Clinical and Developmental Immunology

Systemic Autoimmune Diseases

Guest Editors: Guixiu Shi, Jianying Zhang, Zhixin (Jason) Zhang, and Xuan Zhang

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Editorial Board

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Editorial

Systemic Autoimmune Diseases

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Systemic autoimmune diseases are a broad range of related diseases characterized by dysregulation of immune system which give rise to activation of immune cells to attack autoantigens and resulted in inappropriate inflammation and multitissue damages. They are a fascinating but poorly understood group of diseases, ranging from the commonly seen rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) to the relatively rare systemic sclerosis [1]. The mechanism of pathogenesis of systemic autoimmune diseases is still not very clear. It is now considered that genetic factors, infection, endocrine, and environmental exposure are involved in the pathogenesis of these diseases [2–4]. There is no treatment strategy to cure this kind of disease at present which gives rise to the needs of long-term lasting treatment, making systemic autoimmune diseases be a mounting public health concern for the foreseeable future. Vital organs such as lung and kidney involvement in systemic autoimmune diseases are common and always presented in a progressive pattern with limited treatment strategy, making them be one of the most common causes of death in patients [5].

Based on this background, we assembled this special issue for a better understanding of systemic autoimmune diseases, on aspects of mechanisms of pathogenesis, diagnosis, and treatment, including papers ranging from the basic researches to clinical researches and reviews about systemic autoimmune diseases.

Studies on the basic research of systemic autoimmune disease in this issue provided us new insights into the mechanism of the pathogenesis of systemic autoimmune disease. The role of HMGB1 in the T-cell DNA demethylation was discussed in the paper of Y. Li et al. SNPs with strong RA association signal in the British were analyzed in Han Chinese by H. Li et al., and the methylation status of miR-124a loci in synovial tissues of RA patients was analyzed by Q. Zhou et al. indicating the epigenetic factor in the pathogenesis of RA. Expression of microRNA-155 was studied by L. Long et al. in RA patients. IL-33 status was tested in RA patients by S. Tang et al. D. Lorton et al. and Y. Du et al. indicated the possible role of beta2-adrenergic receptors (β2-AR) and p53 apoptosis effector related to PMP-22(Perp) in the pathogenesis of RA, respectively. It has long been demonstrated that $\gamma\delta$ T cells play important roles in the development of autoimmune diseases; the precise role of $\gamma\delta$ T cells in the pathogenesis of SLE was studied by Z. Lu et al. Z. Gu et al. discussed the role of p53/p21 pathway in the pathogenesis of SLE. X. Gan et al. demonstrated the role of GITR and GITRL in the primary Sjögren's syndrome. The expression of IL-6 and its clinical significance in patients with dermatomyositis was discussed by M. Yang et al. L. Estrada-Capetillo et al. found that DCs from patients with rheumatic inflammatory disease show an aberrant function that may have an important role in the pathogenesis. S. Stratakis et al. studied the mechanisms underlying this beneficial effect of rapamycin in passive and active Heymann nephritis (HN).

Clinical researches and studies included in this issue provided us several useful clinical clues in the diagnosis, treatment, and prediction of some of systemic autoimmune diseases. L. Hongyan et al. studied the clinical and pathologic

features in lupus nephritis with mainly IgA deposits and made a literature review about this topic. Risk factors for interstitial lung disease in patients with idiopathic inflammatory myopathy were analyzed by X. Cen et al. J. Chen et al. defined high resolution chest CT (HRCT) and pulmonary function test (PFT) abnormalities capable of identifying asymptomatic, preclinical RA-ILD. L. Pan et al. made a retrospective study to compare the characteristics of connective tissue disease-associated interstitial lung diseases, undifferentiated connective tissue disease-associated interstitial lung diseases, and idiopathic pulmonary fibrosis. Relationship between Brachial-ankle pulse wave velocity (baPWV) and its associated risk factors in Chinese patients with RA was analyzed by P. Li et al. P. Žigon et al. studied the diagnostic value of antiphosphatidylserine/prothrombin antibodies in systemic autoimmune disease. The correlations of disease activity, socioeconomic status, quality of life, and depression/anxiety in Chinese SLE patients were studied by B. Shen et al. J. Li et al. made a systematic review on efficacy and safety of Iguratimod for the treatment of rheumatoid arthritis.

Review papers also cover many aspects about systemic autoimmune disease. Advances in the knowledge of costimulatory pathways and their role in SLE were discussed by N. Y. Kow and A. Mak H. Draborg et al. summed up existing data about the relationship between epstein-barr virus and autoimmune disease. T. Marchetti et al. discussed obstetrical antiphospholipid syndrome from pathogenesis to the clinical and therapeutic implications. Y. f. Huang et al. summarized the immune factors involved in the pathogenesis, diagnosis, and treatment of Sjogren's syndrome. The role of IL-33 in rheumatic diseases was reviewed by L. Duan et al. T. Ito made a review on advances in the pathogenesis of autoimmune hair loss disease alopecia areata. A. W. J. M. Glaudemans reviewed the use of 18F-FDG-PET/CT for diagnosis and treatment monitoring of inflammatory and infectious diseases. The role of FcγR-mediated trogocytosis in the physiological immune system was discussed by S. Masuda et al.

This special issue covers many important aspects in the systemic autoimmune diseases ranging from novel insights into the pathogenesis of autoimmune disease and the use of newly developed diagnostic strategy in the early diagnosis of autoimmune disease to the treatment of these kinds of diseases, which will surely provide us a better understanding about systemic autoimmune disease.

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Xuan Zhang

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Review Article

Efficacy and Safety of Iguratimod for the Treatment of Rheumatoid Arthritis

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Academic Editor: Xuan Zhang

All randomized controlled trials (RCTs) of iguratimod for rheumatoid arthritis (RA) to assess its efficacy and safety are included in this paper. The Review Manager software was used for meta-analysis to assess risk bias of the studies included, and GRADE profiler software was used for the evidence quality of the studies included. Four RCTs involving 1407 patients with RA were included. Meta-analyses showed that, after 24-week therapy, ACR20, tender joint count, swollen joint count, rest pain, physician and patient global assessment of disease activity, HAQ score, ESR, and CRP in iguratimod group were better than those in placebo group and that the difference between those of iguratimod group and those of other DMARDs (MTX and SASP) group was not significant. GRADE evidence classification of the studies included was moderate. Iguratimod for RA had few adverse events, and its efficacy and safety were the same as those of MTX and SASP for RA. The results of this systematic review suggest that more high-quality and large-scaled RCTs were needed to determine the efficacy of iguratimod for RA and whether iguratimod is as effective as other DMARDs besides MTX and SASP.

1. Introduction

Rheumatoid arthritis (RA) is chronic systemic inflammatory and autoimmune disease of unknown etiology that primarily targets synovial tissue and is characterized by an activation of T lymphocyte, an increase in interleukin and tumor necrosis factor, and severe chronic inflammation of the joints, resulting in erosion and destruction of cartilage, bone, and tendon [1, 2]. It is relatively common, with a prevalence of slightly less than 1% in adults all over the world [1]. Prevalence of moderate and severe disability in adults aging over 60 (in millions) due to rheumatoid arthritis by leading health condition associated with disability is 1.7 in high-income countries and 3.7 in low- and middle-income countries in 2012 [3]. Years lost due to disability (YLD) per 100 000 adults aging over 60 due to rheumatoid arthritis is the 11th in the world in 2012 [3]. Current treatments for RA emphasize the early use of traditional disease-modifying antirheumatic drugs (DMARDs), such as methotrexate (MTX), salazosulfapyridine (SASP), leflunomide, and cyclophosphamide to minimize or prevent joint damage. In addition, biologic agents such as necrosis factor-α blocker, anti-interleukin antibody, and CD20 monoclonal antibody are also used to teat RA. In the recent ten years, iguratimod (T-614) has been used to treat RA as a novel immunomodulator. It functions by suppressing the production of some inflammatory cytokines, including interleukin-1 (IL-1), IL-4, IL-6, IL-17, tumor necrosis factor, nuclear factor-kappaB, and interferon *in vitro* (synovial cells and some cell lines) and *in vivo* (mouse models) [4, 5]. Iguratimod also reduced immunoglobulin production by acting directly on human B lymphocytes without affecting B lymphocyte proliferation [6]. Direct evidence has also showed that iguratimod can dramatically suppress disease progression and markedly protect affected joints

Table 1: Characteristics of the included studies with iguratimod (T-614) for rheumatoid arthritis.

Study	Method	Participants	Intervention	Duration	Outcomes	Allocation concealment
Hara et al. (2007) [14]	Double-blind, randomized, placebo-controlled trial	Country: Japan Site: multicenter Number: 376 Iguratimod group (n = 147) SASP group (n = 156) Placebo group (n = 73) Sex: female/male = 306 : 70	T-614 group: iguratimod 25 mg daily for the first 4 weeks and 50 mg daily for the subsequent 24 weeks SASP group: salazosulfapyridine (SASP) 1000 mg daily Placebo group: placebo tablets	28 weeks of clinical assessment at 0, 4, 6, 12, 18, 24, and 28 weeks.	ACR20, ACR50, tender joint count, swollen joint count, rest pain, physician's and patient's global assessment of disease activity (VAS, mm), physician's global assessment of disease activity, HAQ score, ESR, CRP, and adverse events.	Described in article but not validated because the author could not be reached via email.
Lü et al. (2008) [2]	Double-blind, randomized, placebo-controlled trial	Country: China Site: multicenter Number: 280 T-614 group 1 (n = 93) T-614 group 2 (n = 92) Placebo group (n = 95) Sex: female/male = 231 : 49	Group 1: T-614 25 mg daily for the first 4 weeks and 50 mg daily for the subsequent 20 weeks Group 2: T-614 50 mg daily Placebo group: placebo tablets	24 weeks of clinical assessment at 0, 2, 4, 6, 12, 18, and 24 weeks.	ACR20, ACR50, ACR70, tender joint count, swollen joint count, tender joint score (TJS), swollen joint score (SJS), rest pain, duration of morning stiffness, grip strength, physician and patient global assessment of disease activity, ESR, CRP, rheumatoid factor, HAQ score, radiological damage, and adverse events.	Detailed in article and validity ascertained through telephone.
Lü et al. (2009) [15]	Double-blind, randomized, controlled trial	Country: China Site: multicenter Number: 489 T-614 group 1 (n = 163) T-614 group 2 (n = 163) MTX group (n = 163) Sex: female/male = 418 : 81	T-614 group 1: T-614 25 mg daily for the first 4 weeks and 50 mg daily for the subsequent 20 weeks T-614 group 2: T-614 50 mg per day MTX group: MTX 10 mg weekly for the first 4 weeks and 15 mg weekly for the subsequent 20 weeks	24 weeks of clinical assessment at 0, 4, 10, 17, and 24 weeks.	ACR20, ACR50, ACR70, tender joint count, swollen joint count, tender joint score (TJS), swollen joint score (SJS), rest pain, duration of morning stiffness, grip strength, physician and patient global assessment of disease activity, ESR, CRP, and adverse events.	Detailed in article and validity ascertained through telephone.
Ishiguro (2013) [16]	Double-blind, randomized, controlled trial	Country: Japan Site: multicenter Number: 252 T-614 + MTX group (n = 164) MTX + placebo group (n = 88) Sex: female/male = 204 : 48	T-614 + MTX group: T-614 25 mg daily for the first 4 weeks and 50 mg daily for the subsequent 20 weeks Placebo + MTX group: MTX at low dosages of 6 or 8 mg weekly and folic acid at dosage of 5 mg weekly	28 weeks of clinical assessment at 0, 4, 6, 8, 20, 12, 16, 20, and 24 weeks.	ACR20, ACR50, ACR70, tender joint count, swollen joint count, patient's and physician's global assessment of disease activity, HAQ score, DAS28-CRP, ESR, CRP, and adverse events.	Described in article but not validated because the author could not be reached via email.

Table 2: Summary of the findings for the main comparison: iguratimod compared to placebo for rheumatoid arthritis.

Outcomes	Illustrative comparative risks* (95% CI) Assumed risk Placebo	Corresponding risk Iguratimod	Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)	Comments
ACR20/24 weeks	*219 per 1000*	*514 per 1000* (398 to 660)	RR 2.35 (1.82 to 3.02)	636 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
Tender joint count		The mean tender joint count in the intervention groups was *−0.44 lower* (−0.61 to −0.27 lower)		593 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
Swollen joint count		The mean swollen joint count in the intervention groups was *−0.49 lower* (−0.66 to −0.32 lower)		592 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
Assessment of rest pain		The mean assessment of rest pain in the intervention groups was *−0.71 lower* (−0.89 to −0.54 lower)		590 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
Physician global assessment of disease activity		The mean physician global assessment of disease activity in the intervention groups was *−0.74 lower* (−0.93 to −0.55 lower)		592 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
Patient global assessment of disease activity		The mean patient global assessment of disease activity in the intervention groups was *−0.58 lower* (−0.80 to −0.36 lower)		591 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
HAQ score		The mean HAQ score in the intervention groups was *−0.67 lower* (−0.84 to −0.50 lower)		591 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
CRP		The mean CRP in the intervention groups was *−0.31 lower* (−0.53 to −0.09 lower)		587 (3 studies)	⊕⊕⊕⊖moderate[1]	Important
ESR		The mean ESR in the intervention groups was *−0.64 lower* (−0.82 to −0.45 lower)		530 (3 studies)	⊕⊕⊕⊖moderate[1]	Important

*The basis for the assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI: confidence interval; RR: risk ratio; MTX: methotrexate; SASP: salazosulfapyridine.

GRADE working group grades of evidence.

High quality: further research is very unlikely to change our confidence in the estimate of effect.

Moderate quality: further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.

Low quality: further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.

Very low quality: we are very uncertain about the estimate.

[1]Two studies had unclear selective biases and no intent-to-treat analyses.

against cartilage destruction and bone erosion in collagen-induced arthritis rats [5]. In addition, iguratimod decreased production of matrix metalloproteinases (MMP-1 and MMP-3) and inhibits the migratory expansion of rheumatoid synovial fibroblasts *in vitro* [7]. Although iguratimod has been used to treat RA for 10 years since 2003, no systematic review has been done on its efficacy and safety. Therefore, we conducted this systematic review to assess the efficacy and safety of iguratimod (T-614) for RA.

2. Materials and Methods

2.1. Types of Studies and Inclusion and Exclusion Criteria

2.1.1. Types of Studies. All randomized clinical trials (RCTs) were published in all journals, with a minimum duration of study of at least six months (or 24 weeks).

2.1.2. Types of Participants

(1) Inclusion Criteria. Patients with clinical diagnosis of rheumatoid arthritis (RA) of all eligible RCTs according to the American Rheumatism Association (ARA) criteria are enrolled within the inclusion criteria. Age of patients with only RCTs was at least 18 years old, and sex, race, and region of patients were not limited. These patients must have active disease as shown in the following outcomes: (a) ACR20; (b) tender joint count (TJC); (c) swollen joint count (SJC); (d) assessment of rest pain; (e) physician global assessment of disease activity; (f) patient global assessment of disease activity; (g) health assessment questionnaire (HAQ) score; (h) erythrocyte sedimentation rate (ESR); (i) C-reactive protein (CRP); and (j) adverse events reports.

(2) Exclusion Criteria. The studies on patients with both RA and cancer, abnormal hepatic dysfunction or renal dysfunction, or pregnant women and patients with diabetes mellitus,

Table 3: Summary of the findings for the main comparison: iguratimod compared to the other DMARDs (MTX and SASP) for rheumatoid arthritis.

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk Other DMARDs (MTX and SASP)	Corresponding risk Iguratimod				
ACR20/24 weeks	*581 per 1000*	*505 per 1000* (435 to 592)	RR 0.87 (0.75 to 1.02)	533 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
Tender joint count		The mean tender joint count in the intervention groups was *−0.01 lower* (−0.18 lower to 0.16 higher)		533 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
Swollen joint count		The mean swollen joint count in the intervention groups was *−0.15 lower* (−0.32 lower to 0.02 higher)		533 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
Assessment of rest pain		The mean assessment of rest pain in the intervention groups was *−0.10 lower* (−0.27 lower to 0.07 higher)		530 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
Physician global assessment of disease activity		The mean physician global assessment of disease activity in the intervention groups was *−0.03 higher* (−0.14 lower to 0.20 higher)		533 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
Patient global assessment of disease activity		The mean patient global assessment of disease activity in the intervention groups was *−0.05 lower* (−0.22 lower to 0.12 higher)		531 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
HAQ score		The mean HAQ score in the intervention groups was *−0.09 higher* (−0.08 lower to 0.26 higher)		533 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
CRP		The mean CRP in the intervention groups was *−0.13 lower* (−0.3 to 0.04 lower)		532 (2 studies)	⊕⊕⊕⊖moderate[1]	Important
ESR		The mean ESR in the intervention groups was *−0.05 lower* (−0.22 lower to 0.12 higher)		523 (2 studies)	⊕⊕⊕⊖moderate[1]	Important

*The basis for the assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI: confidence interval; RR: risk ratio; MTX: methotrexate; SASP: salazosulfapyridine.
GRADE working group grades of evidence.
High quality: further research is very unlikely to change our confidence in the estimate of effect.
Moderate quality: further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
Low quality: further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
Very low quality: we are very uncertain about the estimate.
[1]Random sequence of one study cannot mention how to be generated in detail, and no intent-to-treat analysis was included.

hypertension, or abnormal function of gastrointestinal tract were excluded. The studies of duplicate records, non-RCTs, nonclinical trials, the same study, ongoing without outcomes reported, or no full text were excluded.

2.1.3. Types of Intervention. Studies comparing iguratimod treatment (as monotherapy or in combination with other DMARDs) at a dose of 25 or 50 mg/day with placebo or other DMARDs were included. The duration of treatment in the trials must have been at least six months (or 24 weeks).

2.1.4. Types of Outcome Measures

(1) Primary Outcomes. Primary outcome measures were those defined as the ACR core set of disease activity measures for RA for clinical trials, which were endorsed by EULAR and the Outcome Measures in Rheumatology Clinical Trials (OMERACT) [8, 9]. They included (1) tender joint count; (2) swollen joint count; (3) assessment of rest pain; (4) patient global assessment of disease activity; (5) physician global assessment of disease activity; (6) health assessment questionnaire (HAQ) score; (7) acute phase reactants (ESR and CRP); and (8) radiographic change of bone and joint damage for trials lasting at least one year. In addition, the numbers of patients who met the ACR20, ACR50, and ACR70 response criteria were included. The EULAR response criteria are measured as the Disease Activity Score (DAS) according to the EULAR response criteria [10].

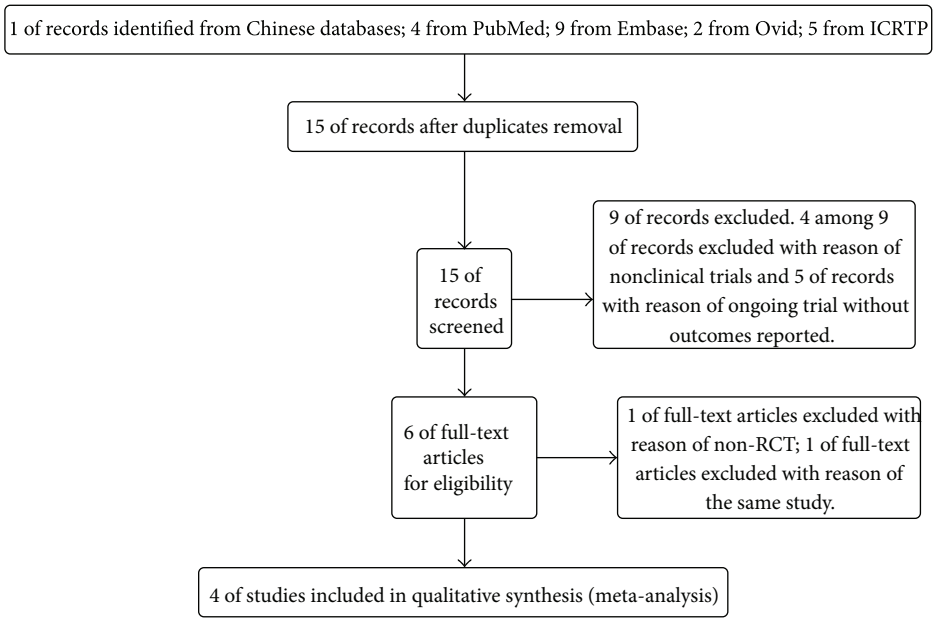


Figure 1: Search study flow diagram.

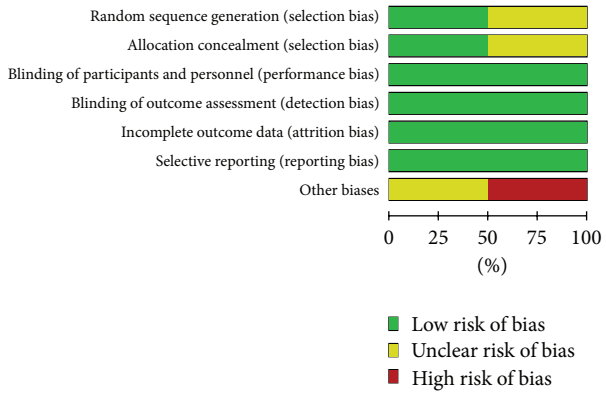


Figure 2: Risk of bias in the 4 studies included.

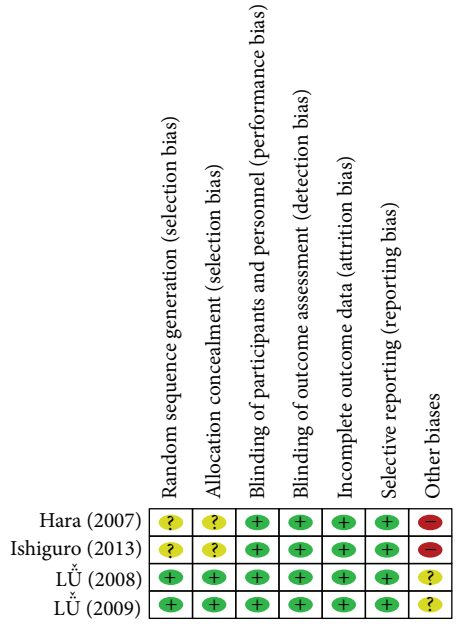


Figure 3: Summary of risk bias in the 4 studies included.

(2) Secondary Outcomes. Secondary outcome measures included health-related quality of life (HRQoL) of the patients, reported side effects, total number of patients withdrawn from the studies, and withdrawals due to adverse events.

We defined serious adverse events according to the ICH Guidelines [11] as any event that led to death, that was life-threatening, and required in-patient hospitalization or prolongation of existing hospitalization, and that resulted in persistent or significant disability and as any important medical events, which might have jeopardized the patient or required intervention to prevent them. We did not consider other adverse events to be serious.

2.2. Search Methods for Identification of Studies

2.2.1. Electronic Search. We searched the Cochrane Central Register of Controlled Trials (the Cochrane Library, 11 July 2012), PubMed (1950 to 29 March 2013), Embase (1980 to 29 March 2013), the Chinese Biomedical Database (CBM) (1975 to 18 March 2013), China National Knowledge Infrastructure (CNKI) (1917 to 18 March 2013), VIP Database (1989 to 18 March 2013), and the WHO ICTRP (18 March 2013).

