple biological activities including cell adhesion, migration, and proliferation. Furthermore, CCN3 can be released out of cytoplasm and serves as a matrix molecule in a number of pathophysiological processes such as wound healing, angiogenesis, and fibrosis [10–13]. In addition, CCN3 also regulates the expression of inflammatory molecules in astrocyte and promotes the regeneration of central nerve system (CNS) myelin. A recent study suggested that CCN3 could be detected in RA and OA synovial tissues [14]. CCN3 knockout mice displayed osteoarthritic changes in knee articular cartilage, suggesting that CCN3 may suppress osteoarthritis progression by maintaining the differentiated phenotype of articular cartilage [15]. However, the role of CCN3 in RA remains unknown.

In the present study, we examined the serum levels of CCN3 by ELISA in RA patients. The correlations with clinical disease activity and inflammatory markers were analyzed. We found that the serum levels of CCN3 were significantly higher in RA patients compared to those in healthy subjects. Particularly, the CCN3 level was positively correlated with the disease activity score. Previous studies showed that the anti-cyclic citrullinated peptide (CCP) antibody is an autoantibody highly specific for RA and is predictive of radiological involvement in RA patients. In this study, we found that CCN3 expressions of RA patients were positively correlated with the serum level of anti-CCP antibody and RA disease activity score. Furthermore, a positive correlation between CCN3 and IL-6 was also observed in RA patients. These data suggest that CCN3 may be an inflammatory factor during the process of RA and may serve as a biomarker or an alternative therapeutic target in managing RA.

2. Materials and Methods

2.1. Patients and Control Subjects. A total of 41 RA patients were recruited from the Department of Rheumatology and Clinical Immunology, Jiangxi Provincial People's Hospital Affiliated to Nanchang University. The RA patients were all diagnosed complying with the 1987 revised diagnostic criteria of the American College of Rheumatology (ACR) [16]. Patients with various types of arthritis including osteoarthritis (OA), septic arthritis, psoriatic arthritis, and reactive arthritis were excluded. We recruited 45 age- and sex-matched healthy volunteers as the control. Informed consent of all the participants and the approval of the medical ethics committee of the hospital were obtained in accordance with the regulation.

2.2. Routine Laboratory Parameters. The clinical and laboratory parameters of RA patients were gathered from the clinical record system. The disease activity parameters were represented by disease activity score 28 (DAS28) (ESR) and DAS28 (CRP) as described previously [17].

2.3. Detection of Cytokines by ELISA. The peripheral blood samples of RA patients and healthy controls were collected into sterile coagulant tubes. Sera were isolated by centrifugation at 3500 rpm for 5 min and then kept frozen at -80°C. Concentrations of CCN3, IL-6, and TNF-α (R&D, Minneapolis, MN) were measured by ELISA kits according to the manufacturer's manuals.

2.4. Immunohistochemical Analysis. The anti-CCN3 antibody was purchased from R&D (Minneapolis, MN). After deparaffinization and rehydration, the sections were treated with 3% H_2O_2 followed by blocking with 10% goat serum in PBS. Then, the sections were stained with anti-CCN3 antibody overnight at 4°C, and afterwards, a hypersensitive two-step immunohistochemical detection reagent (ZSGB-BIO, China) was applied to detect the CCN3 expression under a microscope.

2.5. Statistical Analysis. GraphPad Prism 5 was used for statistical analysis. The Whitney U test was applied to analyze the difference between RA patients and healthy controls. Data were expressed as the mean ± standard deviation ($M \pm$ SD). The Spearmen test was adopted for correlation analysis, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical Characteristics of RA Patients. The clinical characteristics of RA patients were summarized in Table 1. Forty-one patients with RA and forty-five healthy controls were enrolled. The mean age for RA patients was 51.7 years with an age range from 26 to 71, and there were 36 females and 5 males. No significant differences in age and sex were observed between RA patients and healthy controls. The mean of disease duration was 10.6 years with a range of 0.5–20 years. As expected, ESR, the serum levels of C-reactive protein (CRP), rheumatoid factor (RF), and anti-CCP antibody were markedly higher in RA patients than those in healthy controls (Table 1).