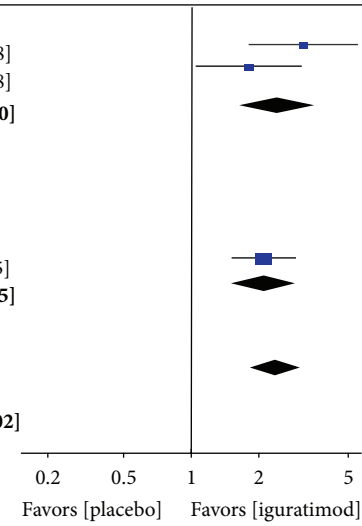
2.2.2. Search Strategy. Search strategy for the Cochrane Library, PubMed, Embase, and so forth, in English is given as follows:

#1 iguratimod

#2 T-614

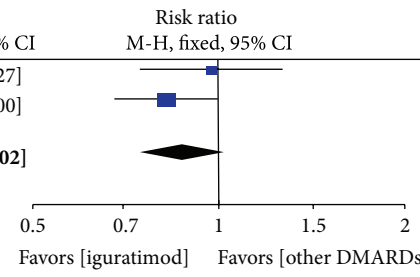
#3 CAS 123663-49-0

Study or subgroup	Iguratimod Events	Iguratimod Total	Placebo Events	Placebo Total	Weight	Risk ratio M-H, fixed, 95% CI
2.2.1 Iguratimod versus placebo						
Hara (2007)	71	132	11	64	22.5%	3.13 [1.79, 5.48]
Lǔ (2008)	28	93	16	95	24.1%	1.79 [1.04, 3.08]
Subtotal (95% CI)		**225**		**159**	**46.6%**	**2.44 [1.65, 3.60]**
Total events	99		27			
Heterogeneity: $\chi^2 = 2.01$; df = 1 ($P = 0.16$); $I^2 = 50\%$						
Test for overall effect: $Z = 4.47$ ($P < 0.00001$)						
2.2.2 Iguratimod + MTX versus placebo + MTX						
Ishiguro (2013)	114	164	27	88	53.4%	2.27 [1.63, 3.15]
Subtotal (95% CI)		**164**		**88**	**53.4%**	**2.27 [1.63, 3.15]**
Total events	114		27			
Heterogeneity: not applicable						
Test for overall effect: $Z = 4.86$ ($P < 0.00001$)						
Total (95%CI)		**389**		**247**	**100.0%**	**2.35 [1.82, 3.02]**
Total events	213		54			
Heterogeneity: $\chi^2 = 2.02$; df = 2 ($P = 0.36$); $I^2 = 1\%$						
Test for overall effect: $Z = 6.59$ ($P < 0.00001$)						
Test for subgroup differences: $\chi^2 = 0.08$; df = 1($P = 0.78$); $I^2 = 0\%$						



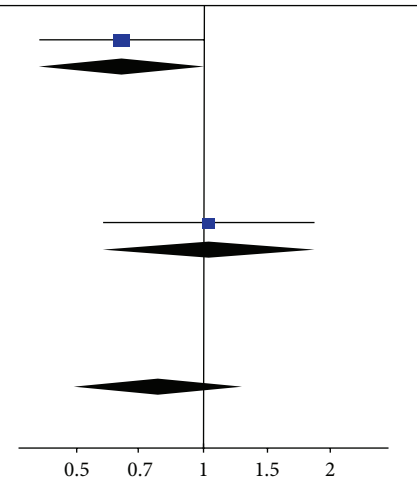
(a)

Study or subgroup	Iguratimod Events	Iguratimod Total	Other DMARDs Events	Other DMARDs Total	Weight	Risk ratio M-H, fixed, 95% CI
Hara (2007)	52	103	54	104	34.7%	0.97 [0.75, 1.27]
Lǔ (2009)	83	163	101	163	65.3%	0.82 [0.68, 1.00]
Total (95% CI)		**266**		**267**	**100.0%**	**0.87 [0.75, 1.02]**
Total events	135		155			
Heterogeneity: $\chi^2 = 1.01$; df = 1 ($P = 0.32$); $I^2 = 1\%$						
Test for overall effect: $Z = 1.69$ ($P = 0.09$)						



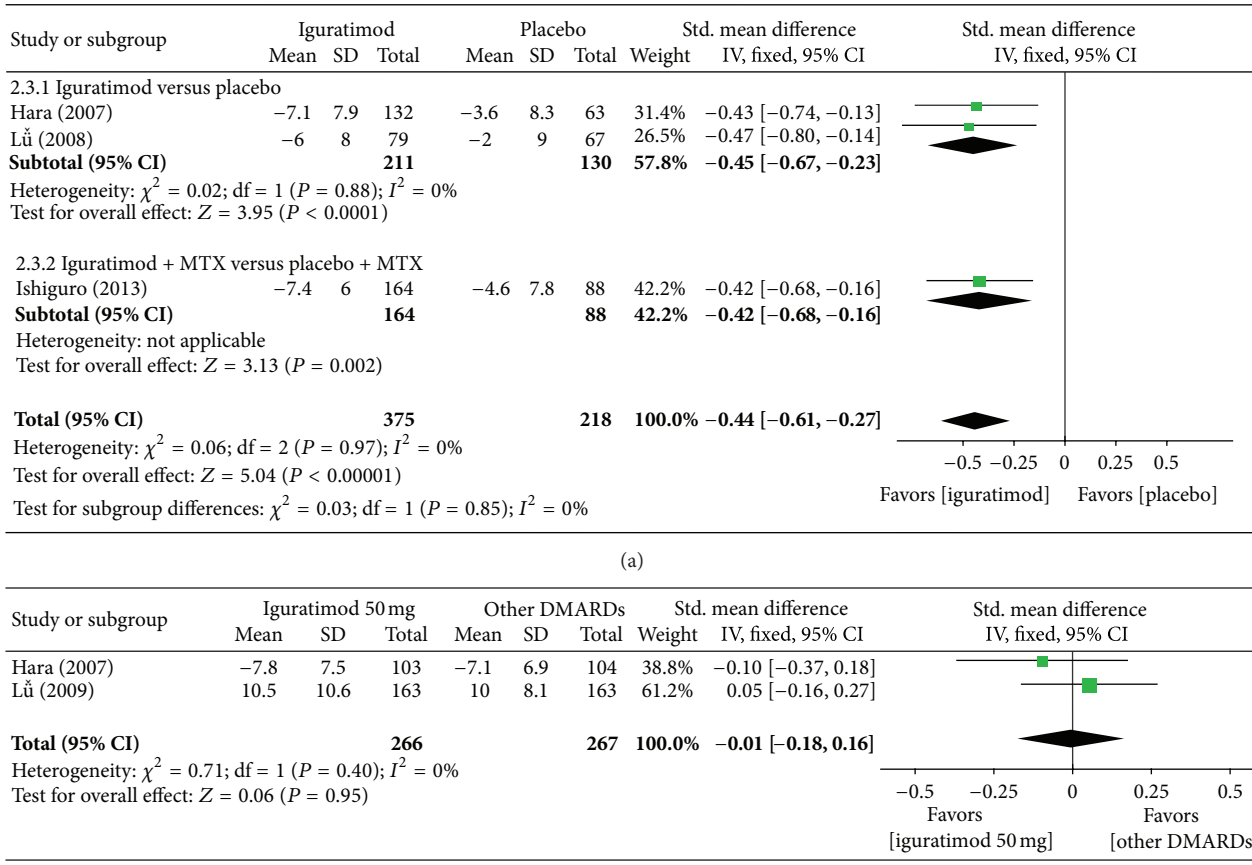
(b)

Study or subgroup	Iguratimod Events	Iguratimod Total	MTX Events	MTX Total	Weight	Odds ratio M-H, random, 95% CI
3.10.1 Iguratimod versus MTX						
Lǔ (2009)	53	163	70	163	57.5%	0.64 [0.41, 1.01]
Subtotal (95%CI)		**163**		**163**	**57.5%**	**0.64 [0.41, 1.01]**
Total events	53		70			
Heterogeneity: not applicable						
Test for overall effect: $Z = 1.94$ ($P = 0.05$)						
3.10.2 Iguratimod versus SASP						
Hara (2007)	35	104	34	103	42.5%	1.03 [0.58, 1.83]
Subtotal (95% CI)		**104**		**103**	**42.5%**	**1.03 [0.58, 1.83]**
Total events	35		34			
Heterogeneity: not applicable						
Test for overall effect: $Z = 0.10$ ($P = 0.92$)						
Total (95%CI)		**267**		**266**	**100.0%**	**0.78 [0.49, 1.24]**
Total events	88		104			
Heterogeneity: $\tau^2 = 0.04$; $\chi^2 = 1.61$; df = 1 ($P = 0.20$); $I^2 = 38\%$						
Test for overall effect: $Z = 1.04$ ($P = 0.30$)						
Test for subgroup differences: $\chi^2 = 1.61$; df = 1 ($P = 0.20$); $I^2 = 38.0\%$						



(c)

Figure 4: (a) Comparison of ACR20 at 24 weeks between iguratimod and placebo groups. (b) Comparison of ACR20 at 24 weeks between iguratimod and other DMARDs (MTX and SASP) groups. (c) Comparison of ACR50 at 24 weeks between iguratimod and other DMARDs (MTX and SASP) groups.



(a)

(b)

Figure 5: (a) Comparison of tender joint count between iguratimod and placebo groups. (b) Comparison of tender joint count between iguratimod and other DMARDs (MTX and SASP) groups.

#4 C17H14N2O6S

#5 Ailamode

#6 N-3-formylamino-4-oxo-6-phenoxy-4H-chromen-7-yl-methanesulfonamide

#7 3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one

#8 or/#1–7

#9 randomized controlled trials

#10 random

#11 control

#12 trials

#13 or/#9–12

#14 rheumatoid arthritis

#15 (#8, #13, and #14).

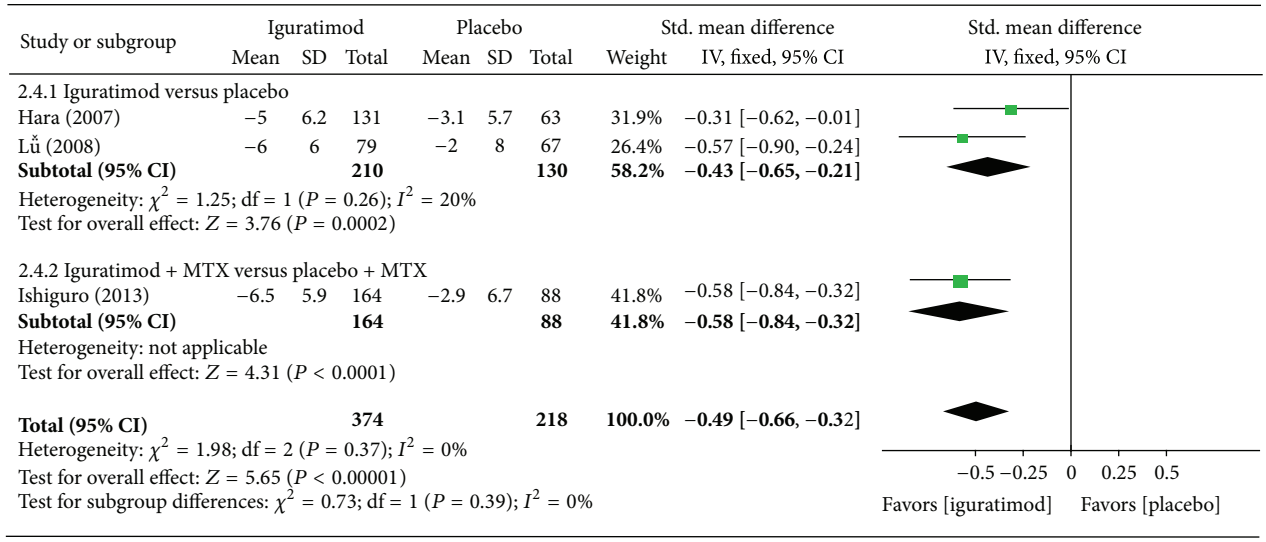
We searched the Chinese Biomedical Database (CBM), China National Knowledge Infrastructure (CNKI), and VIP Database by using the strategy adjusted in Chinese.

2.3. Data Collection and Analysis

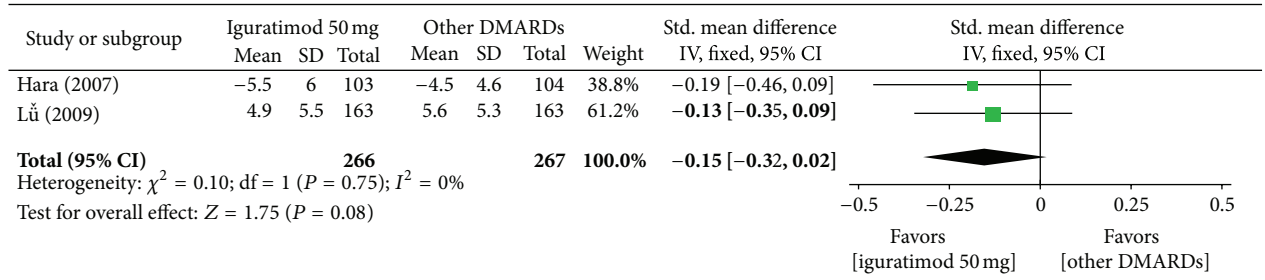
2.3.1. Selection of Studies. Three review authors (Jiangtao Li, Hejuan Mao, and Shuo Chen) independently assessed for inclusion all of the potential studies identified as a result of the search strategy. We resolved disagreements through discussion. We included only those studies that used a strict randomization procedure. We telephoned or wrote letter by email to contact the authors of those articles in which "randomly allocated participants" was mentioned to determine whether the randomization procedure was adequate or not.

2.3.2. Data Extraction and Management. We designed a form to extract data for retrieval of records to meet the needs of the project design. For eligible studies, the three review authors extracted the data using the agreed form. We resolved discrepancies through discussion. Where disagreement could not be resolved even through discussion, experts in the area were contacted to make a decision. We input the data into Review Manager software (RevMan Manager version 5.2.4, 2013) and checked them for accuracy.

2.3.3. Assessments Risk of Bias in Included Studies and Quality of Evidence. According to assessing risk of bias in included



(a)



(b)

Figure 6: (a) Comparison of swollen joint count between iguratimod and placebo groups. (b) Comparison of swollen joint count between iguratimod and other DMARDs (MTX and SASP) groups.

studies in the Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0, updated in March 2011) [12], we assessed each study included in random sequence generation and allocation concealment (selection bias), blinding (performance bias and detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other biases. According to GRADE Handbook for grading quality of evidence and strength of recommendation, version 3.2 (updated in March 2009) [13] and GRADE profiler 3.6 software, we assessed quality of evidence of each study included.

2.3.4. Methods of Statistical Analysis. Statistical analysis was made by using the Review Manager software (Cochrane Collaboration's RevMan 5.2.4, 2013). For continuous variables, mean differences (MD) or standardized mean differences (SMD) were used to describe effect size with confidence interval (CI) set at 95%. For dichotomous variables, odds ratio (OR), relative risk (RR), and risk difference (RD) were used to describe effect with confidence interval (CI) set at 95%. χ^2 test was used to analyze the heterogeneity among results. Where there is no heterogeneity ($P > 0.1$; $I^2 < 50\%$), the fixed effects model analysis was made. If there is heterogeneity between studies, random effects model was used, and the source, cause, and sensitivity of heterogeneity were analyzed in subgroups. Where there is clinical heterogeneity between studies, descriptive analysis was made.

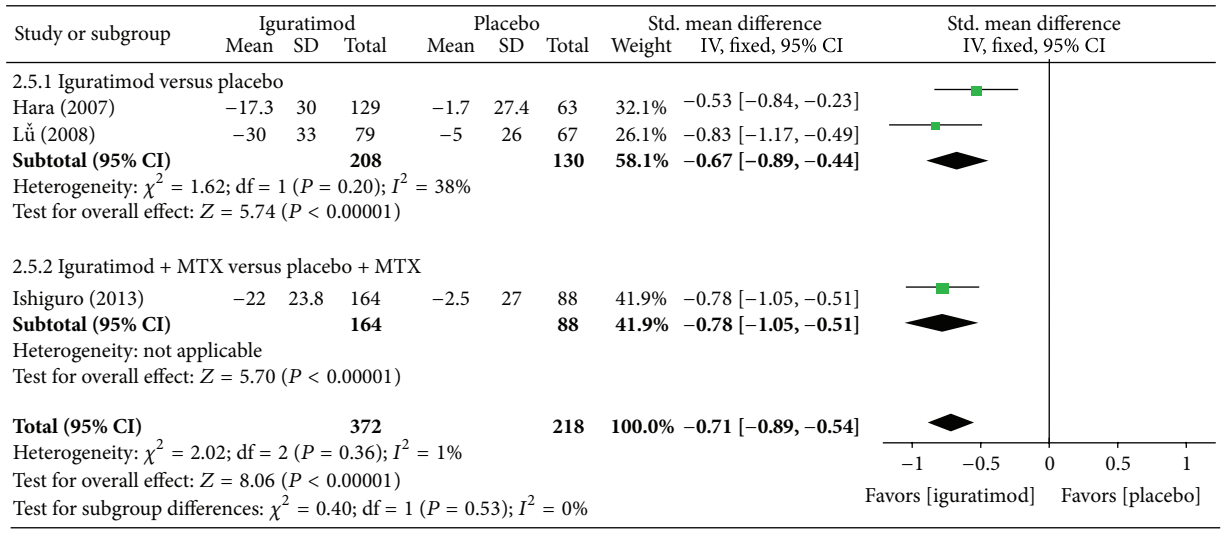
3. Results and Discussion

3.1. Results

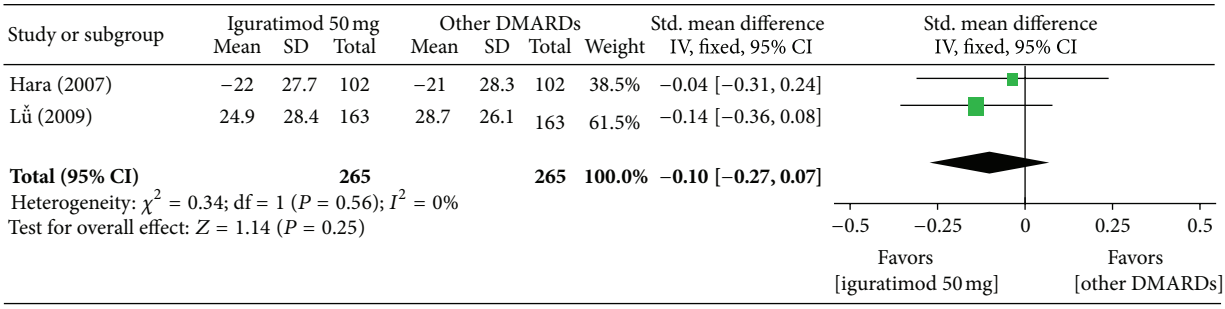
3.1.1. Results of the Search. We identified 21 records by electronic searches and hand searches, including 1 of records indentified from the Chinese database, four from PubMed, nine from Embase, two from Ovid, and five from ICRTP. We excluded 17 records because of duplicate records, non-RCTs, nonclinical trials, the same study, ongoing without outcomes reported, or no full text. At the end, we finalized four studies [2, 14–16] to make quantitative synthesis (meta-analysis) (Figure 1).

3.1.2. Characteristics of Included Studies (Table 1)

(1) Types of Studies. All of the four included studies were multicenter, double-blind, randomized controlled trials. Three of them had a placebo control group. The duration of these studies was about six months (from 24 weeks to 28 weeks).



(a)



(b)

Figure 7: (a) Comparison of assessment of rest pain between iguratimod and placebo groups. (b) Comparison of assessment of rest pain between iguratimod and other DMARDs (MTX and SASP) groups.

The studies were conducted in China ($n = 2$) and Japan ($n = 2$).

(2) Participants of Included Studies. In the four RCT studies included [2, 14–16], in total, 1407 patients with RA were enrolled (1159 females and 248 males; mean age was from 45.9 to 58.2 years old). Diagnosis of RA was based on the American Rheumatism Association (ARA) criteria in 1987 [17] in the two Japanese studies [15, 16] and on the American Rheumatism Association (ARA) criteria in 1991 [18] in the two Chinese studies [2, 15].

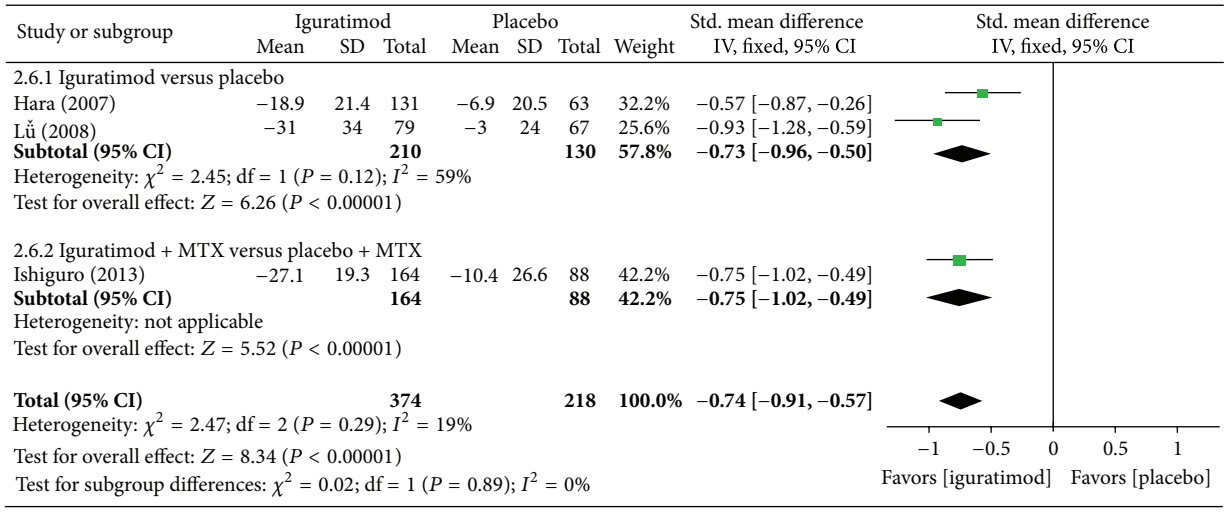
(3) Interventions of Studies Included. Among the four studies included [2, 14–16], four different types of intervention were used: (a) iguratimod versus placebo and iguratimod versus SASP [14], (b) iguratimod versus placebo [2], (c) iguratimod versus MTX, and (d) iguratimod + MTX versus placebo + MTX [16]. The low dose of MTX (6–8 mg per week) was in the study of iguratimod + MTX versus placebo + MTX, and the low dose of MTX (15 mg per week) was in the study of iguratimod versus MTX. Duration of treatment was 24 weeks [2, 15, 16] and 28 weeks in one study [14].

(4) Measures of Outcomes. Primary outcomes (ACR20) and secondary outcomes (tender joint count; swollen joint count; assessment of rest pain; physician global assessment of disease activity; patient global assessment of disease activity; HAQ score; ESR; CRP; adverse events, etc.) were all reported in the four studies included.

3.1.3. Assessment of Methodological Quality of Studies Included [13] (Figures 2 and 3)

(1) Randomized Method and Allocation Concealment. In all of the four studies included [2, 14–16], randomization was described. In two studies [2, 15], random sequence generation and allocation concealment were detailed, and the validity of method was ascertained by telephone. In the other two studies [14, 16], random sequence generation and allocation concealment were described but not ascertained because the author could not be reached via email. For this reason, some degree risk of selection bias of two studies included [15, 16] existed.

(2) Blinding of Participants and Personnel. In all of the four studies [2, 14–16] blinding of participants and personnel was described. Double blinding was detailed in three studies [2, 14, 15] and not mentioned in one study [16]. The author could



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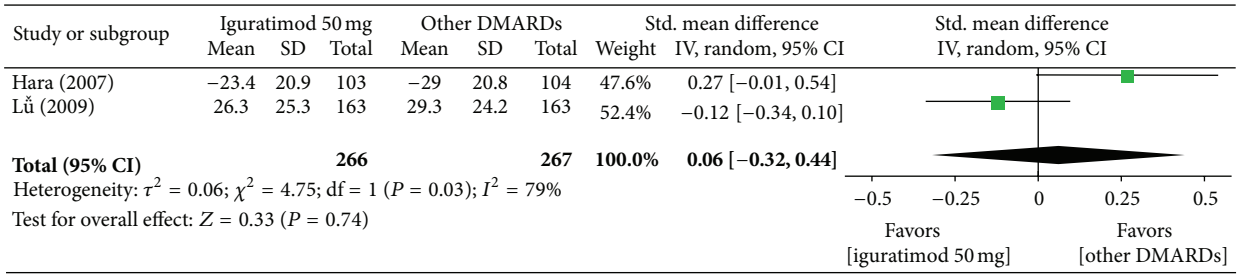


(b)

Figure 8: (a) Comparison of physician global assessment of disease activity between iguratimod and placebo groups. (b) Comparison of physician global assessment of disease activity between iguratimod and other DMARDs (MTX and SASP) groups.

not be reached via email. Thus, low risk of performance bias and detection bias of outcome assessment existed.

(3) Incomplete Outcome Data. Withdrawals, dropouts, loss of followup, and intent-to-treat analysis were reported in detail in two studies included [2, 15] and were not described in the other two studies included [14, 16]. Therefore, high risk of attrition bias in general existed.

(4) Selective Reporting Bias. The four studies [2, 14–16] included reported all outcomes, including adverse events. There was no selective reporting bias.

(5) Other Biases. Baseline information including sex, age and secondary outcomes of the four studies [2, 14–16] was described in detail, and outcomes of different groups had robust comparability. Sample sizes of three studies [2, 15, 16] were estimated. All of the studies included were conducted in general hospitals or good clinical practice bases of medical universities, with all of which having high scientific research level and thus reducing other risk biases of these studies to a large extent.

3.1.4. Meta-Analysis

(1) Primary Outcomes

(a) ACR20, ACR50, and ACR70 at 24 Weeks. Superiority pooled analysis of three studies [2, 14, 16] included shows that ACR20 at 24 weeks among patients with RA in the iguratimod group was significantly superior to that of the placebo group (RR = 2.35, 95% CI: 1.82, 3.02 in Figure 4(a)). Noninferiority pooled analysis of two studies [14, 15] included showed that ACR20 at 24 weeks among patients with RA in iguratimod group did not differ significantly from that of other DMARDs (MTX and SASP) group (RR = 0.87, 95% CI: 0.75, 1.02 in Figure 4(b)). Only in one study [2], ACR50 at 24 weeks did not differ significantly between the iguratimod group and the placebo group (RR = 0.62, 95% CI: 0.21, 1.82), and ACR70 at 24 weeks differed significantly (RR = 0.06, 95% CI: 0.01, 0.51). ACR50 at 24 weeks did not differ significantly between the iguratimod group and the other DMARDs (MTX and SASP) group [14, 15] (RR = 0.78, 95% CI: 0.49, 1.24 in Figure 4(c)). Only in one study [15], ACR70 at 24 weeks did not differ

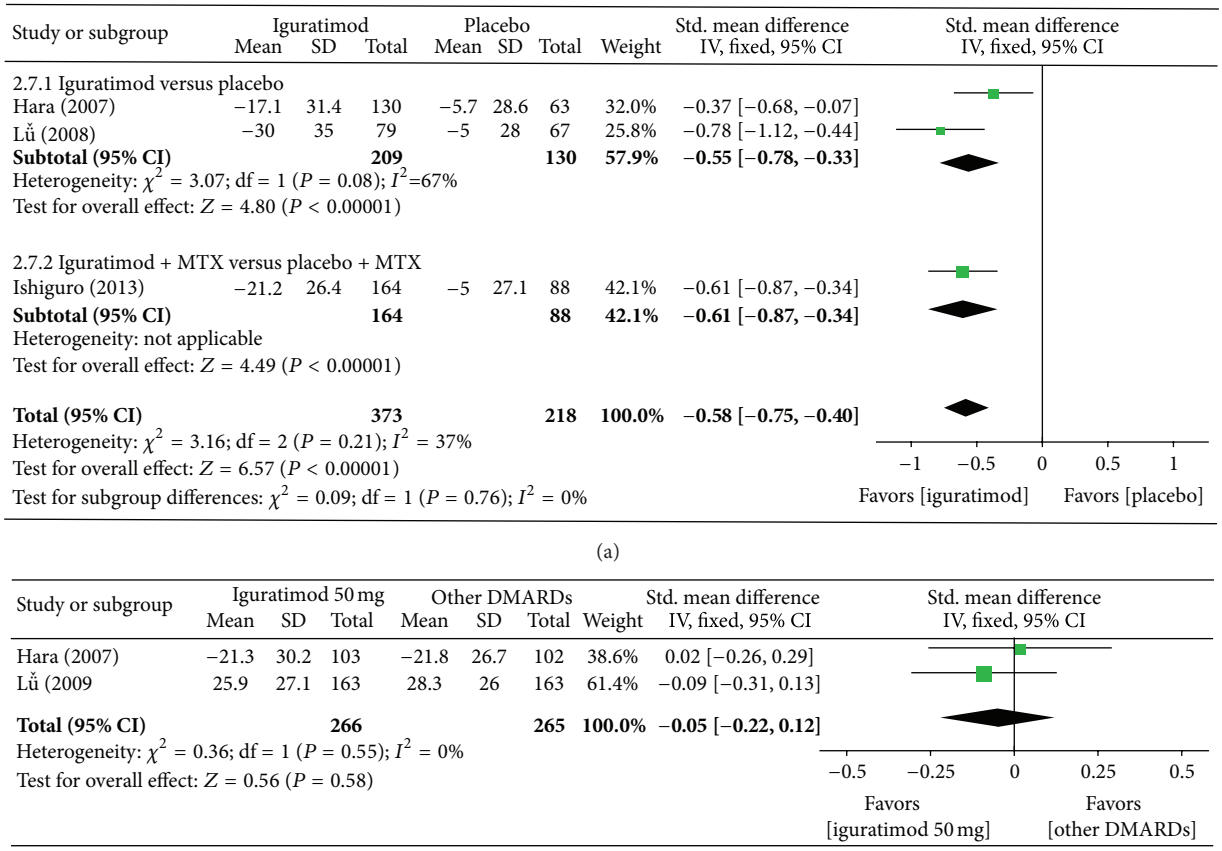


Figure 9: (a) Comparison of patient global assessment of disease activity between iguratimod and placebo groups. (b) Comparison of patient global assessment of disease activity between iguratimod and other DMARDs (MTX and SASP) groups.

significantly between the iguratimod group and the other DMARDs (MTX) group (RR = 0.75, 95% CI: 0.43, 1.32).

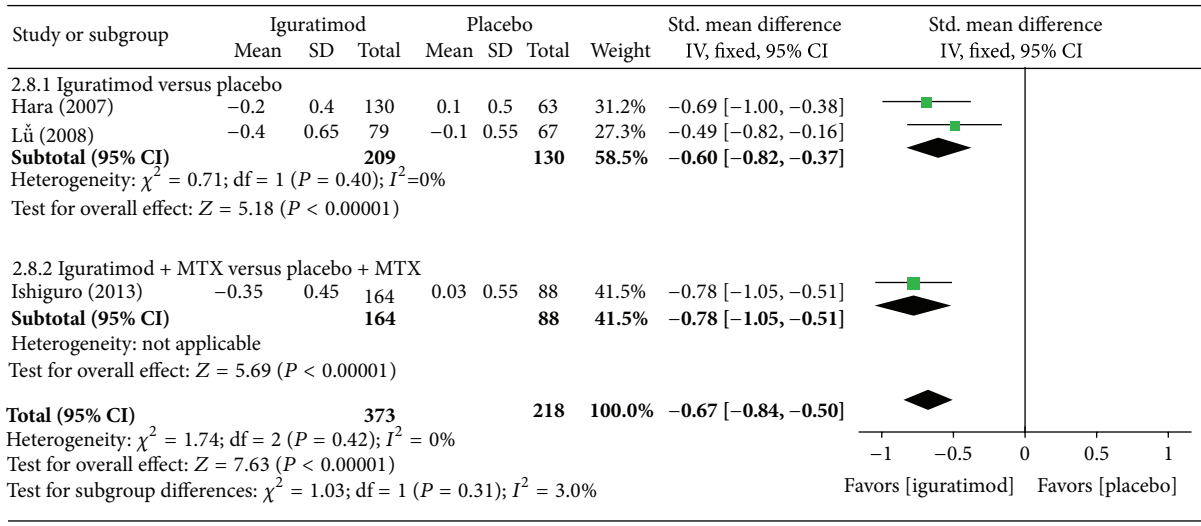
(b) Tender Joint Count. Superiority analysis of three studies [14] included showed that tender joint count among patients with RA in the iguratimod group reduced more than that of the placebo group with a significant difference (MD = −0.44, 95% CI: −0.61, −0.27 in Figure 5(a)). Noninferiority analysis of two studies [14, 15] included showed that tender joint count among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.01, 95% CI: −0.18, 0.16 in Figure 5(b)).

(c) Swollen Joint Count. Superiority analysis of three studies [2, 15, 16] included showed that swollen joint count among patients with RA in the iguratimod group reduced more than that of the placebo group with a significant difference (MD = −0.49, 95% CI: −0.66, −0.32 in Figure 6(a)). Noninferiority analysis of two studies [14, 15] included showed that swollen joint count among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.15, 95% CI: −0.32, 0.02 in Figure 6(b)).

(d) Assessment of Rest Pain. Superiority analysis of three studies included [2, 14, 16] showed that assessment of rest pain among patients with RA in the iguratimod group was worse than that of the placebo group with a significant difference (MD = −0.71, 95% CI: −0.89, −0.54 in Figure 7(a)). Noninferiority analysis of two studies included [14, 15] shows that assessment of rest pain among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.10, 95% CI: −0.27, 0.07 in Figure 7(b)).

(e) Physician Global Assessment of Disease Activity. Superiority analysis of three studies included [2, 15, 16] showed that physician global assessment of disease activity among patients with RA in the iguratimod group reduced more than that of the placebo group with a significant difference (MD = −0.74, 95% CI: −0.93, −0.57 in Figure 8(a)). Noninferiority analysis of two studies included [14, 15] showed that physician global assessment of disease activity among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) did not differ significantly (MD = 0.06, 95% CI: −0.32, 0.44 in Figure 8(b)).

(f) Patient Global Assessment of Disease Activity. Superiority analysis of three studies included [14–16] showed that patient



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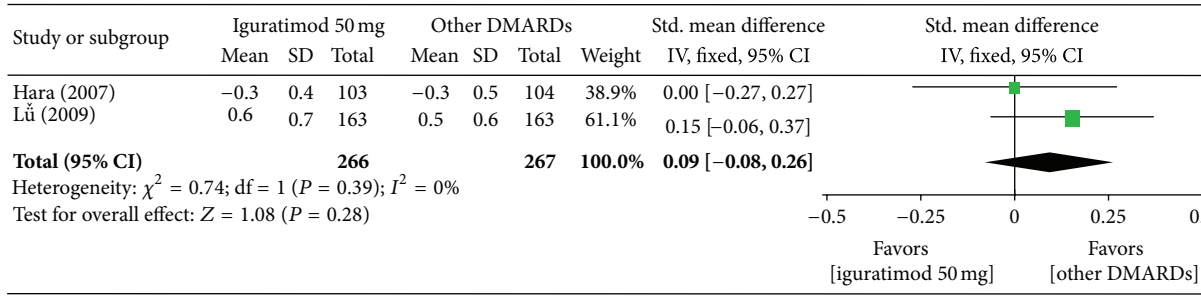


(b)

Figure 10: (a) Comparison of HAQ score between iguratimod and placebo groups. (b) Comparison of HAQ score between iguratimod and other DMARDs (MTX and SASP) groups.

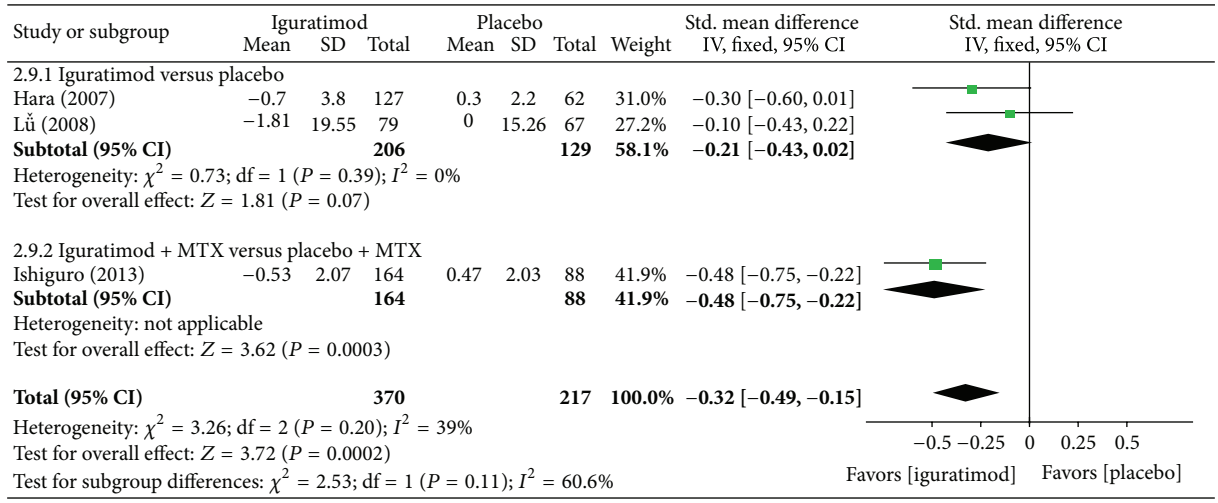
global assessment of disease activity among patients with RA in the iguratimod group reduced more than that of placebo group with a significant difference (MD = −0.58, 95% CI: −0.80, −0.36 in Figure 9(a)). Noninferiority analysis of two studies included [14, 15] showed that patient global assessment of disease activity among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.05, 95% CI: −0.22, 0.12 in Figure 9(b)).

(g) HAQ Score. Superiority analysis of three studies included [14–16] shows that HAQ score among patients with RA in the iguratimod group improved more than that of the placebo group with a significant difference (MD = −0.67, 95% CI: −0.84, −0.50 in Figure 10(a)). Noninferiority analysis of two studies included [14, 15] showed that HAQ score among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = 0.09, 95% CI: −0.08, 0.26 in Figure 10(b)).

(h) C-Reactive Protein (CRP). Superiority analysis of three studies included [14–16] showed that CRP among patients with RA in the iguratimod group decreased more than that of the placebo group with a significant difference (MD = −0.32, 95% CI: −0.49, −0.15 in Figure 11(a)). Noninferiority analysis of two studies included [14, 15] showed that CRP among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.13, 95% CI: −0.30, 0.04 in Figure 11(b)).

(i) Erythrocyte Sedimentation Rate (ESR). Superiority analysis of three studies included [14–16] showed that ESR among patients with RA in the iguratimod group decreased more than that of the placebo group with a significant difference (MD = −0.64, 95% CI: −0.81, −0.46 in Figure 12(a)). Noninferiority analysis of two studies included [14, 15] showed that ESR among patients with RA in the iguratimod group and in the other DMARDs (MTX and SASP) group did not differ significantly (MD = −0.05, 95% CI: −0.22, 0.12 in Figure 12(b)).

(j) Radiographic Change of Bone and Joint Damage. Radiographic change of bone and joint damage was not analyzed for trials of under one year of duration.



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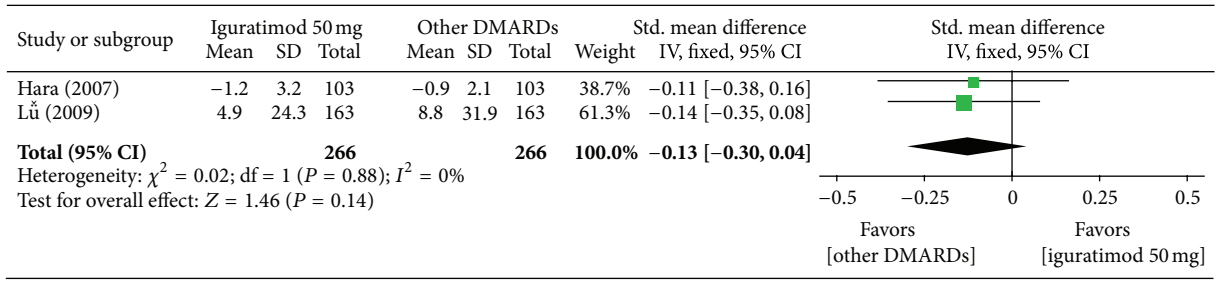


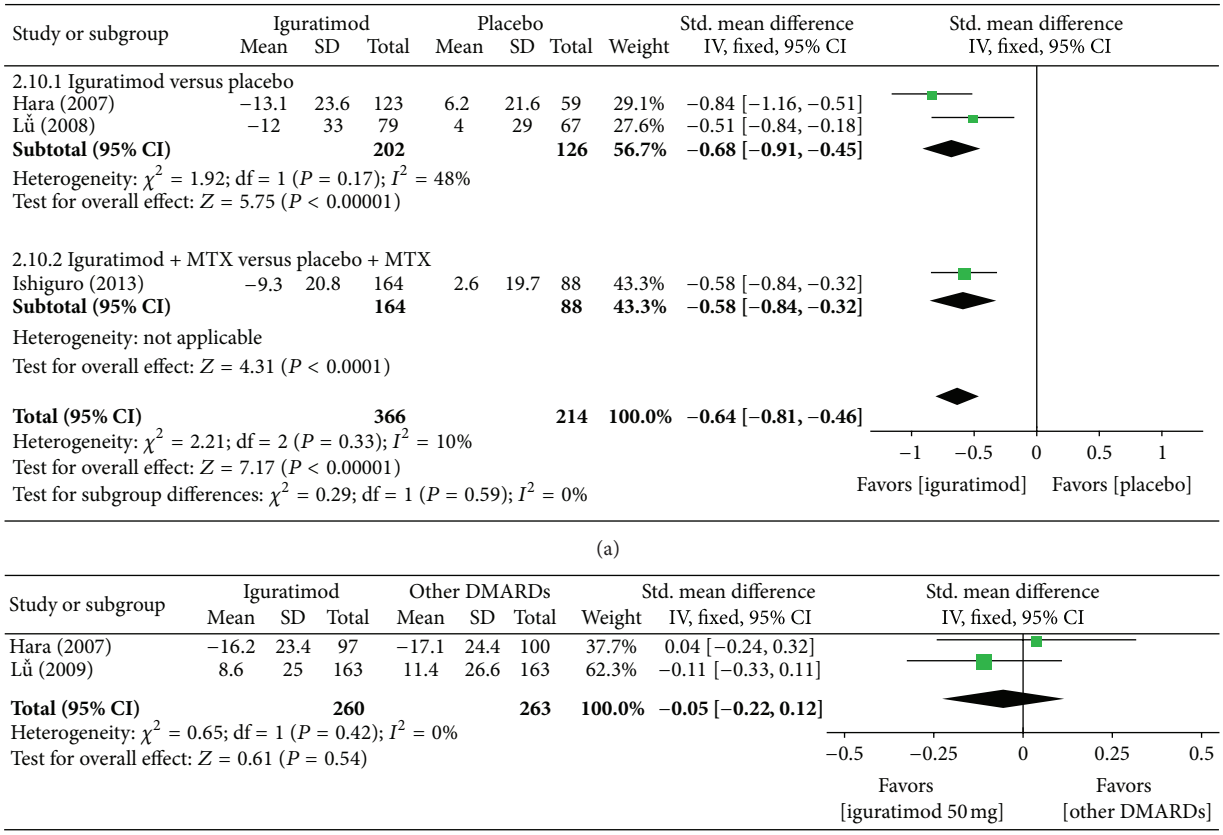
(b)

Figure 11: (a) Comparison of CRP score between iguratimod and placebo groups. (b) Comparison of CRP score between iguratimod and other DMARDs (MTX and SASP) groups.

(2) Secondary Outcomes. Adverse events of the four studies included [2, 14–16] were described in detail. Pooled analysis of these studies showed no significant difference between the iguratimod group and the placebo group (RR = 1.17, 95% CI: 0.41, 3.31 in Figure 13(b)) or between the iguratimod group and the other DMARDs group (MTX and SASP) (RR = 1.14, 95% CI: 0.80, 1.62 in Figure 13(b)). Adverse events mainly included leucopenia, abnormal level function, upper digestive tract disorder, skin rash, or pruritus. No fatal adverse events were reported.

(3) Quality of GRADE Evidence. Assessment for quality of evidence was made using GRADE profiler software recommended by the Cochrane Collaboration. The result of assessment for the four studies included [2, 14–16] was moderate quality (Tables 2 and 3).

3.2. Discussion. This systematic review included four eligible studies [2, 14–16]. In three studies, superiority pooled analysis of outcomes showed a significant difference between the iguratimod group and the placebo group with RA. Results showed that iguratimod obviously improved ACR20 and ACR70 at 24 weeks, and patients' health assessment questionnaire (HAQ) score showed reduced rest pain, tender joint count, and swollen joint count; lowered CRP level and ESR; and lowered physician and patient global assessment level of disease activity, but ACR50 at 24 weeks has no significant difference. In two studies [14, 15], noninferiority pooled analysis showed no significant difference between the iguratimod group and the other DMARDs (MTX and SASP) group. Iguratimod was not superior to the other DMARDs (MTX and SASP) in treating RA. All of the four studies [2, 14–16] reported adverse events of iguratimod in treating RA. No significant difference was found in adverse events between the iguratimod group and the placebo group (RR = 1.38, 95% CI: 0.74, 2.55) or the iguratimod group and the other DMARDs (MTX and SASP) group (RR = 1.14, 95% CI: 0.80, 1.62). Major adverse events included hepatic dysfunction, leucopenia, upper digestive tract disorder, skin rash, and pruritus. No fatal adverse events were reported. A long-term clinical study by Hara on iguratimod intake in RA patients for 1 and 2 years showed that iguratimod had the same efficacy and adverse events as those of SASP [19]. Such finding was confirmed by this systematic review.



(a)

(b)

Figure 12: (a) Comparison of ESR score between iguratimod and placebo groups. (b) Comparison of ESR score between iguratimod and other DMARDs (MTX and SASP) groups.

The units carrying out studies included are all general hospitals or good clinical practice bases of medical universities that have high scientific research diathesis and abilities. Duration and dosage of iguratimod had homogeneity. The evidence of the studies included is moderate quality by carrying out GRADE profiler 3.6 software in spite of some degree-selective biases and no intent-to-treat analyses.

This systematic review has two limitations. First, both the number and the sample size of the studies included are too small. Second, these studies were conducted either in China or in Japan. Similar trials in other countries and regions were not indentified in this review, thus making our review results not representative enough.

In the four studies included [2, 14–16], two described in detail the randomization procedure, allocation concealment, and blinding. We ascertained the validity of the two studies [14, 15] by telephone. For the other two studies [14, 16], we could not reach the authors by email to validate the randomization procedure, allocation concealment, and blinding. Two studies [14, 16] did not have intent-to-treat analysis, and we speculated that participants enrolled were included because of incomplete data. This shortage of two studies [15, 16] made it difficult for us to assess the rationality of study design, and reliability and validity of study results, to some degree, have affected the application of evidence. We hope that future researchers can try to avoid such weaknesses.

4. Conclusions

In summary, this systemic review shows that iguratimod is relatively safe and effective in treating RA and its efficacy is the same as that of MTX and SASP. Nevertheless, due to methodological defects, small number, and small sample size of the studies included, we still do not know whether iguratimod is as effective as other DMARDs besides MTX and SASP. We suggest that more high-quality and large-scaled RCTs should be done to determine the efficacy and safety of iguratimod for RA and whether iguratimod is as effective as other DMARDs besides MTX and SASP.