3.2. Increased Serum CCN3 Level in RA Patient. It has been demonstrated that CCN3 plays a critical role in many diseases, such as central nervous system and cardiovascular diseases [18–20]. However, the role of CCN3 in the development of RA has not been described. To investigate whether CCN3 is involved in the development of RA, the sera of RA patients and healthy controls were collected, and the serum CCN3 level was measured by ELISA. As shown in Figure 1(a), the serum CCN3 level in RA patients was significantly higher compared with that in controls ($p < 0.0001$). The mean level of CCN3 in RA patients was 4288 pg/ml with a range of 1395-9233 pg/ml, while the healthy control was 2506 pg/ml (1409-4691 pg/ml). In addition, the deposition of the CCN3 in paraffin-embedded joint tissues was also determined. We found a considerable deposition of CCN3 in the joint tissues from RA patients, but not in the control tissues collected from OA patients (Figure 1(b)).

3.3. Serum CCN3 Level and Its Correlation with Disease Activity in RA Patients. Previous studies have shown that CCN3 plays an important role in many diseases, including inflammatory diseases [20, 21]. As RA is an inflammatory disease and the expression of CCN3 was highly increased in RA patients, the correlation between CCN3 and disease

Table 1: Baseline characteristics of participants in this study.

Characteristics	RA patients	Healthy controls
Total (female/male)	41 (36/5)	45 (37/8)
Age, mean (SD) (year)	51.7 (16.3)	49.6 (12.1)
Duration of disease, mean (SD) (year)	6.2 (5.6)	—
DAS28 (CRP), mean (SD)	4.4 (1.6)	—
DAS28 (ESR), mean (SD)	4.7 (1.7)	—
CRP (mg/l), mean (SD)	57.5 (61.2)***	4.9 (3.8)
ESR (mm/h), mean (SD)	49.9 (31.0)***	11.3 (9.6)
RF (IU/ml), mean (SD)	580.2 (950.2)***	16.2 (10.9)
Anti-CCP (RU/ml), mean (SD)	66.9 (63.8)***	12.4 (8.1)
IgG (g/l), mean (SD)	14.5 (3.4)	13.8 (6.4)
IgA (g/l), mean (SD)	3.3 (1.3)	3.0 (1.7)
IgM (g/l), mean (SD)	1.5 (0.7)	1.1 (2.0)
WBC (10^9/l), mean (SD)	7.8 (2.4)	6.9 (4.2)
Lym (10^9/l), mean (SD)	1.8 (0.6)	1.9 (1.1)
PLT (10^9/l), mean (SD)	347.9 (102.3)**	196.3 (85.3)
Hb (g/l), mean (SD)	111.2 (14.6)*	145.5 (21.8)

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide; Ig: immunoglobulin; WBC: white blood cell; Lym: lymphocytes; PLT: platelet; Hb: hemoglobin. * indicates RA vs. healthy controls (HC), $p < 0.05$. ** indicates RA vs. HC, $p < 0.01$. *** indicates RA vs. HC, $p < 0.001$.