Conflict of Interests

The authors of the paper have no conflict of interests with the manufacturers of iguratimod, RevMan, and GRADE profiler.

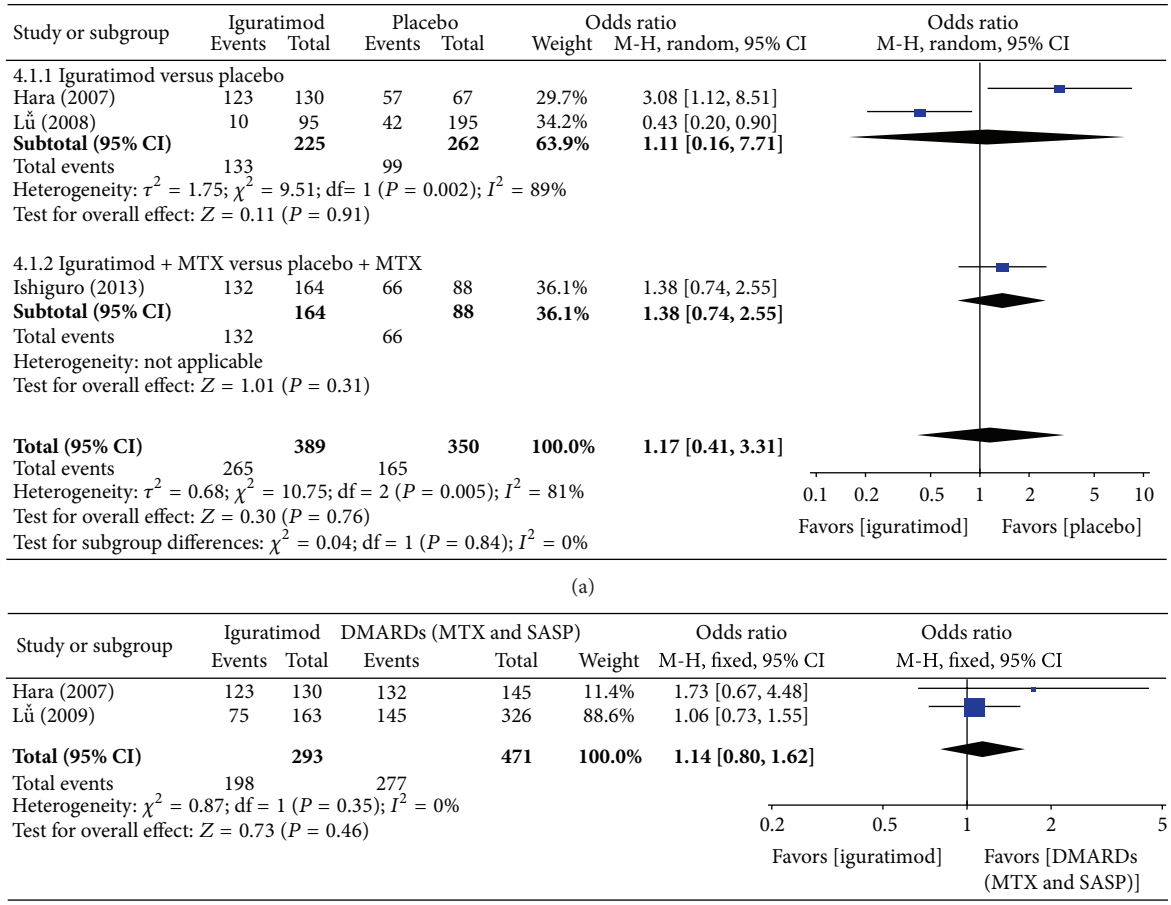


Figure 13: (a) Comparison of adverse events between iguratimod and placebo groups. (b) Comparison of adverse events between iguratimod and other DMARDs (MTX and SASP) groups.

Acknowledgment

The authors thank Professor Dongtao Lin of Sichuan University for copyediting the paper.

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Research Article

Induction of Th17 Lymphocytes and Treg Cells by Monocyte-Derived Dendritic Cells in Patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus

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Dendritic cells (DCs) have a key role in the regulation of immune response. We herein explored, in patients with inflammatory diseases, the role of monocyte derived DC's (mo-DCs) on the generation of Th17 and T regulatory (Treg) lymphocytes. Peripheral blood was obtained from thirty-five patients with rheumatoid arthritis (RA), twelve with systemic lupus erythematosus (SLE), and twenty healthy subjects. Mo-DCs were generated under standard (IL-4/GM-CSF) or tolerogenic (IL-4/GM-CSF plus recombinant P-selectin or PD-1 or IL-10) conditions, and their ability to induce Th17 and Treg lymphocytes was tested. We detected that mo-DCs from patients with RA showed an enhanced release of IL-6 and IL-23 as well as an increased capability to induce Th17 cells. Although mo-DCs from SLE patients also released high levels of IL-6/IL-23, it did not show an increased ability to induce Th17 lymphocytes. In addition, mo-DCs, from patients with RA and SLE generated under the engagement of PSGL-1, showed a defective capability to induce Foxp3+ Treg cells. A similar phenomenon was observed in SLE, when DC's cells were generated under PDL-1 engagement. Our data indicate that DCs from patients with rheumatic inflammatory disease show an aberrant function that may have an important role in the pathogenesis of these conditions.

1. Introduction

The defective regulation of the activation and proliferation of auto-reactive T cells that are not deleted in the thymus may result in different autoimmune disorders [1]. Initially, several inflammatory autoimmune diseases were considered to be entirely mediated by Th1 lymphocytes, which mainly synthesize IL-2 and IFN-γ. However, in recent years, different studies have demonstrated that Th17 lymphocytes also play an important role in the pathogenesis of these conditions, including multiple sclerosis, psoriasis, inflammatory bowel disease, Hashimoto's thyroiditis, and rheumatoid arthritis [2–8]. In addition, Th17 cells and Th17 cytokines are also involved in the pathogenesis of fibrotic autoimmune diseases as primary biliary cirrhosis (PBC) and systemic sclerosis (SSc) [9]. Th17 lymphocytes are a subset of CD4+ T cells characterized by the synthesis of IL-17A, IL-21, and IL-22, and the expression of IL-23R and CCR6 receptors [10]. Interleukin IL-17A is a proinflammatory cytokine that induces the synthesis of TNF-α, IL-1β, IL-6, IL-8, GM-CSF, different metalloproteinases (MMP-1, -3, -9), chemokines (CCL2, 3), and PGE2 by different cell types, including fibroblasts, epithelial and endothelial cells, keratinocytes, osteoblasts, and monocyte/macrophages [11–13]. In addition, in patients with

rheumatoid arthritis (RA), IL-17 is involved in the destruction of the extracellular matrix and juxtaarticular bone resorption, through the induction of synthesis of RANKL and matrix metalloproteases [14, 15].

The differentiation of human Th17 lymphocytes is induced by the combined action of different cytokines, including IL-1β, IL-6, and IL-23 [16], whereas IL-21 promotes the survival and expansion of these cells [17, 18]. In addition, TGF-β seems to also participate in the differentiation of Th17 cells, which may have a conventional or a regulatory phenotype [19, 20].

Dendritic cells (DCs) are professional antigen presenting cells that play a key role in the induction and regulation of T cell mediated responses [21]. Two main DC subsets have been characterized in humans, myeloid, and plasmacytoid DCs (mDCs, $CD11c^+$, pDCs, and $CD11c^-$) [22]. In addition, when monocytes are cultured in the presence of GM-CSF and IL-4, differentiate into a subset of mDCs (monocyte derived DCs or MDDCs or mo-DCs) [23]. DCs express a wide repertoire of membrane receptors, including pattern recognition receptors (PRRs), which upon engagement by their ligands (DAMPS and PAMPs) induce their terminal differentiation and activation [21]. It has been described that a subfamily of PRRs (C-type lectin receptors or CLRs) induce the activation of Syk and CARD9 and preferentially induce the polarization of naive Th0 lymphocytes towards Th17 cells [24–26]. On the other hand, it has also been described that tolerogenic DCs are able to inhibit the differentiation of naïve Th0 lymphocytes, suppressing thus the generation of T cell mediated immune responses [27]. Conversely, tolerogenic DCs are able to induce the generation of T regulatory (Treg) lymphocytes [28]. In this regard, it has been described that different membrane receptors (e.g., PSGL-1 and PDL-1) as well as cytokines (e.g., IL-10) are able to induce the generation of tolerogenic DCs [29, 30].

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by chronic synovial inflammation, which results in cartilage and bone damage, leading to joint destruction. Different cell types and their mediators are involved in the tissue destructive inflammation seen in this condition, including Th17 lymphocytes [31]. In this regard, mice deficient in IL-17 show a decreased induction of collagen induced arthritis (CIA) [32]. Accordingly, in this animal model of RA, the neutralization of IL-17 reduces joint inflammation, cartilage destruction, and bone erosion [33]. In humans, elevated concentrations of IL-17 have been found in synovial fluid and in peripheral blood of RA patients as well as a high proportion of Th17 lymphocytes in their peripheral blood [34]. In fact, different data indicate that the inflamed rheumatoid synovium is a tissue niche that greatly favors the generation of Th17 cells [35]. Recently, Vaknin-Dembinsky et al. showed that mo-DCs from patients with multiple sclerosis show an enhanced synthesis of IL-23 as well as an increased ability to induce the *in vitro* differentiation of Th17 lymphocytes [36]. However, the precise role of IL-23 and DCs on the induction of Th17 cells in patients with RA has not been fully characterized.

SLE is an autoimmune, systemic, inflammatory condition with many different immunologic aberrations, including an enhanced synthesis of IL-10 and type I interferon, a diminished function of natural Treg lymphocytes, and an aberrant phenotype and function of DCs [37]. As in the case of RA, different reports indicate that Th17 lymphocytes are involved in the pathogenesis of SLE, including lupus nephritis [38, 39]. We have herein studied the role of immunogenic and tolerogenic DCs on the *in vitro* induction of Th17 cells and Treg lymphocytes in patients with RA and SLE. We detected that mo-DCs from patients with RA show an enhanced release of IL-6/IL-23 and an increased capability to induce Th17 cells. On the other hand, tolerogenic mo-DCs from patients with RA and SLE showed a defective capability to induce the generation of Foxp3+ Treg cells. These results further support the important role of DCs in the pathogenesis of autoimmune diseases, and indicate that it is interesting to comparatively evaluate the generation of effector and regulatory lymphocytes in these conditions [9].

2. Materials and Methods

2.1. Patients. Thirty-five female patients with RA, with a mean age of 42.3 years, diagnosed according to the criteria of the American College of Rheumatology (ACR) [40] were included in the study. Fifteen patients were untreated at the time of their inclusion in the study, and their mean Disease Activity Score 28 (DAS28) was 4.09. The remaining patients (with a mean DAS28 of 3.45) were under therapy with disease-modifying antirheumatic drugs (DMARDs), receiving mainly low-dose methotrexate (14.9 ± 3.8 mg/week) and sulfasalazine (1.4 ± 0.7 g/day). Twelve female patients with SLE, according to the classification criteria of the American College of Rheumatology [41], were also studied. Their mean age was 33.1 years (range: 16–39), and the mean duration of disease was 19 months. All these patients were not receiving immunosuppressive drugs at the time of the study, and eight of them had active disease, with a MEX-SLEDAI score >3.0 [42]. Three patients were under therapy with low doses of prednisone (10–20 mg/day), and two were receiving hydroxychloroquine (200 mg/day). No patients with renal failure were included. No patients under therapy with biological agents were included in the study. Twenty female healthy individuals with a mean age 35.5 years were included as controls. This study was approved by the local University Ethics Committee and all subjects signed an informed consent.

2.2. Cells. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO). Monocytes and $CD4^+$ T cells were positively isolated from PBMC by using MicroBeads (Miltenyi Biotec) coupled to mAbs, according to manufacturer's instructions. The purity of the isolated cells was verified by flow cytometry analysis and was always greater than 95%. After $CD4^+$ T cells isolation, cells were incubated in complete RPMI culture medium with 2% of fetal calf serum (FCS) until they were used in coculture assays.

2.3. Generation of Monocyte-Derived Dendritic Cells (mo-DC). Monocytes (1×10^6/mL) were plated in RPMI-1640 culture medium supplemented with 10% FCS, 2.0 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY), 1% sodium pyruvate (Gibco), 1% nonessential amino acids (Hyclone Laboratories, South Logan, UT), and 50 mM 2-mercaptoethanol (Gibco) in the presence of 200 ng/mL rhGM-CSF and 15 ng/mL rhIL-4 (eBiosciences) at 37°C and 5% of CO_2. Cells were fed on days 2 and 4 by changing one half of the medium and keeping the same concentration of IL-4 and GM-CSF. At day 6, DCs were induced to mature by adding 200 ng/mL of LPS (Sigma-Aldrich). Forty-eight hours after stimulation, supernatants were collected for cytokine measurement. In the case of the induction of tolerogenic dendritic cells, the differentiation of monocytes was carried out in 24-well tissue culture plates previously coated with recombinant human P-selectin (10 μg/mL) or PD1 (2.5 μg/mL) (BioLegend, San Diego, CA) by overnight incubation at 4°C. In other set of experiments, monocytes were induced to differentiate in the presence of IL-10 (40 ng/mL, Biolegend).

2.4. Lymphocyte-DC CoCultures and Induction of Th17 and Treg Differentiation. For the induction of differentiation of Th17 cells, mature mo-DC and autologous $CD4^+$ T cells were cocultured in 24-well plates at 1 : 10 ratio in complete Iscove's modified Dulbecco medium (IMDM, Gibco BRL). Plates were previously coated with 10 μg/mL anti-CD3 (eBiosciences) and anti-CD28 (Immunotech) mAbs, and Th17 differentiation was induced by adding rhIL-23 (10 ng/mL, R&D systems, Minneapolis, MN), IL-6 (50 ng/mL), IL-1β (10 ng/mL), IL-21 (50 ng/mL), TNF-α (10 ng/mL, all from eBiosciences), and anti-IL-4 and anti-IFN-γ mAbs (5 μg/mL, BioLegend). Under such conditions, cells were cultured during 5 days at 37°C and 5% of CO_2, and then the percent of Th1 and Th1/Th17 cells was determined by flow cytometry. In the case of the induction of Th1/Th17 cells, no anti-IFN-γ antibody was added. For induction of Treg cell differentiation, DCs generated in the presence of tolerogenic stimuli (IL-10, PD1, P-selectin) were cocultured with autologous $CD4^+$ T cells for five days, and then cells were immunostained for CD4, CD25, and Foxp3, and they were analyzed by flow cytometry.

2.5. Flow Cytometry Analysis. Myeloid and plasmacytoid dendritic cells were analyzed by using a mixture of mAbs specific for lineage markers combined with HLA-DR, CD11c, BDCA-2, and BDCA-4 mAbs. The following antibodies were used: FITC labeled anti-CD3, anti-CD14, anti-CD16, anti-CD19, and anti-CD56 (eBiosciences); HLA-DR-APC-Cy7, CD11c-PercPCy5.5 (BioLegend), BDCA-2-APC and BDCA-4-APC (Miltenyi Biotec). Cells were analyzed in a FACSAria II flow cytometer (Becton Dickinson, San José, CA) using the FACSDiva software.

For intracellular cytokine analysis, cells were treated for 5 hours with PMA plus Ionomycin (50 ng/mL, and 1 μg/mL, resp., Sigma-Aldrich) and with Brefeldin A (1 μg/mL, Cytofix/Cytoperm kit, eBioscience) for the last 2 hours. Then, cells were stained for the indicated cell surface markers, fixed, permeabilized (Cytofix/Cytoperm kit, eBiosciences), and labeled for IL17 (anti-IL-17-PE, R&D Systems) and IFN-γ (anti-IFN-γ-FITC, eBiosciences) for 30 min at 4°C. Cells were analyzed in a FACSCalibur flow cytometer (Becton-Dickinson) using the CellQuest Pro software.

Cell surface markers of mo-DCs were analyzed with mAbs directed against CD83, CD86, PSGL-1, PDL-1 (eBiosciences), and HLA-DR (BD Pharmingen). In all analyzes, Fcγ receptors were blocked (FcγR binding inhibitor, eBiosciences) before immunostaining.

2.6. Cytokine Quantification. Levels of IL-23, IL-1β, IL-6, IL-22, IL-17A, and IFN-γ were quantified in cell-free supernatants using Human ELISA kits (eBiosciences and PEPROTECH) according to manufacturer's instructions. All determinations were performed by duplicate, and plates were analyzed by using a Multiskan ELISA reader.

2.7. Statistical Analyses. Differences between two groups were determined using the Student's t test or Mann-Whitney U test. One and two way analysis of variance with the proper post hoc analysis was performed when necessary. Data were analyzed using the GraphPad Prism software (GraphPad, San Diego, CA).

3. Results

3.1. Peripheral Blood DC Subsets and mo-DCs in Patients with RA and SLE. We first analyzed the levels of DCs subsets in the peripheral blood from patients and controls. As shown in Figure 1(a), patients with RA and SLE showed an enhanced proportion of $CD11c^+$ BDCA-2+ myeloid DCs ($P < 0.05$ in both cases). Furthermore, patients with SLE exhibited higher levels of $CD11c^-$ BDCA-4+ plasmacytoid DCs compared to both healthy controls and patients with RA ($P < 0.05$ in both cases). However, no apparent association between disease activity or immunosuppressive therapy and DCs levels was detected in patients with SLE or RA (data not shown). As shown in Figure 1(b), the peripheral blood myeloid DCs from patients with RA and SLE exhibited an enhanced expression of the regulatory receptors PSGL-1 and PDL-1 ($P < 0.05$ in all cases, compared to healthy controls). In the case of plasmacytoid DCs, although the expression of PDL-1 tended to be higher in patients with RA and SLE, no significant differences were observed compared to controls ($P > 0.05$ in both cases, Figure 1(b)). In contrast, the expression of PSGL-1 was significantly higher in plasmacytoid DCs from patients with RA and SLE compared to cells from healthy subjects ($P < 0.05$ in both cases, Figure 1(b)).

We then generated *in vitro* mo-DCs under standard conditions with IL4/GM-CSF. As shown in Figure 1(c), and as we have previously reported [43], in the case of SLE, these myeloid DCs exhibited a diminished density of expressions of CD80, CD86, and HLA-DR (measured as their MFI). In contrast, the phenotype of mo-DCs was similar in patients with RA and healthy controls. In addition, a similar pattern of PSGL-1 and PDL-1 expression was observed in mo-DCs

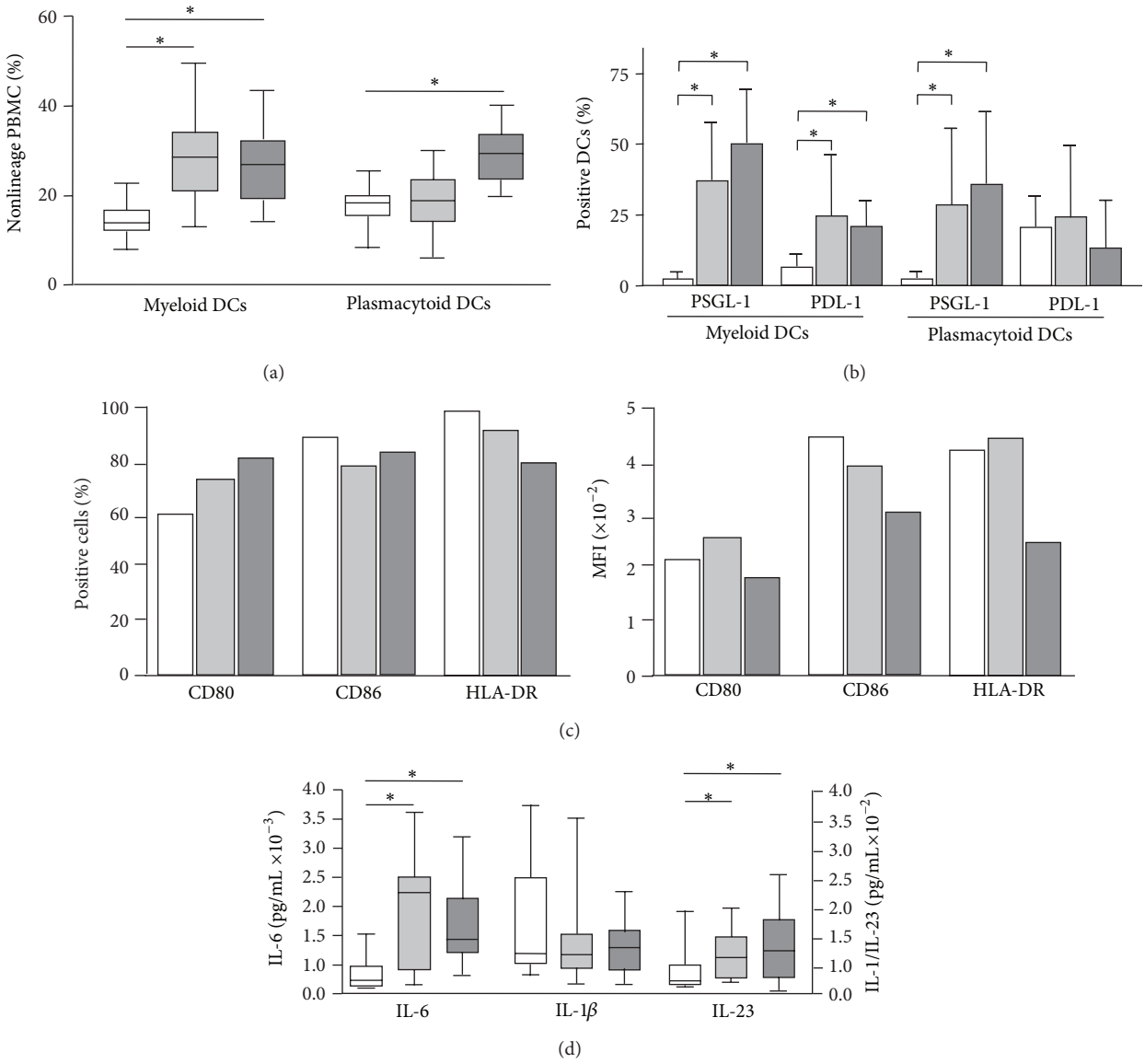

Figure 1: Peripheral blood DCs subsets and mo-DCs in patients with RA and SLE. (a) Myeloid and plasmacytoid DCs levels were determined in freshly isolated peripheral blood mononuclear cells by multiparametric flow cytometry, as stated in materials and methods. Data correspond to the percent of $CD11c^+$ BDCA-2+ nonlineage cells (myeloid DCs), and $CD11c^-$ BDCA-4+ nonlineage cells (plasmacytoid DCs). (b) The expression of the regulatory receptors PSGL-1 and PD-1 by myeloid and plasmacytoid DCs was analyzed by multiparametric flow cytometry in blood samples from patients with RA and SLE, as stated in materials and methods. (c) mo-DCs were generated *in vitro* by culturing peripheral blood monocytes in the presence of IL-4 and GM-CSF, and their maturation was induced by LPS. Then, cells were immunostained for the indicated molecules, and analyzed by flow cytometry. Data correspond to the percent of positive cells (left panel), and mean fluorescence intensity (MFI, right panel) in cells from representative patients with RA and SLE, and a healthy control. (d) IL-1β, IL-6 and IL-23 were quantified in the cell culture supernatants of mo-DCs incubated in the presence of LPS. Median and interquartile range are shown in (a) and (d) and the arithmetic mean and SD in (c). $^*P < 0.05$. In all panels, white boxes correspond to controls, grey light boxes to patients with RA, and grey dark boxes to SLE patients.

from the patients included in this study (data not shown). On the other hand, when different cytokines were measured in the cell culture supernatants of mo-DCs, we observed an enhanced release of IL-6 and IL-23 by cells from patients with RA and SLE, compared to healthy individuals ($P < 0.05$ in all cases, Figure 1(d)). In contrast, similar concentrations of IL-1β were detected in the cell culture supernatants from patients and controls ($P > 0.05$, Figure 1(d)).