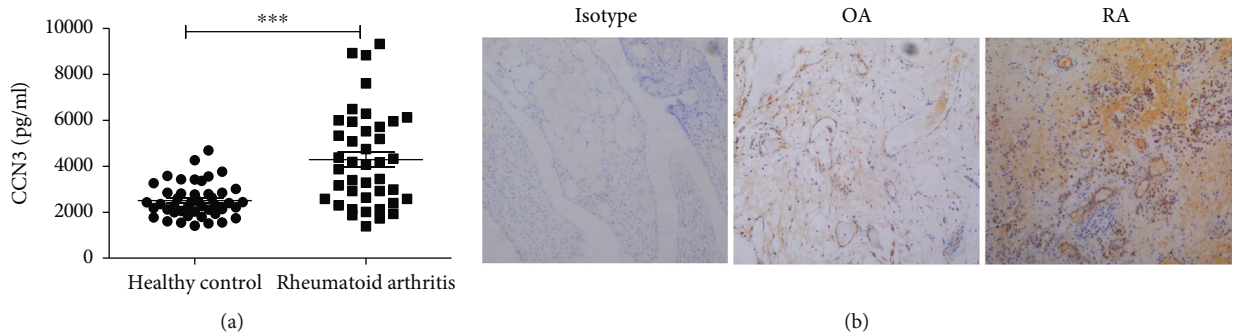


Figure 1: Increased CCN3 expression in RA patients. (a) The sera isolated from rheumatoid arthritis patients (RA, $n = 41$) and healthy controls (HC, $n = 45$) were used for the detection of CCN3 by ELISA. The Mann-Whitney U test was conducted to compare the data between two groups. *** indicates $p < 0.001$. (b) Local expression of CCN3 in joint tissues collected from patients with OA or RA. The expression was examined by immunohistological analysis (magnification 100x).

activity score was analyzed. As expected, the serum CCN3 level was positively correlated with DAS28 (ESR) ($r = 0.483$, $p = 0.0014$) and DAS28 (CRP) ($r = 0.487$, $p = 0.0012$) (Figure 2(a)). We further divided the RA patients into inactive, moderate, and very active groups, according to the DAS28. We found that the patients with a higher disease activity score showed a higher CCN3 level when compared with inactive patients. The serum CCN3 was 2519 pg/ml (1385-4691 pg/ml) in inactive patients (DAS28 (CRP)), 4429 pg/ml (2015-7603 pg/ml) in moderate patients, and 5262 pg/ml (2131-9322 pg/ml) in active patients (Figure 2(b)).

3.4. Association of Serum CCN3 Level with Laboratory Parameters. The inflammatory response during the development of RA often results with the change of a number of laboratory parameters. Although there was a strong positive correlation between CCN3 expression and disease activity score, most laboratory variables did not correlate with the serum CCN3 level (Table 2). It has been demonstrated that the anti-CCP antibody has a higher specifity for RA compared to RF. Among those laboratory parameters, the level of RA-specific autoantibody anti-CCP but not RF was positively correlated with the CCN3 level (anti-CCP: $r = 0.500$, $p = 0.0009$; RF: $r = 0.276$, $p = 0.0801$; Figures 3(a) and 3(b)).

3.5. The Relationship between the Expression of CCN3 and IL-6 in RA Patients. As is well known, inflammatory cytokines such as IL-6 and TNF-α contribute greatly to the pathophysiological process of RA [22]. To explore the effect of CCN3

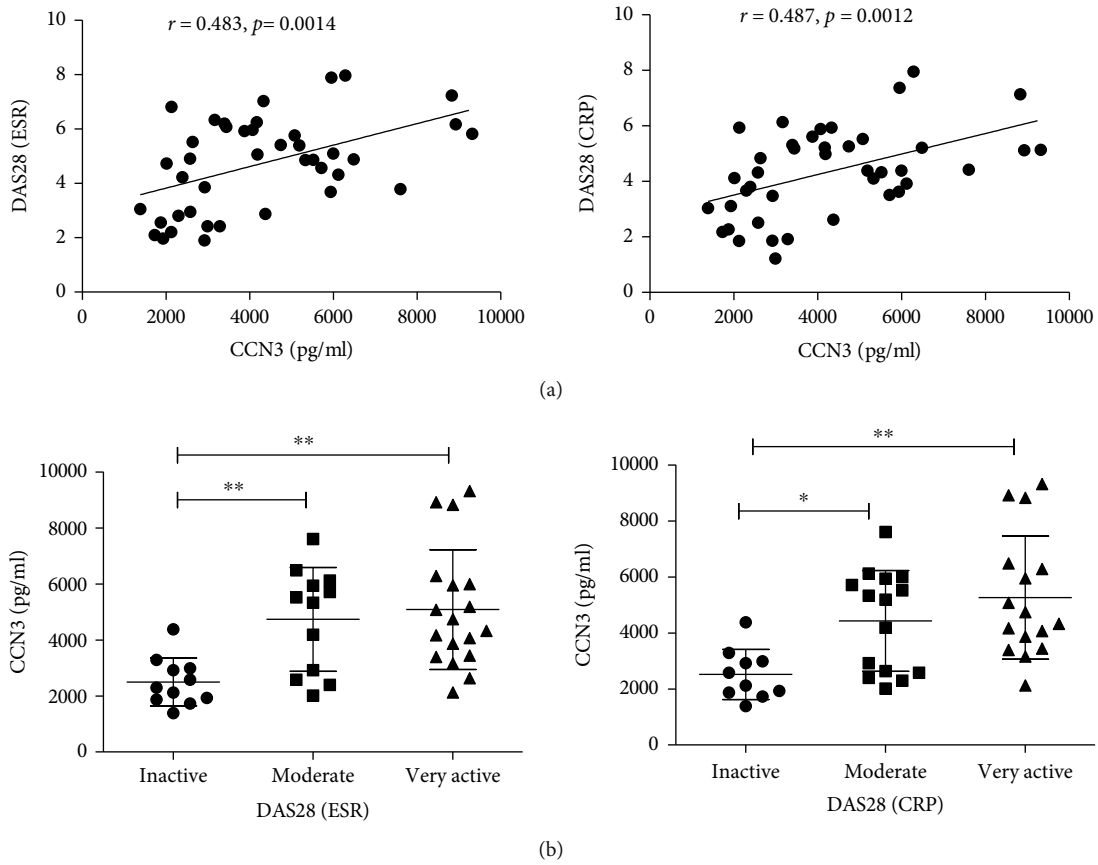


Figure 2: Association of CCN3 with disease activity in RA patients. (a) The determination of linear relationship between serum CCN3 expression and DAS28 in RA patients was performed by the Spearman correlation coefficient. (b) The RA patients were divided into inactive, moderate, and very active groups by the DAS28 (ESR) and DAS28 (CRP). Serum CCN3 levels were compared among these groups, and the difference was evaluated by the Mann-Whitney U test. $*$ indicates $p < 0.05$; $**$ indicates $p < 0.01$.

Table 2: Correlation analysis between CCN3 level and laboratory variables in patients with RA.

Characteristics	WBC	Lym	Hb	PLT	IgG	IgA	IgM
r	0.031	0.158	0.104	0.044	0.045	0.156	0.242
p value	0.859	0.363	0.553	0.803	0.812	0.411	0.198

Spearman's correlation analysis was used to calculate significance.

on the inflammatory cytokine production, the relationships between CCN3 and IL-6 or TNF-α were examined. A positive correlation was observed between CCN3 and IL-6 ($r = 0.4657$, $p = 0.0022$), but not between CCN3 and TNF-α ($r = 0.2247$, $p = 0.1587$) in RA patients (Figure 4). In consistency with this, IL-6 was also positively correlated with RA disease activity DAS28 (ESR) and DAS28 (CRP), suggesting that both CCN3 and IL-6 may serve as a biomarker for inflammation and disease activity in RA (Tables 3 and 4).

4. Discussion

In the present study, a significantly increased serum level of CCN3 was observed in RA patients, and the CCN3 expression was positively correlated with DAS28 (ESR), DAS28 (CRP), and anti-CCP antibody, which are critical clinical and laboratory parameters in RA. Furthermore, we observed a strong correlation between serum CCN3 and IL-6 level in RA patients. IL-6 is a common cytokine that plays a key role in the development of RA. These data suggest that CCN3 might be involved in the development of RA through regulating the inflammatory response.

As a member of matricellular proteins, CCN3 has recently received attention owing to its role in angiogenesis and fibrosis [23]. Treatment with recombinant CCN3 results in neovascularization rat corneas, an angiogenetic effect that might be related to its binding to integrins such as $\alpha v\beta 5$ [24, 25]. In renal fibrosis, CCN3 suppresses TGF-β1-induced extracellular matrix accumulation [26]. In addition, the impact of CCN3 was also observed in systemic scleroderma, an autoimmune rheumatic disease characterized by excessive production and accumulation of collagen in the skin, small arteries, and internal organs [27]. RA is typically characterized by sustained inflammatory synovitis. Previous studies showed that CCN3 was upregulated in RA synovial samples and osteoarthritis [14]. In the present study, we also found the CCN3 expression in the synovial samples from RA and osteoarthritis patients and CCN3 was much higher in the synovial tissue from RA compared to that in OA. Furthermore, the serum level of CCN3 was markedly increased in RA patients when compared with healthy controls.