3.2. Effect of DCs on the In Vitro Differentiation of Th17 Lymphocytes. We first analyzed by flow cytometry the proportion of Th17 cells in the PBMC of healthy controls and patients. As shown in Figure 2, patients with RA and SLE showed enhanced levels of CD4+ IL-17A+ lymphocytes compared to controls ($P < 0.05$ in both cases). In addition, the percentage of Th1/Th17 lymphocytes (defined as CD4+ IL-17A+ IFN-γ+) was also higher in patients with RA or SLE

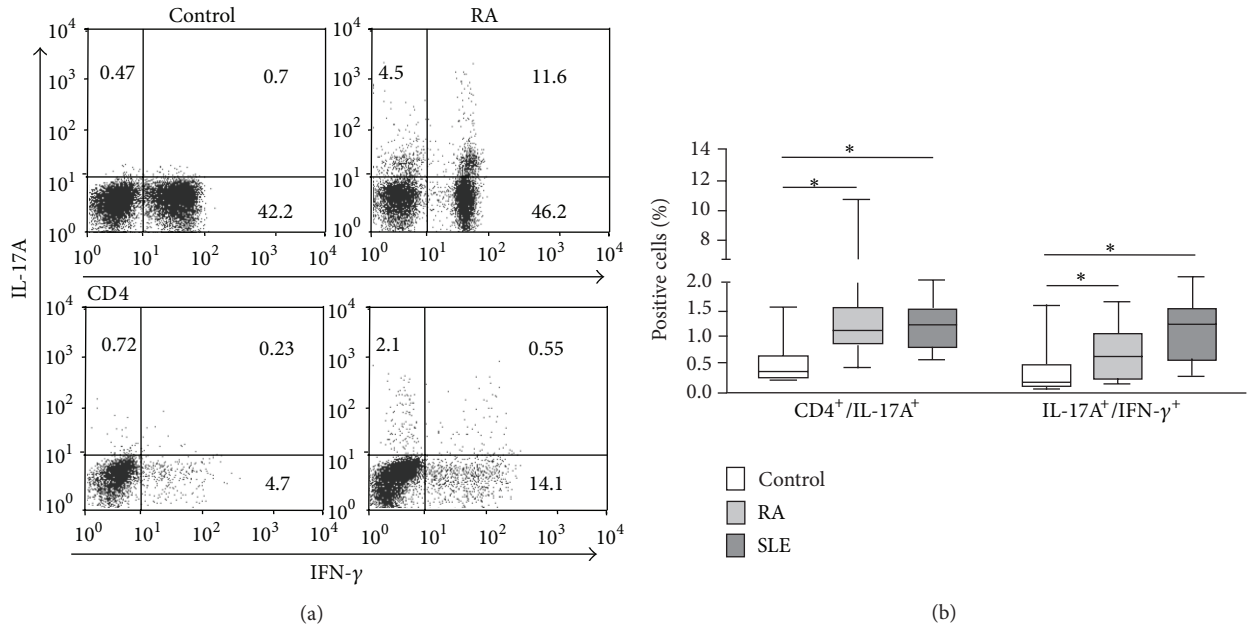

Figure 2: Quantification of Th17 cells in the peripheral blood from patients with RA and SLE. Peripheral blood mononuclear cells from patients with RA and SLE and healthy controls were immunostained for the determination of Th17 (CD4+ IL-17A+) and Th1/Th17 (CD4+ IL-17A+ IFN-γ+) lymphocytes, and analyzed by flow cytometry, as stated in materials and methods. Dot plots from cells of a representative patient with RA and a healthy control are shown in (a), and the median and interquartile range of the percent of positive cells in (b). Numbers in quadrants correspond to the percent of events. $^{*}P < 0.05$.

compared to healthy subjects ($P < 0.05$ in both cases). Similar results were observed in the case of CD4(−) IL-17+ lymphocytes ($P < 0.05$ in both cases, data not shown). Interestingly, patients with RA under therapy with DMARDs showed significantly lower levels of Th17 cells compared to untreated patients ($P < 0.05$, data not shown). However, no significant correlation between disease activity (DAS28 score) and Th17 levels was detected in these patients ($r = 0.17$, $P > 0.05$, data not shown). In contrast, a significant correlation between SLEDAI score and Th17 percentages was observed in patients with SLE ($r = 0.39$, $P < 0.05$, data not shown) .

When the mo-DCs from patients with RA were cocultured with autologous CD4+ T cells, we detected an enhanced generation of Th17 (CD4+ IL-17A+) lymphocytes ($P < 0.05$ compared to controls, Figure 3(a)). As expected, this phenomenon was enhanced when cells were cultured under Th17 polarizing conditions (addition of IL-1, IL-6, IL-21, and TNF-α, with blockade of IL-4 and IFN-γ, Figure 3(a)). However, although the addition of IL-23 alone tended to have a similar effect, no significant differences between RA and controls were reached ($P > 0.05$, Figure 3(a)). Furthermore, similar levels of Th17 induction were observed in cells from patients with SLE and controls, in the different conditions tested ($P > 0.05$ in all cases, Figure 3(a)). Finally, although cell cocultures from patients with RA and SLE tended to show an increased generation of Th1/Th17 lymphocytes (CD4+ IL-17A+ IFN-γ+), no significant differences were observed when compared to cells from healthy controls ($P > 0.05$ in all cases, Figure 3(b)). In agreement with the above results, we observed a significant enhanced release of Th17 cytokines (IL-17A and IL-22) in the mo-DC/T lymphocyte cocultures from patients with RA (Figure 4).

3.3. Effect of Tolerogenic DCs on the Induction of Treg Lymphocytes. An additional analysis of the functional status of mo-DCs in SLE and RA was performed, by measuring their capability to induce Treg lymphocytes. When mo-DCs were generated under standard conditions (IL-4/GM-CSF), those derived from SLE patients showed a diminished capability to induce the generation of autologous CD4+ CD25+ Foxp3+ Treg cells ($P < 0.05$ compared to controls, Figure 5). Interestingly, when mo-DCs were generated under the engagement of the tolerogenic receptor PSGL-1 (by the addition of exogenous P-selectin), a diminished capability to promote the generation of Treg lymphocytes was observed in patients with RA and SLE ($P < 0.05$ compared to controls, in both cases, Figure 5). A similar phenomenon was observed in the case of mo-DCs from patients with SLE, when they were generated in the presence of recombinant PD-1 ($P < 0.05$ compared to controls, Figure 5). Although mo-DCs from patients with RA generated under PDL-1 engagement tended to show a diminished ability to induce Treg cells, no significant difference was reached in this case ($P > 0.05$, Figure 5).

4. Discussion

Different data indicate that in the complex pathogenesis of RA and SLE, DCs as well as Th17 and Treg lymphocytes have

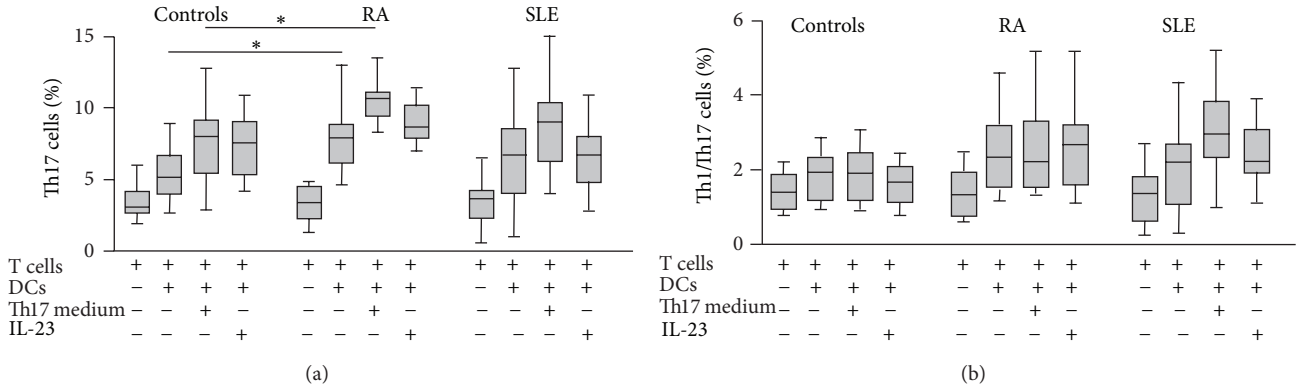

Figure 3: Induction of Th17 and Th1/Th17 lymphocytes by DCs from patients with RA and SLE. Monocyte derived DCs were cocultured with autologous CD4+ cells for five days in the presence or not of a Th17 polarizing medium (IL-1β, IL-6, IL-21, and TNF-α plus anti-IL-4 and anti-IFN-γ blocking antibodies) or IL-23. Then, cells were immunostained for CD4, IL-17A and IFN-γ and analyzed by flow cytometry. Data correspond to the median and interquartile range of the percent of positive cells. $^*P < 0.05$.

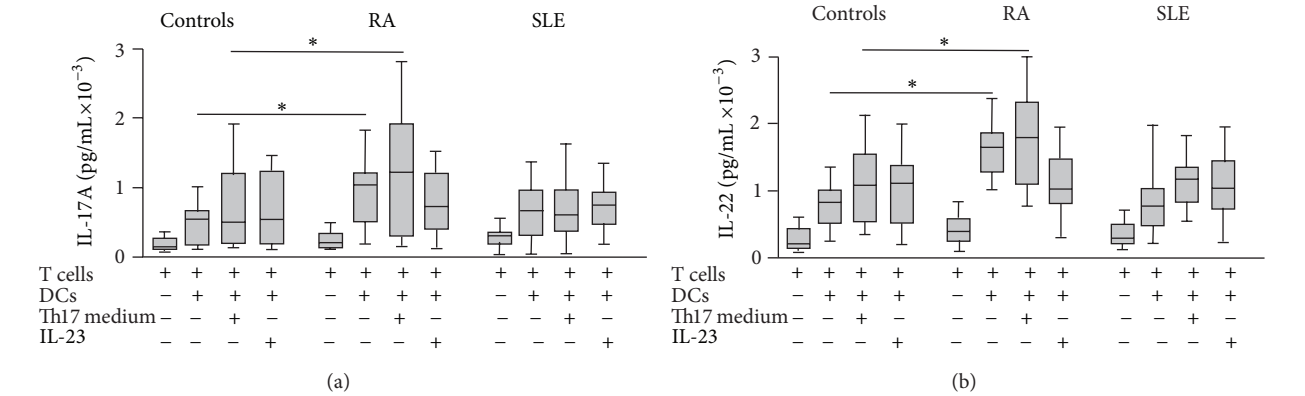

Figure 4: Th17 cytokine synthesis by cocultures of DCs and CD4+ lymphocytes in patients with RA and SLE. IL-17A and IL-22 were quantified in the supernatants of the cell cultures shown in Figure 3. Data correspond to the median and interquartile range of cytokine concentration. $^*P < 0.05$.

an important role [8, 31, 36]. In this work, we first analyzed the levels of Th17 lymphocytes and the two main subsets of peripheral blood DCs in samples from these patients and healthy controls. In agreement with previous reports [12, 44, 45], and as has been observed in other conditions such as PBC or SSc [9], we detected enhanced levels of Th17 lymphocytes in the peripheral blood from patients with RA and SLE. However, it is of interest that other authors have not observed increased numbers of these T helper cells in patients with RA [43]. It is feasible that these apparent contradictory results could be due to differences in the therapy, time of disease evolution, level of disease activity or genetic background of patients. In this regard, we have detected that those patients under therapy with DMARDs exhibit lower percentages of Th17 cells than those in untreated patients. In addition, we found a significant association between disease activity and Th17 cell levels in patients with SLE. However, in patients with RA this correlation was not detected. All these data indicate that it would be interesting to perform a longitudinal study of Th17 cell levels in a cohort of patients with RA and SLE.

In order to further explore the role of DCs in the induction of Th17 lymphocytes in patients with RA and SLE, we performed *in vitro* experiments with mo-DCs. In this regard, it is worth mentioning that although this type of DCs is not detected in the peripheral blood of healthy subjects, different evidences indicate that this subset of antigen presenting cells is generated *in vivo* [46]. Our results indicate that the mo-DCs from patients with RA have an increased capability to induce the *in vitro* differentiation of Th17 lymphocytes as well as an enhanced potential to release cytokines that allow the generation of these cells. These data further support the important role of DCs in the pathogenesis of RA and indicate that this functional feature is preserved during their *in vitro* generation from peripheral blood monocytes. In addition, our results also support that the inflamed rheumatoid synovium is a privileged site for the generation of Th17 cells [35]. Since this enhanced capability of mo-DCs to induce Th17 lymphocytes was not observed in patients with SLE, our data indicate that, under our experimental conditions, the overproduction of IL6/IL-21/IL-23 by DCs is not enough to increase the differentiation of Th17 lymphocytes. In this regard, it is of interest that we have corroborated [47] that mo-DCs from patients with SLE show a defective expression of HLA-DR and the costimulatory molecule CD86. Thus,

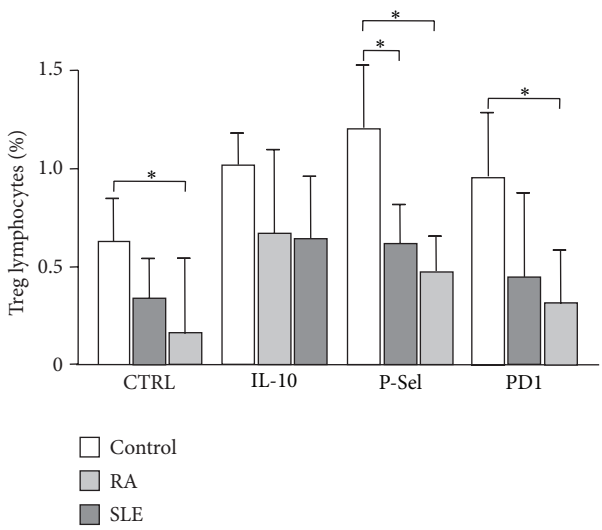

Figure 5: Induction of Treg lymphocytes by mo-DCs from patients with RA and SLE. Monocyte derived DCs generated under tolerizing conditions (PDL-1, PSGL-1 engagement, or IL-10 addition) or not, were cocultured with autologous CD4+ lymphocytes for five days. Then, the percent of CD4+ CD25+ Foxp3+ lymphocytes was determined by flow cytometry. Data correspond to the median and interquartile range of the percent of positive cells. $^{*}P < 0.05$.

it is feasible that this defective phenotype of SLE mo-DCs accounts, at least in part, for their diminished ability to induce Th17 cells, compared to cells from patients with RA.

We have detected that the levels of peripheral blood mDCs are enhanced in patients with RA, and this finding is in disagreement with a previous report [48]. As in the case of Th17 cells, it is feasible that different factors could account for this apparent contradictory results. In this regard, it seems evident that the levels of DCs in peripheral blood are strongly influenced by their rates of generation in bone marrow and their extravasation to different tissues, phenomena that are not easy to analyze *in vivo*, in human beings. Thus, we consider that it would be easy and interesting to perform a prospective study on the levels of the different DC subsets in the peripheral blood in patients with RA and SLE.

Patients with RA and SLE show a defective function of nTreg cells, and abnormal numbers and proportions of Treg lymphocytes have been reported in these patients [49–51]. Since DCs have an important role in the generation of induced Treg (iTreg) cells, we decided to generate *in vitro* tolerogenic DCs and test their ability to trigger the differentiation of these regulatory lymphocytes. We have detected that upon engagement of the tolerogenic receptor PSGL-1, the mo-DCs from patients with RA and SLE showed a defective capability to induce the generation of iTreg cells. We consider that this is an interesting finding since further supports the important role of DCs in the defective immune-regulation observed in these patients. In addition, it is also of interest that this aberrant functional feature is observed after their *in vitro* differentiation from peripheral blood monocytes. In this regard, it would be of interest to determine whether this type of mo-DCs are generated *in vivo*, in patients with RA and SLE, in their diseased, and to explore their possible contribution to the defective regulation of the inflammatory phenomenon. The possible cause of the enhanced expression of PSGL-1 and PDL-1 by the mo-DCs from these patients also remains as an interesting point to be explored.

Since it has been previously reported that patients with SLE and RA show an enhanced synthesis of IL-10 [36, 52], it is of interest that this cytokine had showed a tolerogenic effect on mo-DCs that was not significantly different from that found in patients compared to controls. Thus, this finding suggests that although the overproduction of IL-10 may contribute to the pathogenesis of SLE and RA [37, 52]. It is also feasible that the enhanced synthesis of this cytokine could favour the generation of tolerogenic DCs *in vivo* in these patients. In contrast, our findings indicate that the other tolerogenic stimulus employed in this study (PD-1) is not able to exert a significant effect on the mo-DCs from patients with SLE. This finding further suggests that the defective immune-regulatory function observed in patients with SLE is the result of many different factors, including the diminished activity of several tolerogenic receptors expressed by DCs.

5. Conclusions

Our findings strongly suggest that mo-DCs may significantly contribute to the enhanced generation of Th17 lymphocytes, and the diminished number and activity of Treg cells observed in patients with RA and SLE. Thus, the possible functional role of this subset of DCs *in vivo*, in the pathogenesis of autoimmune inflammatory conditions, is an interesting point to be further explored.

Conflict of Interests

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

Authors' Contribution

Lizbeth Estrada-Capetillo and Berenice Hernández-Castro contributed equally to this work.

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Research Article

Research of the Methylation Status of miR-124a Gene Promoter among Rheumatoid Arthritis Patients

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Objective. To analyze the methylation status of miR-124a loci in synovial tissues of rheumatoid arthritis (RA) patients using methylation-specific polymerase chain reaction (MSP). *Materials and Methods*. DNA obtained from the frozen tissue of 7 RA samples, 6 osteoarthritis (OA) samples, and 3 healthy controls were undergoing bisulfite conversion and then analyzed for miR-124a promoter methylation using MSP assay. *Results*. miR-124-a1 and miR-124-a2 promoter methylation were both seen in 71.4% of RA samples compared to 16.7% of OA samples. miR-124-a3 promoter methylation was seen in 57.1% of RA samples and 0% of OA samples. All the three loci were unmethylated in 3 healthy controls. *Conclusion*. The methylation status of miR-124a seen in this study concurs with that reported in tumor cells, indicating epigenetic dysregulation constituents, a mechanism in the development of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, symmetrical polyarticular autoimmune disease affecting ~1% of the population worldwide [1, 2]. The main characteristic features of RA are persistent inflammation, synovium hyperplasia, lymphocyte infiltration, and abnormal proliferation of fibroblast-like synoviocytes (FLS), which eventually lead to progressive cartilage erosion and bone destruction [3]. Although the pathogenesis of RA is not clear, much evidence demonstrates that microRNAs (miRNAs) display important roles in immune response [4, 5]. miRNAs are endogenous, short (about 19–25 nucleotides long) single-stranded, and noncoding RNAs that can influence the target mRNA processing at the posttranscriptional level [6] and play important roles in cell processes such as proliferation, apoptosis, differentiation, or even in tumorigenesis [7]. One of these important miRNAs is miR-124a. Accumulating evidence shows that miR-124a is downregulated in synovial tissues of RA patients compared with that of osteoarthritis (OA) patients [8]. For example, Nakamachi et al. found that its level in RA FLS was less than one-sixth of that seen in OA FLS [9]. However, the mechanism of the aberrant expression of miR-124a in RA synovial tissues is still unknown. It is found that miR-124a has three genomic loci (miR-124a-1 (8p23.1), miR-124a-2 (8q12.3), and miR-124a-3 (20q13.33)) that encode for the same mature miRNA. Interestingly, the miR-124a-1 and miR-124a-3 genes are located within CpG islands, whereas miR-124a-2 is 760 bp downstream of a CpG island. Studies in several cancer cells demonstrated that all the three loci are silenced by the hypermethylation of its promoter [10, 11]. Agirre et al. pointed out that the corresponding CpG islands of miR-124a-1 and miR-124a-3 are frequently methylated in acute myeloid leukemia [12]. Since reported data showed that the expression of miR-124a was suppressed in RA synovial tissues, it would be of interest to investigate whether epigenetic mechanism, especially DNA methylation, has played a role in it.

2. Materials and Methods

2.1. Synovial Samples. Synovial tissues were obtained from RA patients, OA patients, and joint trauma patients (healthy control specimens) undergoing total knee arthroplasty from

Table 1: Clinical characteristics of the patients*.

Patient	Age	Sex	Disease duration (years)	Presurgical CRP (mg/dL)	Presurgical ESR (mm/h)	DAS 28	Medications
RA1	72	F	17	1.9	17	2.42	MTX 10 mg qw LEF 20 mg qd
RA2	58	F	9	1.5	21	2.16	MTX 12.5 mg qw LEF 10 mg qd
RA3	63	M	21	2.7	13	1.87	Pred. 7.5 mg qd MTX 12.5 mg qw
RA4	65	F	15	<1	2	1.21	Pred. 5 mg qd LEF 20 mg qd
RA5	59	M	6	3.2	5	1.49	Pred. 5 mg qod MTX 10 mg qw
RA6	66	F	8	<1	9	1.23	LEF 20 mg qd
RA7	61	F	13	5.3	28	2.62	MTX 15 mg qw
OA1	72	F	—	<1	3	—	—
OA2	75	M	—	<1	2	—	—
OA3	69	M	—	<1	4	—	—
OA4	74	M	—	3.3	9	—	—
OA5	68	F	—	<1	3	—	—
OA6	70	M	—	2.8	11	—	—
C1	39	M	—				
C2	45	M	—				
C3	43	F	—				

*RA: rheumatoid arthritis; OA: osteoarthritis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS: disease activity score; MTX: methotrexate; Pred.: prednisone; LEF: leflunomides; C: control.

October 2012 to April 2013 in Sichuan Provincial People's Hospital. Tissue was snap-frozen and stored at −80°C. RA and OA were diagnosed according to the criteria of the American College of Rheumatology [13, 14]. The clinical characteristics of the patients are shown in Table 1. Samples were obtained in accordance with the Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects, as approved by the World Medical Association. All patients signed informed consent forms, and the study was approved by the Ethics Committees of Sichuan Provincial People's Hospital.

2.2. DNA Extraction and Bisulfite Conversion. Genome DNA was extracted from 25 mg of frozen synovial tissue using the PureLink Genomic DNA mini kit (Invitrogen, USA). DNA was quantitated using the Nanodrop (Nanodrop technologies, USA). 2 μg DNA was used for bisulfite conversion as described [15]. Modified DNA was purified using the QIAEX II Gel Extraction kit (QIAGEN, Germany) according to the manufacturer.

2.3. Methylation-Specific PCR. The DNA methylation status was analyzed by methylation-specific PCR (MSP) using primers specific for either the methylated or bisulfate modified unmethylated DNA. 1.5 μL bisulfite-converted DNA was amplified using 0.15 μL primers (Sangon Biotech, Shanghai), 0.45 μL $MgCl_2$ (25 mM), 1.8 μL dNTP (2.5 mM each), 6.75 μL 2x GC buffer (I) (Takara, Japan), and 1.6 μL *Takara LA taq* polymerase (Takara, Japan). Each step included methylated (RKO, ATCC CRL-2577) and unmethylated (MGC-803, ATCC) controls along with nontemplate control. The amplified products were run on a 2% agarose gel with an expected size of 164, 185, and 150 bp (three loci each) for a methylated product and 165, 180, and 155 bp (three loci each) for an unmethylated product.

2.4. Statistical Analysis. Statistical comparisons between groups were carried out by Chi square test or Fisher's exact test. P values less than 0.05 were considered significant.

3. Results

3.1. General Clinical Features. The mean age of the 7 RA patients was 63 (range 58–72) years, with a female to male ratio of 2.5 : 1. The mean presurgical CRP and ESR were 2.4 ± 1.5 mg/dL and 14 ± 9 mm/h, respectively. The mean DAS28 was 1.86 ± 0.57. The three healthy control patients underwent surgery because of car accident, falling off, and fighting, respectively.