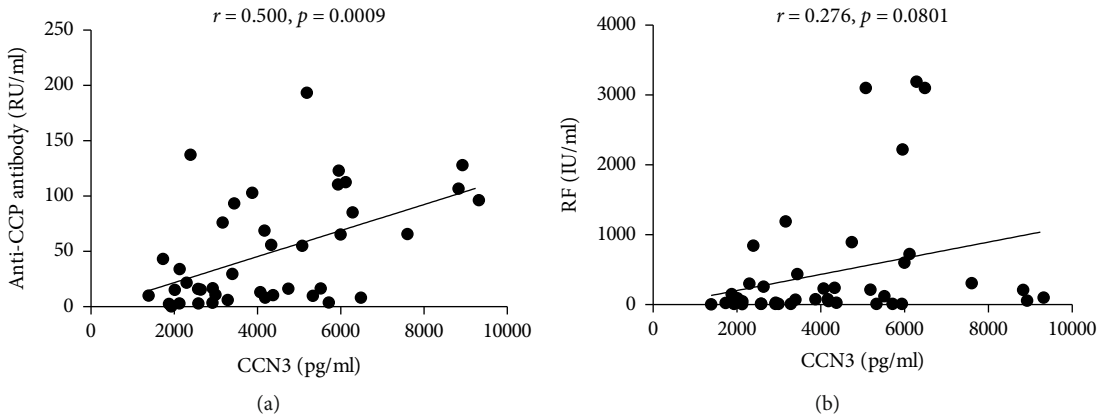


Figure 3: Positive correlation of CCN3 with anti-CCP and RF in RA patients. Serum CCN3 was positively correlated with anti-CCP (a) but not with RF (b) in RA patients. Spearman correlation analysis was conducted (anti-CCP: $r = 0.500$, $p = 0.0009$; RF: $r = 0.276$, $p = 0.0801$).

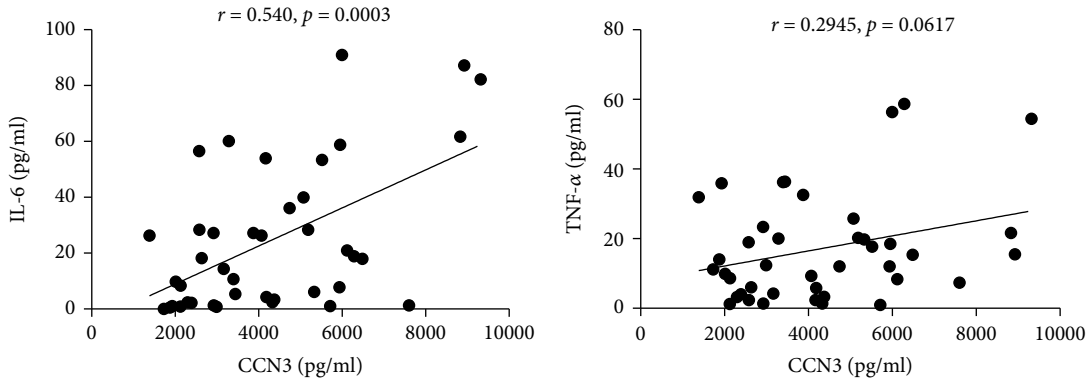


Figure 4: The correlation of CCN3 with inflammatory cytokines in RA patients. The ELISA was used to detect inflammatory cytokines from RA patients' sera. The determination of linear relationship between CCN3 and IL-6 or TNF-α was performed by the Spearman correlation coefficient. CCN3 is positively correlated with IL-6 ($r = 0.4657$; $p = 0.0022$).

Table 3: Correlation analysis between DAS28 (ESR) and laboratory variables in RA patients.