3.2. Methylation Status of miR-124a. The methylation status of miR-124a was evaluated in 7 RA, 6 OA, and 3 control synovial tissues (Figure 1). All of the RA tissues were hypermethylated: 5 for miR-124a-1 (5/7), 5 for miR-124a-2 (5/7), and 4 for miR-124a-3 (4/7). Among them, 4 tissues (4/7) showed promoter methylation of all three gene loci, and

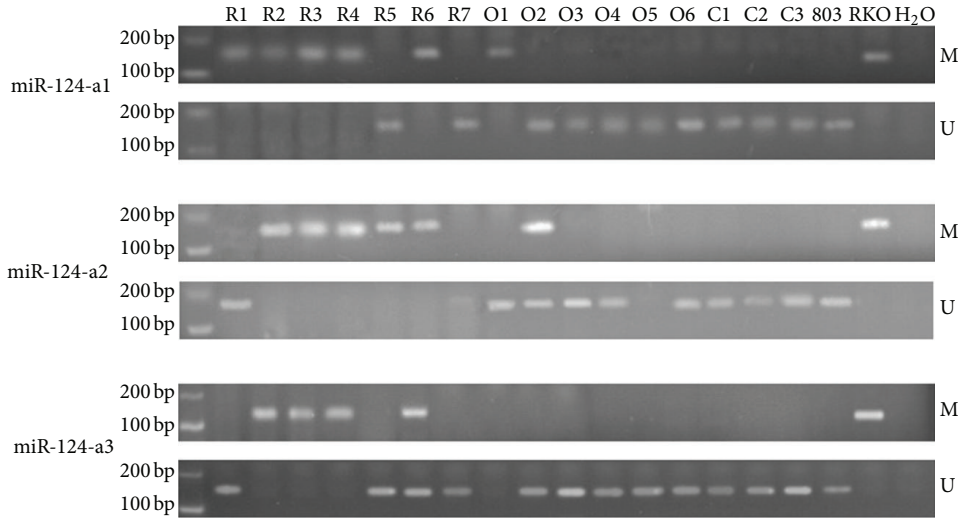

Figure 1: Gel electrophoresis of the MSP products for miR-124-a1, miR-124-a2, and miR-124-a3 in RA, OA patients, and healthy controls. R: rheumatoid arthritis; O: osteoarthritis; C: healthy control; 803 is unmethylated positive control; RKO is methylated positive control; H_2O is blank control.

Table 2: Methylation status of miR-124a in synovial tissues of RA and OA*.

Gene	Methylation frequency			*P* value	
	RA	OA	C	RA versus OA	RA versus C
miR-124-a1	71.4%	16.7%	0	0.07	0.08
miR-124-a2	71.4%	16.7%	0	0.07	0.08
miR-124-a3	57.1%	0	0	0.05	0.17
All three loci	57.1%	0	0	0.05	0.17

*RA: rheumatoid arthritis; OA: osteoarthritis; C: healthy control.

the frequency was 57.1%, higher than that of OA (0, 0/6) and control (0, 0/3) (Table 2). The methylation frequency of the three genes in RA synovial tissues has no statistical significance compared to each other.

4. Discussion

Rheumatoid arthritis is one of the common autoimmune diseases, and the molecular mechanism of its pathogenesis is unclear now. miRNAs have been implicated in the pathogenesis of malignant and nonmalignant disease. The microRNA miR-124a was initially identified as a crucial regulator involved in neurogenesis [16]. In neuronal tissues, miR-124a contributes to the differentiation of neural progenitors into mature neurons [17]. Pierson et al. reported that the 3′-UTR of CDK-6 mRNA is a direct target of miR-124 and that CDK-6 expression is suppressed by miR-124 overexpression in medulloblastoma cell lines [18]. Nakamachi et al. confirmed that the expression of CDK-6 protein was higher in RA FLS than in OA FLS and that CDK-6 expression was suppressed when pre-miR-124a was transfected into RA FLS; thus, the cell cycle was arrested at the G1 phase [9]. Since CDK-6 is known as a G1/S phase regulator, it is speculated that miR-124a is an important regulator of the G1/S transition in synovial tissue as well as in tumors.

We decided to focus on the methylation status of miR-124a in RA synovial tissues for several reasons. (a) The expression of miR-124a is downregulated in RA synovial tissues. (b) Hypermethylation of gene promoters is a frequent mechanism of gene silencing. (c) The three loci of miR-124a are either located within a CpG island or somewhere downstream a CpG island. (d) It is reported that miR-124a has been silenced by gene promoter hypermethylation in several cancer cell lines. The results of our study support the approach, showing that the gene promoter of miR-124a is hypermethylated, and this might associate with its downregulation in RA synovial tissues.

The methylation frequencies of the three gene loci observed among RA synovial tissues (57.1%~71.4%) were much higher than that observed among OA (0~16.7%) and control synovial tissues (0). However, the *P* values were not so significant considering the small sample size.

We obtained good-quality DNA from all frozen tissues, yet there were some RA samples that showed both methylated and unmethylated band. We considered the following reasons: (a) although we carefully removed all connective tissue and fat, the area chosen might still have very little nonsynovial tissue to confound the results. It would be pertinent to point out that small amounts of contaminating nonsynovial tissues could become a source of contamination and could provide false negative results. This issue emphasizes the need to carefully choose samples for detection of methylation status, therefore avoiding potential confounders. (b) Regions within the synovial tissue might be variably methylated, even within the individual clones of the same tissue sample, and the synovial tissue could be a mosaic of variable degrees of methylation. Thus we speculate mosaicism could have contributed to our findings here. This issue could be solved by bisulfite-sequencing.

This study highlights the methylation pattern of RA synovial tissues compared with that of OA and healthy control. Although it has some limitations such as the number of samples analyzed, the study still provides useful information on promoter methylation pattern of miR-124a. This epigenetic dysregulation of miR-124a in RA synovial tissue constitutes an emerging mechanism implicated in the development of RA and will provide us with new excellent targets for DNA demethylating agents.

Conflict of Interests

None of the authors had any conflict of interests.

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Research Article

Altered Sympathetic-to-Immune Cell Signaling via β_2-Adrenergic Receptors in Adjuvant Arthritis

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Adjuvant-induced arthritic (AA) differentially affects norepinephrine concentrations in immune organs, and *in vivo* β-adrenergic receptor (β-AR) agonist treatment distinctly regulates *ex vivo* cytokine profiles in different immune organs. We examined the contribution of altered β-AR functioning in AA to understand these disparate findings. Twenty-one or 28 days after disease induction, we examined β_2-AR expression in spleen and draining lymph nodes (DLNs) for the arthritic limbs using radioligand binding and western blots and splenocyte β-AR-stimulated cAMP production using enzyme-linked immunoassay (EIA). During severe disease, β-AR agonists failed to induce splenocyte cAMP production, and β-AR affinity and density declined, indicating receptor desensitization and downregulation. Splenocyte β_2-AR phosphorylation (pβ_2-AR) by protein kinase A (pβ_2-AR$_{PKA}$) decreased in severe disease, and pβ_2-AR by G protein-coupled receptor kinases (pβ_2-AR$_{GRK}$) increased in chronic disease. Conversely, in DLN cells, pβ_2-AR$_{PKA}$ rose during severe disease, but fell during chronic disease, and pβ_2-AR$_{GRK}$ increased during both disease stages. A similar pβ_2-AR pattern in DLN cells with the mycobacterial cell wall component of complete Freund's adjuvant suggests that pattern recognition receptors (i.e., toll-like receptors) are important for DLN pβ_2-AR patterns. Collectively, our findings indicate lymphoid organ- and disease stage-specific sympathetic dysregulation, possibly explaining immune compartment-specific differences in β_2-AR-mediated regulation of cytokine production in AA and rheumatoid arthritis.

1. Introduction

The sympathetic nervous system (SNS) via its modulation of the immune system is an important player in determining the disease onset, progression, and severity in rheumatoid arthritis (RA) and animal models of RA [1, 2]. Autonomic dysregulation is reported in RA, juvenile chronic arthritis, and arthritic animals (reviewed in [3]) that support increased sympathetic activity. Treatment of rodent RA models with adrenergic drugs suggests both a permissive and suppressive role for the SNS in the pathogenesis of RA [4–6]. Blocking sympathetic signaling at disease initiation with an arthrotigenic challenge or disease onset exacerbates or attenuates disease severity [5, 7], respectively, suggesting a complex role for the SNS in RA.

SNS dysregulation in RA patients is supported by reports of reduced numbers of β_2-AR receptors and functional responses of peripheral blood mononuclear cells (PBMCs) to sympathomimetic agents compared with nonarthritic, age-matched controls [8–12]. The anti-CD3 antibody, OKT3-induced PBMC proliferative responses in the presence of catecholamines are attenuated in RA compared with healthy subjects [8]. Diminished expression and activity of G protein-coupled receptor kinases (GRKs) in PBMCs occur in RA patients [13] and in animal models of RA [14]. In RA patients, PBMC

GRK-2 and GRK-6 levels are reduced, whereas, GRK-5 levels are not altered. Given that receptor ligand concentration and GRKs regulate β-AR functions, these findings indicate that sympathetic signaling in targeted immune cells is dysregulated in RA and animal models of RA.

Sympathetic nerves signal immune cells in lymphoid organs primarily through the release of norepinephrine (NE). The primary adrenergic receptors (ARs) expressed on lymphocytes are β_2-ARs. β-ARs are G coupled-protein receptors that classically use the cAMP/protein kinase A (PKA) signaling pathway. The expression of β_2-AR on target cells is dynamically regulated, in part by ligand availability, but also intracellularly by PKA, β-arrestins, and different GRKs [15].

β_2-AR function is regulated by PKA and GRK through β_2-AR phosphorylation (pβ_2-AR) at different serines [16–18]. Desensitization is induced by PKA, and at higher agonist concentrations, by PKA and GRK [19–21]. PKA-induced pβ_2-AR (pβ_2-AR$_{PKA}$) alters receptor conformation, impairing Gs and enhancing Gi protein coupling to induce transient activation of extracellular signal-regulated kinases (ERK 1/2) [21]. pβ_2-AR by GRK (pβ_2-AR$_{GRK}$), specifically GRK2, leads to β-arrestin binding to the β_2-AR [21–23], altering the conformation of the β_2-AR, further impeding Gs protein coupling [21] and inducing receptor internalization. Internalized β_2-AR are dephosphorylated and recycled to the plasma membrane or degraded [24, 25].

Additionally, β_2-AR signaling can induce sustained ERK 1/2 signaling in a β-arrestin- [15, 20, 26] and GRK-dependent manner [27, 28]. pβ_2-AR$_{GRK}$ occurs in an agonist concentration-dependent manner by GRK 2, 5, and 6 to induce different receptor functions [18, 28, 29]. High agonist concentrations induce PKA- and GRK5/6-mediated pβ_2-AR and subsequent binding to β-arrestin [30], a scaffold/adaptor protein for mitogen-activated protein kinase (MAPK) activation [31–33]. In this manner, high agonist concentrations can induce sustained ERK activation independent of the G protein pathway [27, 34, 35]. Therefore, chronically elevated sympathetic tone in patients with RA [36–39] may provide conditions for differential pβ_2-AR that result in β_2-AR-induced ERK 1/2 signaling.

Previously, our laboratory has reported reduced and elevated NE concentration in the spleen and draining lymph nodes (DLN) of adjuvant-induced arthritis (AA) rats, respectively, compared with non-AA controls [40]. These findings suggest differential activational states of splenic and DLN sympathetic nerves in AA, which may induce differential β_2-AR signaling in immune cells from these two lymphoid organs. Consistent with this hypothesis, in rats challenged to induce AA, a model of RA, we find that TH1 versus TH2 cytokine production differs depending on the source of immune cells cultured *ex vivo* [41]. Despite findings that support the downregulation and desensitization of β_2-ARs in peripheral blood leukocytes from RA patients, no studies have examined the extent to which leukocyte β_2-ARs in animal models of RA mimic the receptor changes observed in RA. It is also not clear if leukocytes from relevant lymphoid organs, where autoreactive T cells differentiate, also demonstrate changes in β_2-AR indicative of altered signaling and how such changes impact immune functions in different lymphoid organs in RA or animal models of this disease. In the present study, we tested the hypothesis that disparate TH cell cytokine profiles in different lymphoid organs reflect disease-induced and organ-specific changes of β_2-AR functions. We examined β_2-AR numbers, affinity, signaling, and phosphorylation patterns in immune cells from spleens and DLN of AA rats challenged with complete Freund's adjuvant (CFA) and non-AA rats challenged with components of the CFA (mineral oil or bacterial cell wall) or saline vehicle. We report here immune organ-dependent changes in β_2-AR expression, intracellular signaling, and receptor phosphorylation in AA rats and nonarthritic rats treated with one of the components of CFA, mineral or the heat-killed bacterial cell wall in saline.

2. Materials and Methods

2.1. Materials. Dried and heat-killed *Mycobacterium butyricum* (*M. butyricum*) and mineral oil were obtained from Difco (Detroit, MI) and Sigma-Aldrich Company (St. Louis, MO), respectively. CFA and *M. butyricum* in saline were prepared as previously described [5]. All tissue culture media and supplements were obtained from Gibco BRL (Rockville, MD), unless otherwise stated. EIA kits for cAMP determination and (^{125}I)CYP (2200 Ci/mMole) were purchased from R&D Systems and Amersham International (Amersham Bucks, UK), respectively. OptEIA kits for the detection of interferon-γ (IFN-γ) were obtained from BD Pharmingen (Los Angeles, CA). Antibodies recognizing the β_2-AR (no. sc-569) and pβ_2-AR at serines 345 or 346 (PKA site; no. 16718) or 355 or 356 (GRK site; no. 16719) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CGP-12177, propranolol, and all chemicals, reagents, and buffers used for the western blotting were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

2.2. Animals. Male Lewis rats (200–250 g) were obtained from Charles River Laboratory (Wilmington, MA). Rats were housed two per cage in plastic bottom cages with soft CareFRESH bedding (Absorption, Bellingham, WA), allowed access to water and food pellets *ad libitum*, and placed on a 12 h-on 12 h-off light schedule. Rats were allowed to adjust to conditions in the Banner Sun Health Research Institute's AAALAC accredited vivarium for one week prior to starting the experiments. For AA rats, food (Purina Lab Diet 5001) was placed in the bottom of the cage, and water was supplied using long-stemmed sipper tubes to ensure easy access to food and water. All rats were observed to eat and drink throughout the study. Animals were weighed weekly and observed daily to verify adequate weight gain and overall good health. Other than the development of arthritis, the general health of the animals was maintained during the course of the experiments. Protocols for the use and care of animals in this study were approved prior to beginning the experiments by the Banner Health Research Institute's Institutional Animal Care and Use Committee and complied

with NIH guidelines for the humane use and care of research animals.

2.3. Induction of AA and Experimental Design. Rats were randomly assigned to 1 of 4 groups ($n = 4$ per group per sacrifice day) based on the treatment they received. The experiment was repeated twice per time point. No differences were seen between the experiments so the data for the two experiments was collapsed giving an $n = 8$ per group, per time point. Rats were given 0.1 mL (1) complete Freund's adjuvant (CFA); (2) mineral oil (MO); (3) *M. butyricum* (SMB; 30 mg/10 mL sterile endotoxin-free saline); or (4) sterile endotoxin-free saline by intradermal injection into the base of the tail. Only rats that received CFA developed AA. Control rats treated with MO or SMB served to control for the CFA vehicle or the effects of mycobacterium challenge in the absence of inflammatory arthritis, respectively. Saline-treated rats controlled for the stress of the injection and handling. A single preparation of CFA and SMB was used for this study. All CFA-immunized animals developed arthritis with similar timing of disease onset and severity. After overdose-induced anesthesia with 8% chloral hydrate, rats were sacrificed on day (D) 21 or 28 after CFA challenge or control treatments. The DLN (popliteal and inguinal lymph nodes) and spleens were harvested, and single cell suspensions were prepared. D21 and D28 represent acute and severe disease, respectively.

A separate experiment was performed to evaluate whether altered β_2-AR phosphorylation in immune organs from AA rats affects the production of a cytokine that is regulated by β_2-AR under normal conditions and in AA. The effects of *in vivo* treatment of arthritic rats with a β_2-AR agonist, terbutaline, or vehicle on *ex vivo* production of IFN-γ by spleen and DLN cells were examined. Terbutaline (1200 μg/day) was administered by intraperitoneal (i.p.) injections twice a day in a total volume of 250 μL per injection ($n = 8$). Control arthritic rats were given i.p injections of the same volume of vehicle (0.01 mM ascorbic acid in 0.9% sterile, endotoxin-free saline; $n = 8$). Terbutaline or vehicle treatments were started on day 12 after immunization, the time of disease onset, and continued until sacrifice. The animals were sacrificed using an overdose of 8% chloral hydrate 10.0 mL/kg body weight. On day 28, DLN and spleen were dissected and collected in preparation for culturing immune cells.

2.4. Disease Assessment. The onset and progression of arthritis was apparent based on the development of inflammation and swelling of the hind and fore limbs. The inflammatory response in arthritic rats was assessed by routine methods, as previously described [42]. Dorsoplantar widths of the hind feet were measured using a dial thickness gauge (Mitutoyo Corporation, Chicago, IL), beginning before CFA immunization and continuing approximately every other day until sacrifice. After sacrifice, the hind limbs were removed, and radiographs were taken to assess disease severity using the following settings: 400 nN, 50 kvp, and 0.4 s exposure time at 40 cm. The digitized images are printed using a Fujifilm model FM-DPL printer for analysis.

X-rays were evaluated using a grading scale, as previously reported [5]. In brief, the radiographs were coded to obscure the treatment groups, and then two independent observers subjectively rated each of the radiographs using the scale: 0 (normal), 1 (slight), 2 (mild), 3 (moderate), and 4 (severe) abnormalities in the tissue. The radiographs were scored using the 4 point scale for each of the following characteristics: (i) swelling as indicated by the width of soft tissue shadows and changes in the normal configuration of the soft tissue planes; (ii) osteoporosis as measured by bone density (recognized by increases in radiolucency relative to uninvolved adjacent bone); (iii) cartilage loss shown by narrowing of the joint spaces; (iv) heterotopic ossification defined as proliferation of new bone tissue (fine ossified line paralleling normal bone but not contiguous with calcified area of the bone itself); and (v) bone erosions. The radiographic scores for each category were added for both hind limbs giving a maximum score of 40.

2.5. Harvesting Spleen and Lymph Node Cells. After anesthetic overdose, the spleens and DLN were aseptically removed. Spleens were placed into stomacher bags containing 10 mL Hank's balanced salt solution (HBSS) and homogenized for 2 × 30 sec. Spleen cells were triturated with a 10 mL pipette, then washed with an additional 10 mL of HBSS, and passed through a 70 μm nylon mesh (Marsh Industries, Plano, TX) to remove the extraneous connective tissue. The collected cells were centrifuged at 3,000 g for 7 min and then resuspended in 5 mL of a NH_4Cl hypotonic buffer for 3 min to lyse the red blood cells. The immune cells were washed 2X with 10 mL HBSS, centrifuged, and resuspended into complete media (RPMI 1640 supplemented with 5% fetal calf serum and 1% antibiotic/antimycotic). Splenocytes were counted using a hemocytometer and then brought to a final concentration of 2×10^6 cells/mL in complete media. Single cell lymph node suspensions were prepared by teasing apart the harvested lymph nodes in 5 mL HBSS using fine forceps. These cells were then triturated with a 5 mL pipette to disperse immune cells and washed with an additional 5 mL HBSS. Cells and tissue debris were separated by filtering the cell suspension through a 70 μm nylon mesh. The cells were pelleted and resuspended in 5 mL HBSS. The cells were pelleted and resuspended into complete media, counted using a hemocytometer, and brought to a final concentration of 2×10^6 cells/mL.

2.6. Measurement of cAMP by Enzyme Immunoassay (EIA). To measure cAMP production, spleen cells were incubated in the presence or absence of 10^{-5} M isoproterenol (ISO) or forskolin, a direct activator of adenylyl cyclase that bypasses the G-coupled protein receptors, in 1.0 mL of HBSS containing 1.0 mM IBMX (a cAMP phosphodiesterase inhibitor) at 37°C for 10 min. The reaction was then stopped by addition of 3 mL ice cold RPMI 1640 to the tubes. Samples then were centrifuged at 4°C for 8 min at 1,000 rpm, and the supernatants decanted. The cell pellets were resuspended in 0.5 mL 50 mM sodium acetate buffer. Splenocytes were lysed by two cycles of freezing (dry ice) and boiling (100°C water

bath) for 5 min each. The samples were centrifuged for 10 min at 3,000 rpm to remove cellular debris, and the supernatants were collected and stored at −70°C until cAMP analysis by EIA according to the manufacturer's instructions.

2.7. Radioligand Binding Assays. Radioligand binding studies were performed on whole splenocytes from CFA- and saline-treated rats (n of 8 per group) on D21 after challenge. Binding studies were completed using the ligand (^{125}I)cyanopindolol ((^{125}I)CYP), a β-AR antagonist with equal affinity for the β_1- and β_2-AR subclasses. Spleen cells used for β-AR binding assays were resuspended to 5×10^6 cells/mL RPMI 1640. (^{125}I)CYP (2200 Ci/mMole) was diluted in 1% ethanol, 5 mM HCl, and 0.2% bovine serum albumin (BSA). Assays were performed in duplicate in 13 × 100 mm polypropylene tubes containing 1×10^6 spleen cells with 8 concentrations of (^{125}I)CYP ranging from 15.8 to 333 pM. Nonspecific binding was determined using parallel assays incubated in the presence of 10^{-6} M CGP-12177, a hydrophilic β-AR antagonist. Tubes were incubated at 37°C for 60 min in a shaking water bath (100 oscillation/min) to ensure that equilibrium was reached. The reaction was terminated with the addition of 3 mL of ice cold hypotonic buffer (3.8 mM KH_2PO_4, 16.2 mM K_2HPO_4, and 4 mM $MnSO_4$) for 20 min to lyse the cells. The reaction mixture was filtered using a cell harvester (Brandel Corp., Gaithersburg, MD) and bound radioactivity collected on Whatman fiberglass filters (GF/B) (Brandel Corp., Gaithersburg, MD). Filters were washed with 16 mL (4 × 4 mL) of ice-cold Tris-EGTA buffer to remove the unbound radioligand. Filters were removed, placed in 12 × 75 mm tubes, and counted in a gamma counter (Whatman) at 82% efficiency.

Specific binding was defined as the difference between binding of the radioligand at each concentration in the absence or presence of 1 μM propranolol. Nonspecific binding ranged from 8 to 10% of total binding. Receptor density ($B_{\max}$) and affinity (K_D) were determined using an iterative nonlinear regression curve fitting program, Prism, version 4, to a model of a single class of homogenous binding sites. Data were transformed into linear form by Scatchard analysis. Lines of best fit were generated using the $B_{\max}$ and K_D to determine the X and Y axis intercepts. The maximal number of binding sites per cell was calculated based on simple stoichiometric assumptions (1 molecule of ligand binding to 1 receptor site) and expressed as sites/cell.