Characteristics	WBC	Lym	Hb	PLT	IgG	IgA	IgM	IL-6	TNF-α	CCN3
r	0.142	-0.141	-0.026	0.128	0.098	0.410	0.454	0.481	0.205	0.483
p value	0.413	0.418	0.879	0.463	0.604	0.024*	0.011*	0.001**	0.198	0.001**

Spearman's correlation analysis was used to calculate significance. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Table 4: Correlation analysis between DAS28 (CRP) and laboratory variables in RA patients.

Characteristics	WBC	Lym	Hb	PLT	IgG	IgA	IgM	IL-6	TNF-α	CCN3
r	0.089	-0.140	0.045	0.115	0.050	0.430	0.586	0.427	0.224	0.487
p value	0.610	0.420	0.793	0.510	0.790	0.017*	0.001**	0.005**	0.158	0.001**

Spearman's correlation analysis was used to calculate significance. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Recently, the immunoregulatory role of CCN3 has become a rising concern. It has been demonstrated that blockade of CCN3 activity inhibits cell inflammation and apoptosis through downstream TGF-β/p-Smad and NF-κB pathway in human alveolar epithelial cells [28]. Furthermore, recent studies also showed that CCN3 was a novel adipokine, and it had emerged as a potential metabolic regulator in type 2 diabetes [29]. In this study, we also found a positive correlation between CCN3 and disease activity score in RA patients, suggesting that CCN3 may play a critical role in the development of RA. In contrast, it has been shown that CCN3 exhibits a protective role in osteoarthritis and disruption of CCN3 in aged male mice results in osteoarthritis-like disease [30]. In addition, CCN3 is identified as a protective

factor in cartilage by inhibiting the PI3K/AKT/mTOR pathway, alleviating the deterioration of osteoarthritis [31]. Therefore, CCN3 may play distinct roles in RA and osteoarthritis, two common types of chronic arthritis. As a chronic and systemic autoimmune disease, the inflammatory processes of RA involve immune cells, cytokines, chemokines, proteases, and matrix metalloproteinases (MMPs). In contrast, osteoarthritis (OA), an age-related degenerative joint disease, is pathologically characterized by articular cartilage degeneration. Infiltration of immune cells, especially T cells and macrophages in the synovial membrane of RA patients, leads to fibroblast expansion, osteoclast stimulation, and eventually tissue remodeling [32]. In consistency with this, CCN3 is recently identified as a Treg-derived mediator and plays a role in remyelination in multiple sclerosis [13].

In addition to T cells, macrophages also play a key role in the inflammation of RA. CCN3 is also associated with macrophage polarization into M1 (proinflammatory) and M2 (anti-inflammatory). In atherosclerosis, CCN3 inhibits macrophage foam cell formation [33]. Interestingly, the absence of CCN3 gene was associated with a change in macrophage profile (M1-like to M2-like) and proinflammatory cytokine/-chemokine expression in adipose tissues. Macrophage is the main producer of TNF-α and IL-6. In our study, a positive correlation between CCN3 and inflammatory cytokine IL-6 was observed. There was also a trend of positive correlation between CCN3 and TNF-α, although it was not statistically significant. Besides, the CCN3 expression is also positively correlated with the anti-CCP antibody, which has been shown to promote macrophage polarization into M1 subset. These data show that CCN3 plays an important role in inflammation by regulating macrophage polarization and cytokines, resulting in a deleterious role in joint tissue destruction.

In summary, these results suggest that the serum CCN3 could be a sensitive marker for disease activity in RA patients. In addition, CCN3 was associated with inflammatory cytokines and anti-CCP antibody in RA, suggesting that the CCN3 may play a critical role in the pathogenesis of RA. Of course, further studies are required to explore the specific regulatory mechanism and the pathogenic role of CCN3 in RA.

Data Availability

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

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

YW and JC performed the experiments. LP, YW, and JC analyzed data and wrote the manuscript. KS and NZ contributed to the clinical patients' sample collection. LL provided technical support. LD and JZ provided critical revision of the manuscript. JZ and JC designed the research and revised the article. All authors have read and approved the final manuscript. Yingying Wei and Linan Peng contributed equally to this work.

Acknowledgments

We thank all the volunteers who participated in this study. This work was supported by the National Natural Science Foundation of China (81871286, 81960296, 81974254, 81671544, and 81670431), Natural Science Foundation of Jiangxi Province (20192ACB21006), Interdisciplinary Innovation Team, Frontier Science Key Research Project of Jiangxi Provincial People's Hospital (19-008).