2.8. pβ_2-AR and β_2-AR Western Blots. Spleen and DLN cell pellets containing 5×10^6 cells were lysed by addition of 1 mL of ice cold RIPA buffer (50 mM Tris 1% IGEPAL, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EGTA, and 1 mM NaF; pH 7.4) containing a cocktail of protease inhibitors (Complete EDTA-free, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor (Phosstop, Roche Diagnostics). Cell samples were placed on ice, vortexed every 10 min for 30 min, and then centrifuged at 14,000 rpm for 5 min. Supernatants were collected for western blot analysis and Bradford protein determination. Samples (20 μg protein) were treated with reducing agent, denatured at 95°C for 5 min, and then cooled on ice. The samples were loaded onto a 4–12% acrylamide gel, ran at a constant 70 V through stacking, and then increased to 200 V for resolving of the protein bands. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 400 mA for 1 h. The membranes were blocked for 1 h using Tris-buffered saline and Tween 20 (TBST, 10 mM Tris HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.4)/5% milk and washed in TBST for 5 min (six times). The membranes were then placed in TBST/5% milk containing primary antibody (β_2-AR$_T$) (catalog number, SC569; 1 : 200), pβ_2-AR (Ser 345/346; catalog number, SC16718; 1 : 300), or pβ_2-AR (Ser 355/356; catalog number, SC16719R; 1 : 300) and then incubated overnight at 4°C. The blots were washed in TBST for 5 min (six times) at room temperature and then placed into TBST/5% milk containing biotinylated anti-rabbit (1 : 3,000) secondary antibody for 1 h. Next, the blots were washed in TBST for 5 min (six times) and then incubated with Supersignal Chemi Substrate (Pierce Chemical Company, Rockford, IL) for 5 min at room temperature then apposed to CL-Xposure Film (Pierce Chemical Company). Antibodies were stripped from the blots by applying Restore Stripping Buffer (Pierce Chemical Company) for 15 min at 37°C with gentle agitation. The blots were then washed twice in TBST for 10 min each and blocked for 1 h with TBST/5% milk. The blots were then reprobed for β-actin (1 : 5,000) as a loading control. Analysis of blots was completed using MCID 7.0 software (Image Research Inc., ON, Canada), and GraphPad PRISM software was used for data analyses.

2.9. Cell Culture for Cytokine Production. Spleen and DLN cells suspended at a final concentration of 2×10^6 cells/mL were plated into 24-well plates (Falcon, Oxnard, CA) and then placed in an incubator set at 7% CO_2 and 37°C for 24 h. After 24 h, the supernatants were harvested and stored in the freezer at −80°C. ELISAs for IFN-γ were completed with standards run on each plate and according to the manufacturer's directions. Plates were read at 450 nm within 30 min with λ correction 570 nm using an ELISA plate reader (Ceres 900 HDI, Bio Tek Instruments Incorporated, Winooski, VT) and cytokine levels determined by comparing optical densities from a standard curve created using known concentrations of IFN-γ present on each plate.

2.10. Statistical Analysis

2.10.1. Disease Outcome Measures. The average of the right and left foot pad measurements was obtained for each animal, and then these individual means were averaged within each group, expressed as a mean ± standard error of the mean (SEM), and analyzed using a two-way analysis of variance (ANOVA) with repeated measures. Significant ANOVA ($P < 0.05$) values were subjected to Bonferroni posthoc testing. The sum of the radiographic scores for the hind limbs was averaged within the treatment groups and expressed as a group mean ± SEM and subjected to Kruskal-Wallis statistical analysis (nonparametric statistic equivalent to an ANOVA; $P < 0.05$) followed by Dunn posthoc testing.

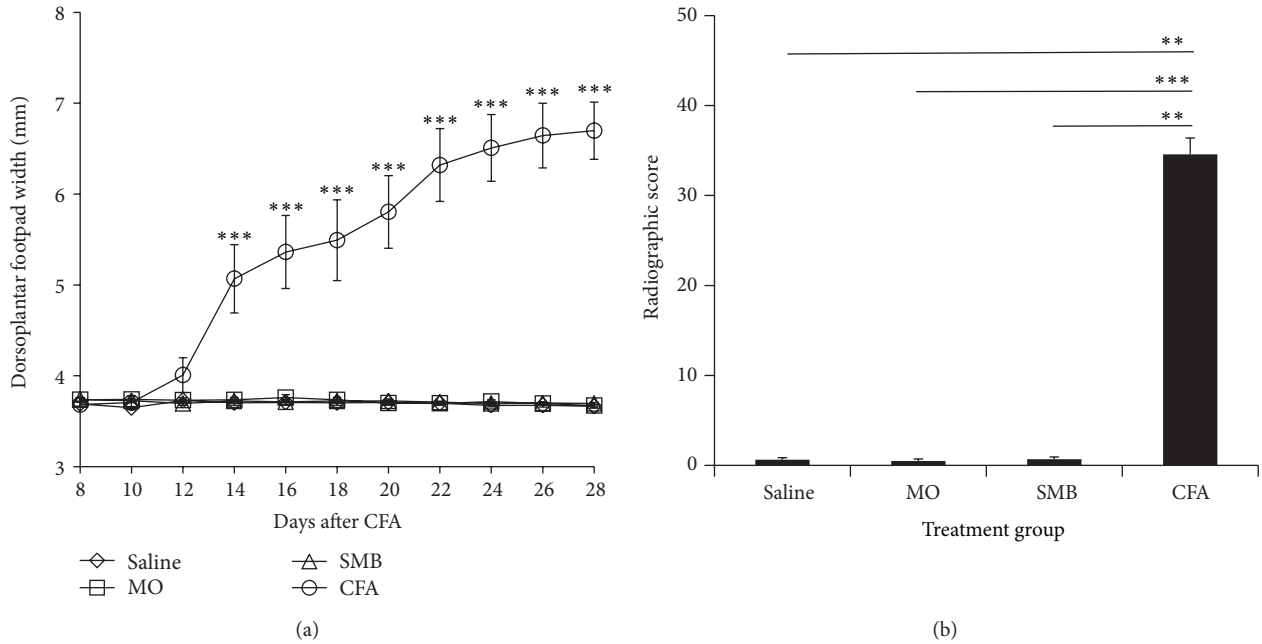

Figure 1: Mean hind limb dorsoplantar widths (a) and radiographic scores (b) for rats treated with CFA, SMB, MO, or Saline. (a) A significant increase in mean dorsoplantar widths, indicative of soft tissue swelling, was observed in CFA (O)-challenged rats compared with groups treated with SMB (Δ), MO (□) or Saline (◊) between D12–28 after CFA immunization. No differences were observed in dorsoplantar widths between SMB-, MO- or Saline-treated rats. Values represent the mean dorsoplantar width in millimeters (mm) ± SEM with an n of 8 rats per treatment group. Statistics: repeated measures two-way ANOVA with Bonferroni multiple comparison tests ($^{***}P < 0.001$). (b) Rats treated with CFA had significantly higher radiographic scores compared with rats treated with SMB, MO, or Saline on D28 post-immunization. No differences were observed in radiographic scores between SMB-, MO- or Saline-treated rats. Data are expressed as the mean radiographic score ± SEM with an n of 8 rats per treatment group. Statistics: Kruskal-Wallis analysis followed by Dunn post hoc testing ($^{**}P < 0.01$; $^{***}P < 0.001$).

2.10.2. cAMP Production, β_2-AR Binding Assays, Cytokine Production, and β_2-AR Protein Levels. Replicated individual optical densities (OD) from β_2-AR western blots normalized to β-actin, β_2-AR K_D and B_{max}, or fmol cAMP/2×10^6 cells were averaged for each animal and then for each treatment group. Data were expressed as a mean ± SEM and analyzed using one-way ANOVA with Bonferroni posthoc testing. OD obtained for pβ_2-AR_{PKA} and pβ_2-AR_{GRK} were also expressed as a ratio of the β_2-AR_T OD. IFN-γ concentrations from duplicate wells were averaged, group means were calculated, and the data are expressed as a mean in pg/mL. Significant differences between treatment groups for mean β_2-AR, pβ_2-AR_{PKA} and pβ_2-AR_{GRK} OD and the ratios for pβ_2-AR_{PKA} and pβ_2-AR_{GRK} to β_2-AR_T were determined using one-way ANOVA ($P < 0.05$) followed by Bonferroni post hoc analysis. Differences between treatment groups for production of IFN-γ were determined using a Student's t-test ($P < 0.05$). Only data where significant differences ($P < 0.1$) between treatment groups were observed will be described.

3. Results

3.1. Disease Severity. Animals challenged with saline, SMB, or MO developed no clinical signs of arthritis throughout the duration of the experiment (Figure 1(a)). Erythema was apparent in CFA-challenged rats by D8 after CFA challenge. Dorsoplantar foot pad width increased in the CFA-challenged animals, reaching significance ($P < 0.0001$) between D12–14 compared with Saline-, SMB-, or MO-treated rats (Figure 1(a)) (CFA versus Saline, CFA versus SMB, and CFA versus MO: $P < 0.0001$ at each time point). Dorsoplantar foot pad widths of CFA-challenged rats continued to increase through D21–24 (severe disease) and then stabilized through D28 (chronic disease). Radiographic analysis of the ankle joints (Figure 1(b)) revealed significant differences between treatment groups ($P < 0.0004$). CFA-challenged rats had increased soft tissue swelling, bone loss, periosteal bone formation, narrowing of their joint spaces, and reduced bone density by D28 after CFA challenge compared with the Saline- ($P < 0.01$), MO- ($P < 0.001$), or SMB- ($P < 0.01$) challenged rats.

3.2. β-AR-Stimulated cAMP Production in Spleen Cells. On D21, unstimulated splenocytes from Saline- and CFA-challenged rats contained similar baseline levels of cAMP. Treatment with forskolin (Figure 2) significantly increased splenocyte cAMP production in nonarthritic and CFA-treated rats from basal levels (40 and 54%, resp., $P < 0.05$). Similarly, isoproterenol-stimulated cAMP production rose 40% ($P < 0.05$) higher than in unstimulated spleen cells from nonarthritic rats. In contrast, adding isoproterenol to the spleen cell suspension from arthritic rats had no effect on cAMP production.

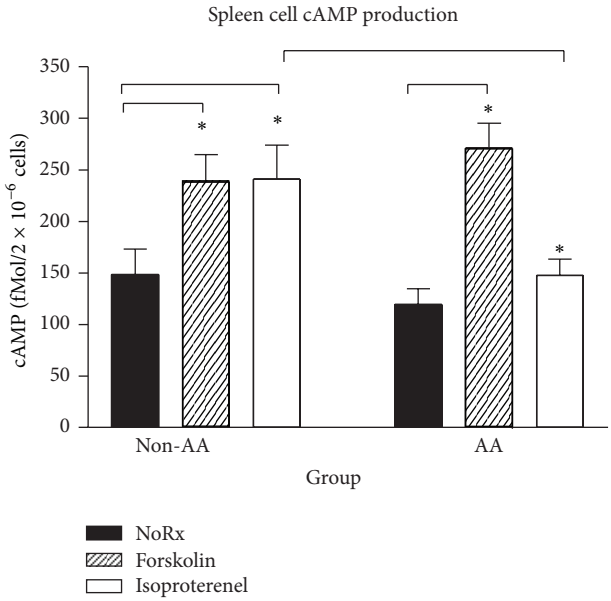

Figure 2: Forskolin and isoproterenol elevated cAMP production compared with untreated spleen cells *in vitro* in saline-treated non-AA rats. In contrast, forskolin, but not isoproterenol treatment, increased cAMP production compared with untreated splenocytes in AA rats. The lower cAMP production observed in splenocytes treated with isoproterenol in AA rats was also significantly different compared with non-AA rats. Data are expressed as a mean ± SEM with an n of 8 rats per treatment group. Statistics: one-way ANOVA with Bonferroni multiple comparison tests ($^*P < 0.05$).

3.3. Receptor Binding Assays. On D21 after treatment with Saline, MO, SMB, or CFA, saturation isotherms obtained from binding experiments with splenocytes are shown in Figures 3(a)–3(d). Binding of the radioligand was rapid, saturable, and of high affinity in all treatment groups and at each time point. Specific binding was greater than 90% of the total binding at near saturating radioligand concentrations. Scatchard plots are shown in the insets of Figures 3(a)–3(d). ANOVA indicated significant differences in the mean B_{max} between treatment groups ($P < 0.0002$). Mean B_{max} (Figure 3(a)) was significantly reduced in both SMB- and CFA-challenged rats compared with the Saline and MO treatment groups (SMB versus Saline or MO, $P < 0.001$; CFA versus Saline or MO, $P < 0.05$). The mean K_D differed significantly between treatment groups ($P < 0.0009$). K_D was significantly higher ($P < 0.01$) for MO- or CFA-treated compared with SMB-treated rats. There was a trend ($P < 0.1$) for an increased mean K_D in splenocytes from MO- and CFA-compared with Saline-treated rats.

3.4. β_2-AR Expression Is Reduced in Splenocytes, but Not in DLN Cells in Established AA. To determine if β_2-AR expression is altered in animals with established AA, splenocyte β_2-AR expression was assessed by western blot (Figures 4(a)–4(c)). On D21, ANOVA revealed significant differences ($P < 0.0005$) in splenocyte β_2-AR density between treatment groups (Figure 4(a)). Immune challenge with CFA ($P < 0.01$) or SMB ($P < 0.05$) significantly elevated the splenocyte β_2-AR density compared with Saline or MO treatment. There also was a significant difference ($P < 0.005$) in splenocyte β_2-AR density between treatment groups on D28 (Figure 4(b)). Spleen cells from CFA-challenged rats had significantly decreased β_2-AR density compared with Saline (35%, $P < 0.001$), MO (26%, $P < 0.05$), or SMB (29%, $P < 0.01$) treatment groups. A representative immunoblot depicting β_2-AR expression in splenocytes from each treatment group on D28 after CFA challenge is shown in Figure 4(c). No difference in β_2-AR expression in DLN cells was observed between any of the treatment groups at either time point after CFA challenge (Figures 5(a)–5(c)).

3.5. AA Reduces Phosphorylation of Splenocyte β_2-AR at Ser 345-346 (PKA Site). To determine if the lack of isoproterenol-induced cAMP response observed in splenocytes from rats with established arthritis could be due to pβ_2-AR$_{PKA}$ with subsequent uncoupling of the receptor to Gs proteins, splenocyte pβ_2-AR$_{PKA}$ expression was assessed using western blots. On D21 and D28 after immunization, significant main effects of treatment ($P < 0.0001$ at both time points) were observed (Figures 6(a)–6(c)). On D21, expression of pβ_2-AR$_{PKA}$ in splenocytes from CFA-challenged rats was significantly reduced ($P < 0.001$) by 43, 47, and 42% compared with Saline-, MO-, and SMB-challenged rats, respectively (Figure 6(a)). Similarly, on D28, pβ_2-AR$_{PKA}$ in splenocytes from CFA-challenged rats were significantly reduced ($P < 0.001$) by 52, 52, and 49% compared with Saline-, MO-, and SMB-challenged rats, respectively (Figure 6(b)). A representative immunoblot depicting pβ_2-AR$_{PKA}$ expression in splenocytes from each treatment group on D28 after CFA challenge is shown in Figure 6(c).

ANOVA revealed significant differences in the pβ_2-AR$_{PKA}$ on D21 ($P < 0.0001$) and D28 ($P < 0.0042$), when expressed as a pβ_2-AR$_{PKA}$/β_2-AR$_T$ (Figures 6(d) and 6(e)). There was a trend ($P < 0.1$) towards lower pβ_2-AR$_{PKA}$/β_2-AR$_T$ expression for SMB- compared with Saline- or MO-treated on D21 (Figure 6(d)). pβ_2-AR$_{PKA}$/β_2-AR$_T$ were significantly reduced by 58 ($P < 0.01$), 61 ($P < 0.01$), and 48% ($P < 0.05$) in arthritic CFA- compared with Saline-, MO-, and SMB-treated rats on D21, respectively. Similarly, pβ_2-AR$_{PKA}$/β_2-AR$_T$ were significantly decreased on D28 by 26 ($P < 0.05$), 34 ($P < 0.01$), and 28% ($P < 0.05$) in arthritic rats compared with the Saline, MO, and SMB treatment groups, respectively (Figure 6(e)).

3.6. Spleen Cell pβ_2-AR at Ser 355-356 (GRK Site) Is Increased in AA Rats. Analysis of western blots revealed significant treatment effects on expression of pβ_2-AR$_{GRK}$ for both D21 and D28 after immune challenge ($P < 0.0154$ and $P < 0.0134$, resp.; Figures 7(a)–7(c)). At D21, pβ_2-AR$_{GRK}$ in splenocytes from rats challenged with MO, SMB, or CFA were significantly increased (43%; $P < 0.05$) compared with Saline-treated rats (Figure 7(a)). On day 28, pβ_2-AR$_{GRK}$ trended towards being reduced ($P < 0.1$) in SMB- compared with Saline-treated rats (Figure 7). Only pβ_2-AR$_{GRK}$ in splenocytes from arthritic rats were significantly increased compared with MO (28%) and SMB (32%; $P < 0.05$). A representative

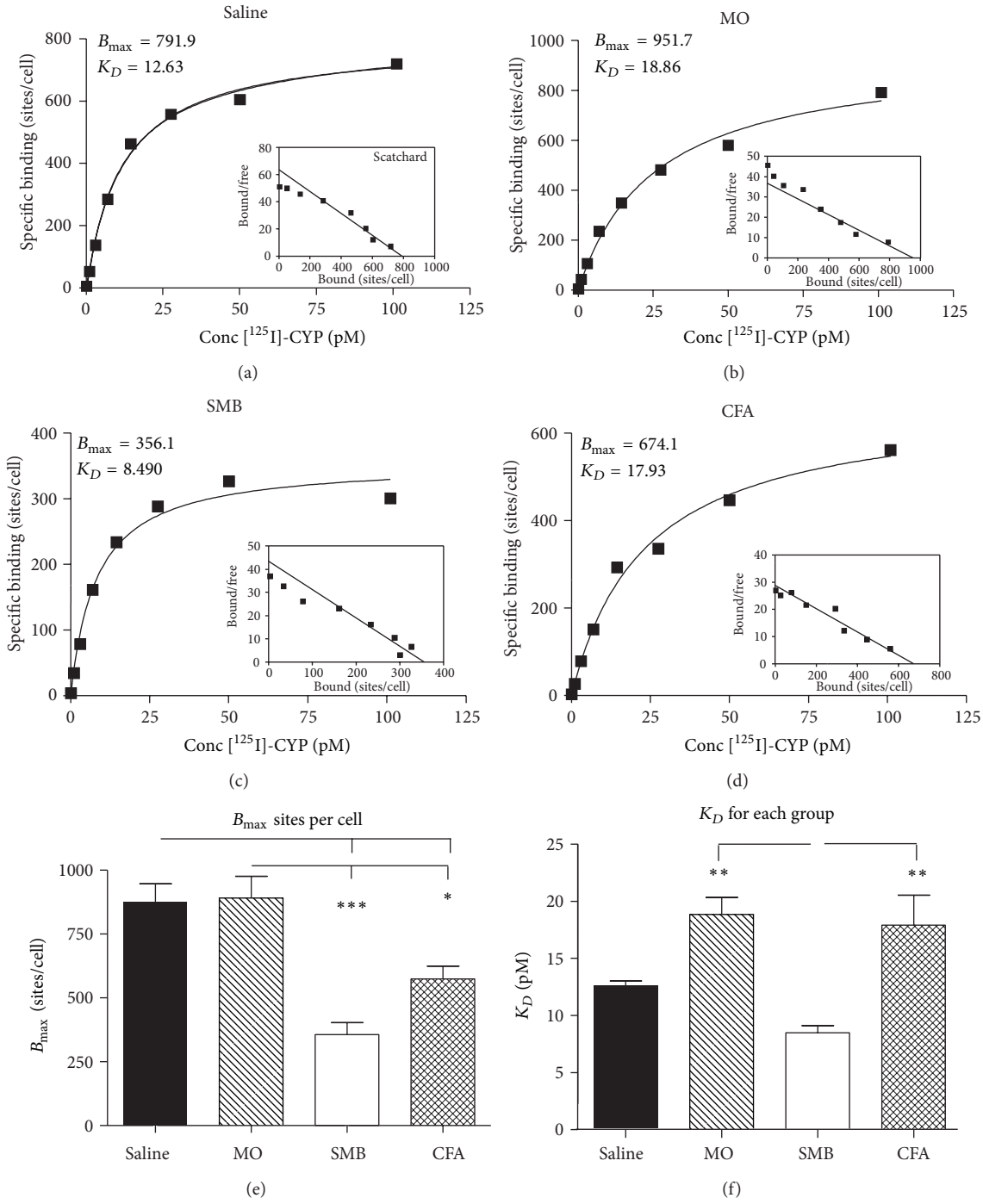

FIGURE 3: Specific binding and Scatchard plots of (−)-(^{125}I)cyanopindolol (^{125}ICYP) in whole spleen cells from rats treated with (a) Saline, (b) MO, (c) SMB, and (d) CFA. Spleen cells were incubated under equilibrium binding conditions at 37°C with ^{125}ICYP (15.8–333 pM) for 60 min, then the reaction was stopped, and the radioactivity was quantified by gamma scintillation counting. Binding assays were run in duplicate. Specific binding (sites/cell) and inset Scatchard plots (bound/free) represent mean values obtained from 8 rats in each treatment group. (e)-(f). The mean density of β-AR (B_{max}) expressed as sites/cell (e) and K_D (f) on spleen cells from Saline-, MO-, SMB-, and CFA-treated rats. (e) The number of β_2-AR sites per splenocyte is significantly decreased in SMB- and CFA-treated rats compared with both Saline- and MO-treated rats. (f) The mean K_D was increased in MO- and CFA-treated rats compared with rats treated with SMB. Mean values were calculated for the B_{max} and K_D determined from specific binding curves generated for each rat from each treatment group. Data are expressed as a mean B_{max} or K_D ± SEM with an n of 8 rats per treatment group. Statistics: one-way ANOVA with Bonferroni multiple comparison tests ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

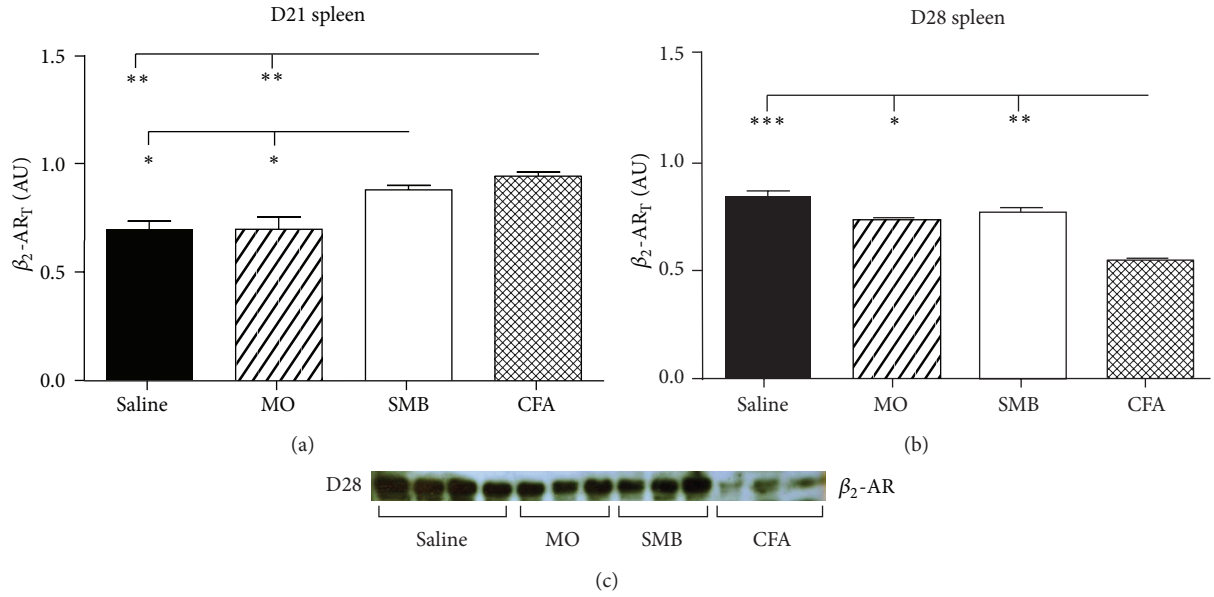

FIGURE 4: Total β_2-AR expression in splenocytes from AA and non-AA rats at (a) D21 and (b) D28 after immune challenge. (a) Expression of β_2-AR was increased on D21 in splenocytes from SMB- and CFA-treated rats compared with Saline- and MO-treated rats. (b) In contrast, on D28, β_2-AR expression was significantly decreased in CFA-treated compared with all other treatment groups. (c) Representative immunoblot depicting β_2-AR expression in splenocytes from each treatment group on D28 after CFA challenge. Spleen cells were harvested, lysed, and proteins resolved by SDS-PAGE. The data were normalized to β-actin. Cellular extracts were probed with a β_2-AR receptor antibody to determine β_2-AR expression and quantified by densitometry. Each bar represents the mean optical density ± SEM with an n of 8 rats per treatment group. Statistics: one-way ANOVA with Bonferroni multiple comparison tests. ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