References

[1] J. S. Smolen, D. Aletaha, A. Barton et al., "Rheumatoid arthritis," *Nature Reviews Disease Primers*, vol. 4, no. 1, article 18001, 2018.

[2] D. L. Scott, F. Wolfe, and T. W. Huizinga, "Rheumatoid arthritis," *The Lancet*, vol. 376, no. 9746, pp. 1094–1108, 2010.

[3] A. J. Silman and J. E. Pearson, "Epidemiology and genetics of rheumatoid arthritis," *Arthritis Research*, vol. 4, Supplement 3, pp. S265–S272, 2002.

[4] M. C. Boissier, E. Assier, G. Falgarone, and N. Bessis, "Shifting the imbalance from Th1/Th2 to Th17/treg: the changing rheumatoid arthritis paradigm," *Joint, Bone, Spine*, vol. 75, no. 4, pp. 373–375, 2008.

[5] L. Codarri, G. Gyulveszi, V. Tosevski et al., "RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation," *Nature Immunology*, vol. 12, no. 6, pp. 560–567, 2011.

[6] M. C. Boissier, "Cell and cytokine imbalances in rheumatoid synovitis," *Joint, Bone, Spine*, vol. 78, no. 3, pp. 230–234, 2011.

[7] D. M. Roeleveld and M. I. Koenders, "The role of the Th17 cytokines IL-17 and IL-22 in rheumatoid arthritis pathogenesis and developments in cytokine immunotherapy," *Cytokine*, vol. 74, no. 1, pp. 101–107, 2015.

[8] H. M. Knochelmann, C. J. Dwyer, S. R. Bailey et al., "When worlds collide: Th17 and Treg cells in cancer and autoimmunity," *Cellular & Molecular Immunology*, vol. 15, no. 5, pp. 458–469, 2018.

[9] P. A. Klenotic, C. Zhang, and Z. Lin, "Emerging roles of CCN proteins in vascular development and pathology," *Journal of Cell Communication and Signaling*, vol. 10, no. 3, pp. 251–257, 2016.

[10] V. Joliot, C. Martinerie, G. Dambrine et al., "Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas," *Molecular and Cellular Biology*, vol. 12, no. 1, pp. 10–21, 1992.

[11] B. L. Riser, F. Najmabadi, B. Perbal et al., "CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic pathway in an *in vitro* model of renal disease," *The American Journal of Pathology*, vol. 174, no. 5, pp. 1725–1734, 2009.

[12] D. Janune, T. Abd El Kader, E. Aoyama et al., "Novel role of CCN3 that maintains the differentiated phenotype of articular

cartilage," *Journal of Bone and Mineral Metabolism*, vol. 35, no. 6, pp. 582–597, 2017.

[13] Y. Dombrowski, T. O'Hagan, M. Dittmer et al., "Regulatory T cells promote myelin regeneration in the central nervous system," *Nature Neuroscience*, vol. 20, no. 5, pp. 674–680, 2017.

[14] M. Komatsu, Y. Nakamura, M. Maruyama, K. Abe, R. Watanapokasin, and H. Kato, "Expression profiles of human CCN genes in patients with osteoarthritis or rheumatoid arthritis," *Journal of Orthopaedic Science*, vol. 20, no. 4, pp. 708–716, 2015.

[15] A. Peidl, "A friend in knee: CCN3 may inhibit osteoarthritis progression," *Journal of Cell Communication and Signaling*, vol. 12, no. 2, pp. 489-490, 2018.

[16] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[17] P. L. van Riel and L. Renskers, "The disease activity score (DAS) and the disease activity score using 28 joint counts (DAS28) in the management of rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 34, no. 5, Supplement 101, pp. S40–S44, 2016.

[18] B. Y. Su, W. Q. Cai, C. G. Zhang, V. Martinez, A. Lombet, and B. Perbal, "The expression of ccn 3 (nov) RNA and protein in the rat central nervous system is developmentally regulated," *Molecular Pathology*, vol. 54, no. 3, pp. 184–191, 2001.

[19] J. Pakradouni, W. Le Goff, C. Calmel et al., "Plasma NOV/CCN3 levels are closely associated with obesity in patients with metabolic disorders," *PLoS One*, vol. 8, no. 6, article e66788, 2013.

[20] J. Liu, Y. Ren, L. Kang, and L. Zhang, "Overexpression of CCN3 inhibits inflammation and progression of atherosclerosis in apolipoprotein E-deficient mice," *PLoS One*, vol. 9, no. 4, article e94912, 2014.

[21] L. Kular, C. Rivat, B. Lelongt et al., "NOV/CCN3 attenuates inflammatory pain through regulation of matrix metalloproteinases-2 and -9," *Journal of Neuroinflammation*, vol. 9, no. 1, article 36, 2012.

[22] S. T. Wei, Y. H. Sun, S. H. Zong, and Y. B. Xiang, "Serum levels of IL-6 and TNF-α may correlate with activity and severity of rheumatoid arthritis," *Medical Science Monitor*, vol. 21, pp. 4030–4038, 2015.

[23] J. I. Jun and L. F. Lau, "Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets," *Nature Reviews Drug Discovery*, vol. 10, no. 12, pp. 945–963, 2011.

[24] C. G. Lin, S. J. Leu, N. Chen et al., "CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family," *Journal of Biological Chemistry*, vol. 278, no. 26, pp. 24200–24208, 2003.

[25] C. G. Lin, C. C. Chen, S. J. Leu, T. M. Grzeszkiewicz, and L. F. Lau, "Integrin-dependent functions of the angiogenic inducer NOV (CCN3): implication in wound healing," *The Journal of Biological Chemistry*, vol. 280, no. 9, pp. 8229–8237, 2005.

[26] H. F. Liu, H. Liu, L. L. Lv et al., "CCN3 suppresses TGF-β1-induced extracellular matrix accumulation in human mesangial cells *in vitro*," *Acta Pharmacologica Sinica*, vol. 39, no. 2, pp. 222–229, 2018.

[27] P. Henrot, M. E. Truchetet, G. Fisher, A. Taieb, and M. Cario, "CCN proteins as potential actionable targets in scleroderma," *Experimental Dermatology*, vol. 28, no. 1, pp. 11–18, 2019.

[28] H. P. Zhu, H. Y. Huang, D. M. Wu et al., "Regulatory mechanism of NOV/CCN3 in the inflammation and apoptosis of lung epithelial alveolar cells upon lipopolysaccharide stimulation," *Molecular Medicine Reports*, vol. 21, no. 4, pp. 1872–1880, 2019.

[29] J. Y. Li, Y. D. Wang, X. Y. Qi et al., "Serum CCN3 levels are increased in type 2 diabetes mellitus and associated with obesity, insulin resistance and inflammation," *Clinica Chimica Acta*, vol. 494, pp. 52–57, 2019.

[30] K. A. Roddy and C. A. Boulter, "Targeted mutation of NOV/CCN3 in mice disrupts joint homeostasis and causes osteoarthritis-like disease," *Osteoarthritis and Cartilage*, vol. 23, no. 4, pp. 607–615, 2015.

[31] X. Huang, B. Ni, Z. Mao et al., "NOV/CCN3 induces cartilage protection by inhibiting PI3K/AKT/mTOR pathway," *Journal of Cellular and Molecular Medicine*, vol. 23, no. 11, pp. 7525–7534, 2019.

[32] S. Alivernini, G. Peluso, A. L. Fedele, B. Tolusso, E. Gremese, and G. Ferraccioli, "Tapering and discontinuation of TNF-α blockers without disease relapse using ultrasonography as a tool to identify patients with rheumatoid arthritis in clinical and histological remission," *Arthritis Research & Therapy*, vol. 18, no. 1, article 39, 2016.

[33] H. Shi, C. Zhang, V. Pasupuleti et al., "CCN3 regulates macrophage foam cell formation and atherosclerosis," *The American Journal of Pathology*, vol. 187, no. 6, pp. 1230–1237, 2017.