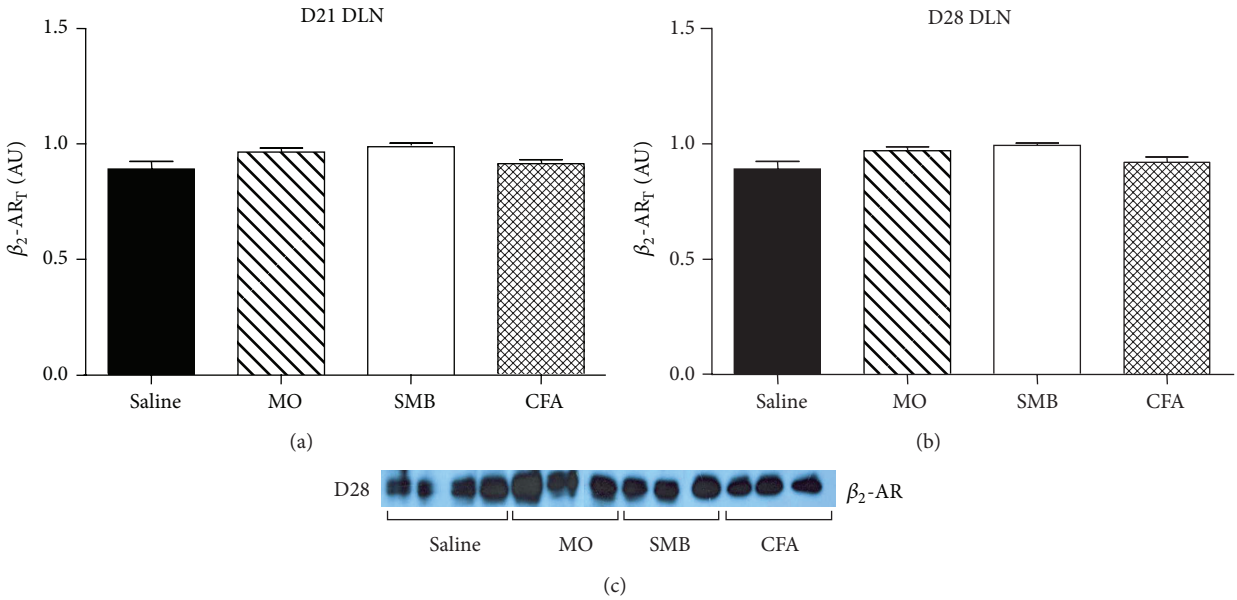

FIGURE 5: Total β_2-AR expression in DLN cells from AA and non-AA rats at (a) D21 and (b) D28 after immune challenge ((a)–(c)). In contrast to splenocytes, expression of β_2-AR was unchanged in DLN cells from CFA-, SMB-, and MO-challenged rats on D21 or D28 compared with Saline-treated controls. DLN cells were harvested, lysed, and proteins resolved by SDS-PAGE. The data were normalized to β-actin. Cellular extracts were probed with a β_2-AR receptor antibody, and β_2-AR expression was quantified by densitometry. An example of the western blots at D28 is shown in (c). Each bar represents the mean optical density ± SEM with an n of 8 rats per treatment group. A one-way ANOVA with Bonferroni multiple comparison tests was used to determine statistically significant differences between treatment groups.

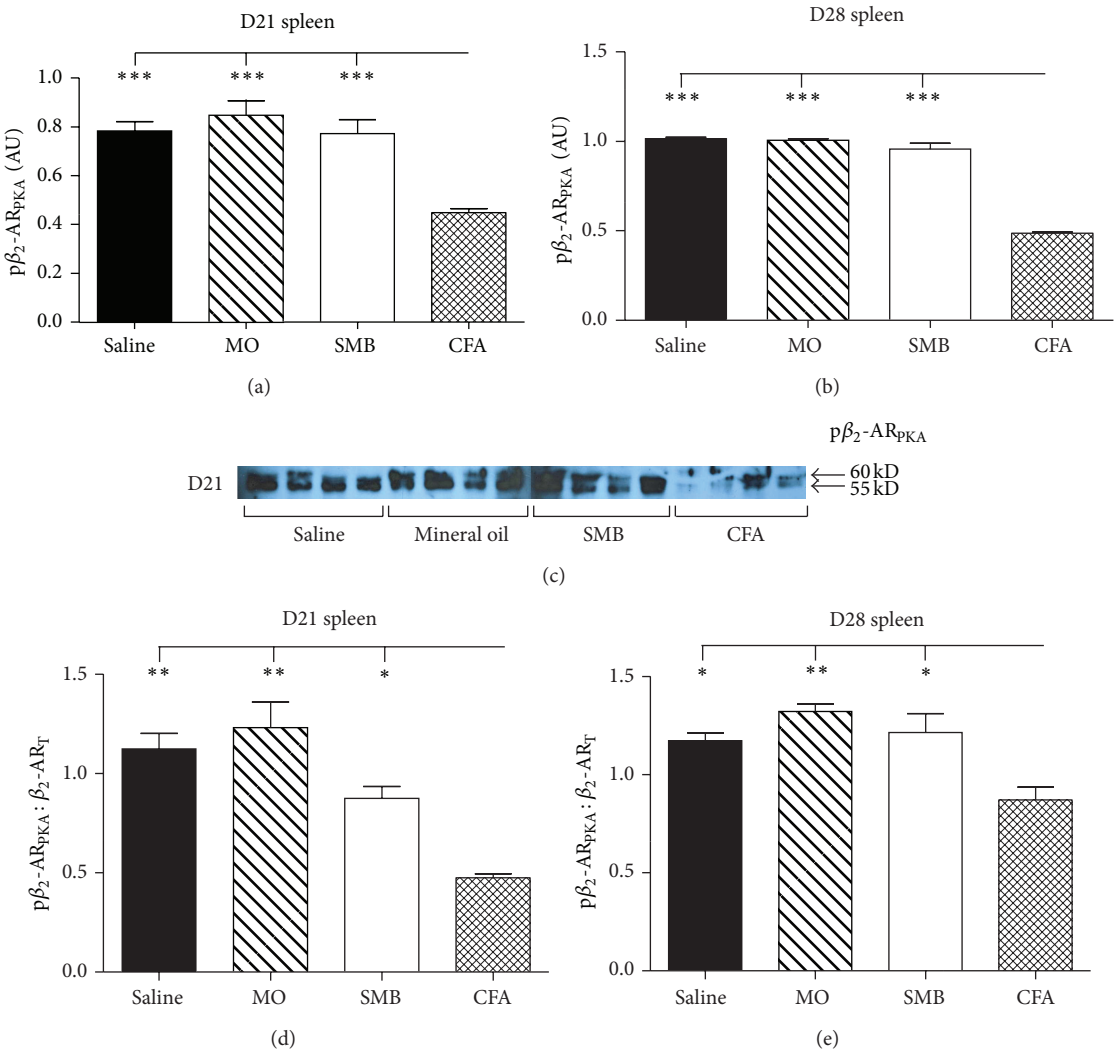

Figure 6: Expression of PKA-phosphorylated-β_2-AR ($p\beta_2$-AR_{PKA}) in splenocytes from arthritic and nonarthritic control rats. ((a)–(e)). Expression of $p\beta_2$-AR_{PKA} in splenocytes was reduced in CFA- compared with SMB-, Saline-, and MO-challenged rats D21 (a) and D28 (b) after immune challenge. Expression of $p\beta_2$-AR_{PKA} in splenocytes from AA and non-AA rats at D21 (d) and D28 (e) after immune challenge was normalized to total receptor (β_2-AR_T) levels. β_2-AR_{PKA}/β_2-AR_T was reduced in CFA-challenged rats compared with SMB-, MO-, and Saline-treated rats at both time points examined, indicating a disease-specific effect. Spleen cells were harvested, lysed, and total protein collected, then resolved by SDS-PAGE. The data were normalized to β-actin. Cellular extracts were probed with an antibody against phosphorylated Ser345, Ser346 of the β_2-AR and quantified by densitometry. An example of the western blots at D21 is shown in (c). Each bar represents the mean optical density ± SEM (a)-(b) or mean $p\beta_2$-AR_{PKA} normalized to β_2-AR_T ± SEM (d)-(e) with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

immunoblot depicting $p\beta_2$-AR_{GRK} expression in splenocytes from each treatment group on D28 after CFA challenge is shown in Figure 7(c).

Western blot data for $p\beta_2$-AR_{GRK} were also expressed as $p\beta_2$-AR_{GRK}/β_2-AR_T (Figures 7(d) and 7(e)). ANOVA revealed significant differences in the $p\beta_2$-AR_{GRK}/β_2-AR_T on D21 ($P < 0.0181$) and D28 ($P < 0.0001$) (Figure 7(d)). On D21, $p\beta_2$-AR_{GRK}/β_2-AR_T were elevated 27% in MO-compared with Saline- or CFA-treated rats ($P < 0.05$) (Figure 7(d)). In MO-treated rats, this ratio trended toward an increase compared with SMB-treated rats (23%, $P < 0.1$). $p\beta_2$-AR_{GRK}/β_2-AR_T in CFA-treated rats significantly increased ($P < 0.001$) compared with Saline- (44%), MO- (46%) or SMB- (52%) treated rats on D28 after immune challenge (Figure 7(e)).

3.7. Phosphorylation of DLN Cell β_2-AR at Ser 345-346 (PKA Site) Increased at Peak Disease. Expression of $p\beta_2$-AR_{PKA} was also assessed in DLN cells from arthritic and nonarthritic treatment groups (Figures 8(a) and 8(b)). ANOVA revealed significant differences in the $p\beta_2$-AR_{PKA}/β_2-AR_T on D21 ($P < 0.0002$) and D28 ($P < 0.0136$). On day 21, $p\beta_2$-AR_{PKA}/β_2-AR_T in DLN cells increased in SMB-treated rats compared with Saline or MO treatment ($P < 0.05$) and in CFA-challenged rats compared with Saline- or MO-treated rats

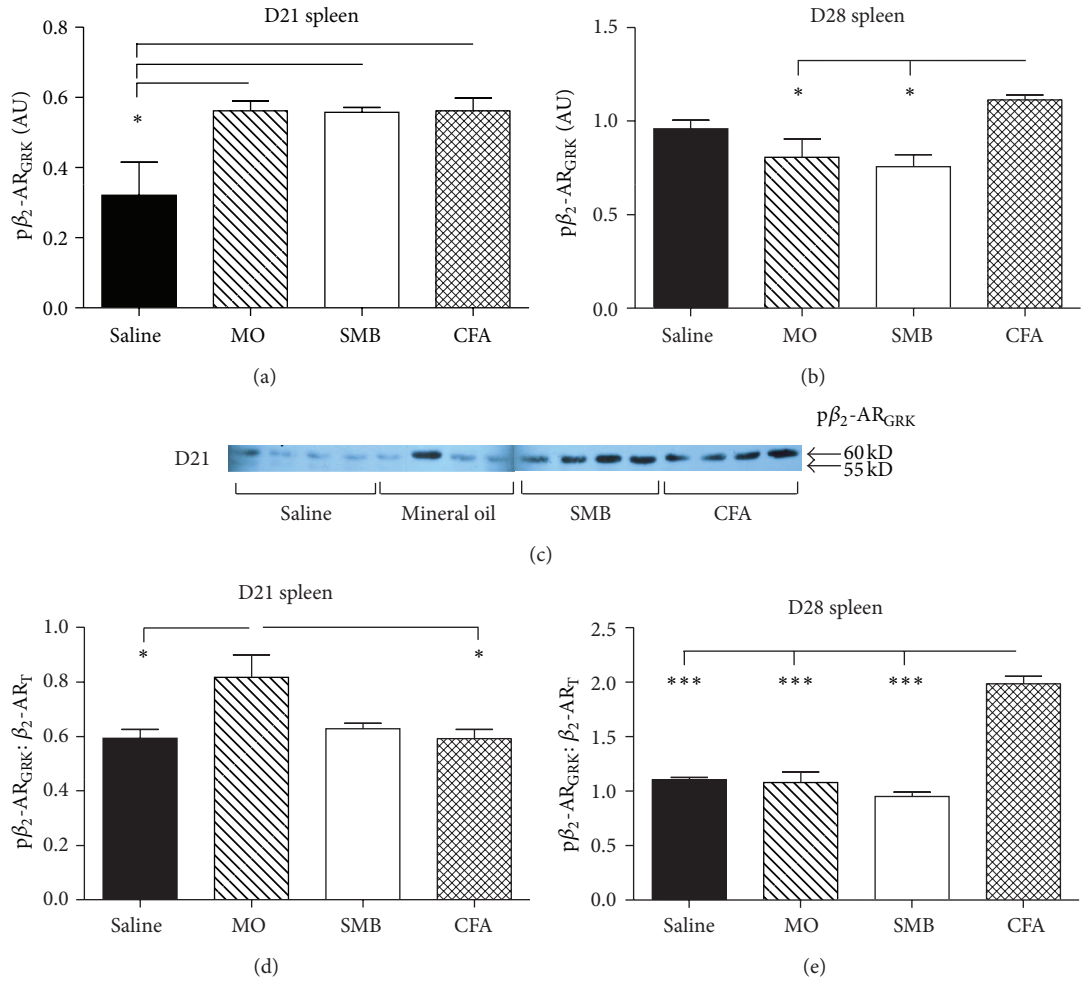

FIGURE 7: Expression of GRK phosphorylated-β_2-AR ($p\beta_2$-AR_{GRK}) in splenocytes from arthritic and nonarthritic control rats. (a) Expression of $p\beta_2$-AR_{GRK} in splenocytes was increased in CFA-, SMB-, and MO-challenged rats compared with Saline-treated rats on D21. (b) By D28, $p\beta_2$-AR GRK expression in spleen cells from SMB- and MO-challenged rats was reduced compared with CFA-challenged rats. No difference in $p\beta_2$-AR_{GRK} expression between CFA- and Saline-treated rats was observed. (d) Expression of $p\beta_2$-AR_{GRK} in splenocytes from AA and non-AA rats normalized to β_2-AR_T levels. MO-treated rats expressed greater $p\beta_2$-AR_{GRK}/β_2-AR_T levels compared with Saline- and CFA-treated rats on D21 after immune challenge. (e) On D28 after challenge, $p\beta_2$-AR_{GRK}/β_2-AR_T was significantly increased in CFA-challenged rats compared with all other treatment groups. Splenocytes were harvested, lysed, and proteins resolved by SDS-PAGE. Cellular extracts were probed with an antibody against phosphorylated Ser355/Ser356 of the β_2-AR. (c) Western blot shown is representative of the blots within each treatment group. The data were normalized to β-actin. Data are expressed as a mean $p\beta_2$-AR_{GRK} ± SEM (a)-(b) or mean $p\beta_2$-AR_{GRK} normalized to β_2-AR_T ± SEM (d)-(e) with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

($P < 0.001$ or $P < 0.01$, resp.) (Figure 8(a)). In contrast, $p\beta_2$-AR_{PKA}/β_2-AR_T in DLN cells from CFA- and SMB-challenged rats were decreased ($P < 0.05$) on D28 compared with Saline-treated rats (Figure 8(b)).

3.8. Phosphorylation of β_2-AR at Ser 355-356 (GRK Site) in DLN Cells Is Increased in AA Rats. Analysis of western blots revealed significant treatment effects on expression of $p\beta_2$-AR_{GRK}/β_2-AR_T for both D21 and D28 after immune challenge ($P < 0.0001$ and $P < 0.0004$, resp.; Figures 9(a)–9(c)). $p\beta_2$-AR_{GRK}/β_2-AR_T was significantly increased in SMB- and CFA-challenged rats compared with either the Saline- or MO-treated rats on D21 (SMB versus Saline or MO, $P < 0.001$; CFA versus Saline, $P < 0.001$ or MO, $P < 0.01$; Figure 9(a)). On D28, $p\beta_2$-AR_{GRK}/β_2-AR_T significantly increased ($P < 0.05$ or $P < 0.001$, resp.) in CFA-challenged rats compared with either the Saline- or MO-treated rats (Figure 9(b)). $p\beta_2$-AR_{GRK}/β_2-AR_T also significantly ($P < 0.01$) increased in SMB- compared with MO-treated rats. There was a trend ($P < 0.1$) for a higher ratio in SMB- compared with Saline-treated rats.

3.9. Terbutaline Treatment Differentially Affected IFN-γ Production in Spleen and DLN Cells. Figure 10 shows the *ex vivo* secretion of IFN-γ in spleen (a) and DLN (b). IFN-γ production did not differ significantly between spleen cells obtained from arthritic rats treated with terbutaline compared with arthritic rats treated with the vehicle

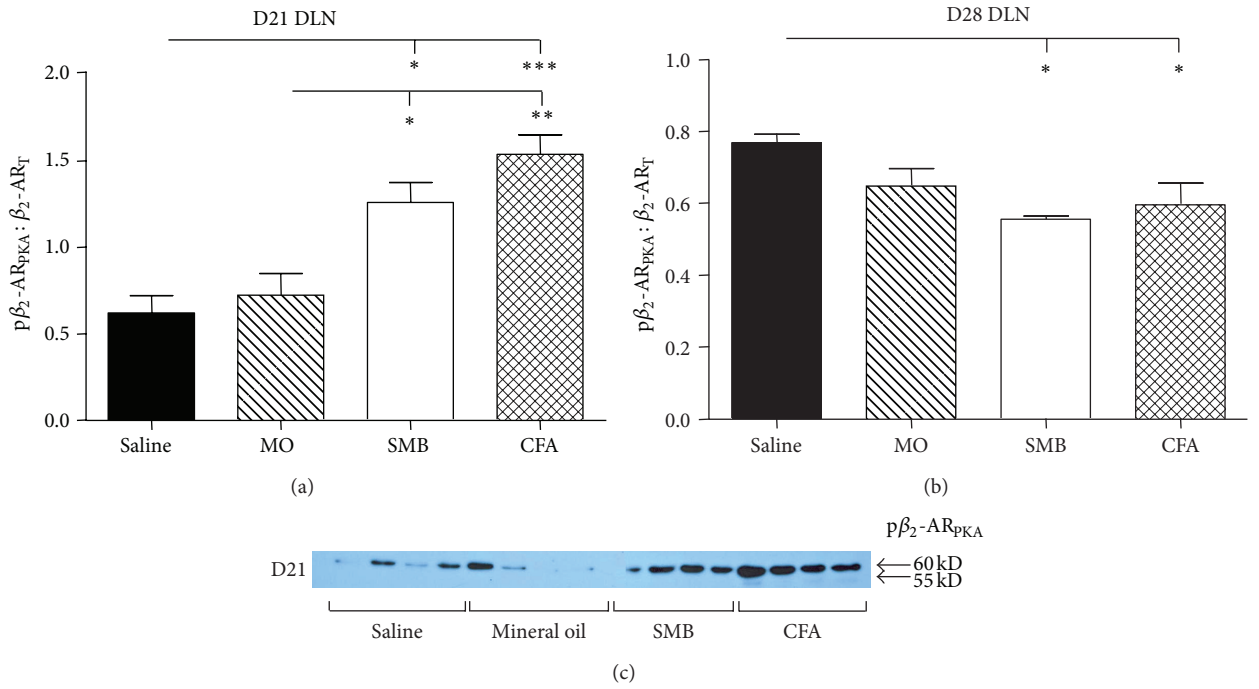

Figure 8: Expression of PKA-phosphorylated-β_2-AR ($p\beta_2$-AR_{PKA})/β_2-AR_T in DLN cells from rats challenged with CFA, SMB, or MO compared with Saline-treated controls. Levels of $p\beta_2$-AR_{PKA}/β_2-AR_T were not significantly different in Saline- and MO-treated rats on D21 (a) or D28 (b). In contrast, $p\beta_2$-AR_{PKA}/β_2-AR_T in DLN cells from CFA- and SMB-challenged rats were significantly increased on D21, but significantly decreased on D28 compared with Saline- or MO-treated rats. DLN cells were harvested, lysed, and total protein collected and resolved by SDS-PAGE. Cellular extracts were probed with an antibody against the phosphorylated Ser345, Ser346 of the β_2-AR to determine $p\beta_2$-AR_{PKA} expression and quantified by densitometry. A western blot is shown (c) that is representative of the blots seen within each treatment. The data were normalized to β-actin, then $p\beta_2$-AR_{PKA} was normalized to β_2-AR_T levels. Each bar represents the mean $p\beta_2$-AR_{PKA}/β_2-AR_T ± SEM with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

(Figure 10(a)). In contrast, terbutaline treatment in arthritic rats significantly increased DLN cell IFN-γ production compared with vehicle treatment (Figure 10(b); $P < 0.05$).

4. Discussion

In the present study, we have determined the effects of CFA, and its components, on β_2-AR expression and signaling in immunocytes from lymphoid organs that mediate disease processes for RA. We report that in AA, during severe disease, β-AR agonists fail to induce cAMP production in splenocytes. Loss of β-AR-induced cAMP is accompanied by reduced affinity and expression of β-AR, indicative of receptor desensitization and downregulation. $p\beta_2$-AR_{PKA} and $p\beta_2$-AR_{GRK} are strikingly different in splenocytes and DLN cells from AA rats. In splenocytes, $p\beta_2$-AR_{PKA} is reduced regardless of the time point examined, while in DLN cells, $p\beta_2$-AR_{PKA} is increased during severe AA and reduced during chronic disease. Similarly, in splenocytes, $p\beta_2$-AR_{GRK} is unchanged and increased with severe and chronic disease stages, respectively, but increased in DLN cells during both disease stages. Differential $p\beta_2$-AR support disparate regulation of spleen and DLN cell functions by the SNS in AA rats. These findings are consistent with previous findings from our lab [41] and in the present study, with the findings from *in vivo* terbutaline treatment showing increased IFN-γ production by DLN cells, but no change in splenocyte IFN-γ production *ex vivo*.

In the present study, basal levels of cAMP were similar in AA and nonarthritic rats, but β-AR stimulation failed to alter cAMP levels in splenocytes from AA rats during peak disease severity (D21) compared with controls. Synthesis of cAMP was not impaired, because forskolin induced intracellular cAMP production in AA rats. Since β_2-AR-Gs protein coupling is required for adenylyl cyclase-induced cAMP production, these findings support an uncoupling of β-ARs to Gs proteins in splenocytes from AA rats. Loss of catecholamine-induced cAMP production indicates altered receptor function and is supported by findings from the receptor binding studies. Receptor affinity ($1/K_D$) was reduced in splenocytes from AA rats by D21 after CFA challenge compared with controls (Saline and SMB). Receptor affinity was also decreased in MO-treated controls, indicating that the MO component of CFA induced this effect. B_{max} values were reduced in splenocytes from SMB and AA compared with Saline- and MO-treated rats. The effects of SMB treatment on β-AR density support a role for the bacterial cell wall component of CFA in this response. Collectively, our data indicate that splenocyte β_2-ARs are desensitized and downregulated in severe AA, effects that are mediated via MO and SMB, respectively.

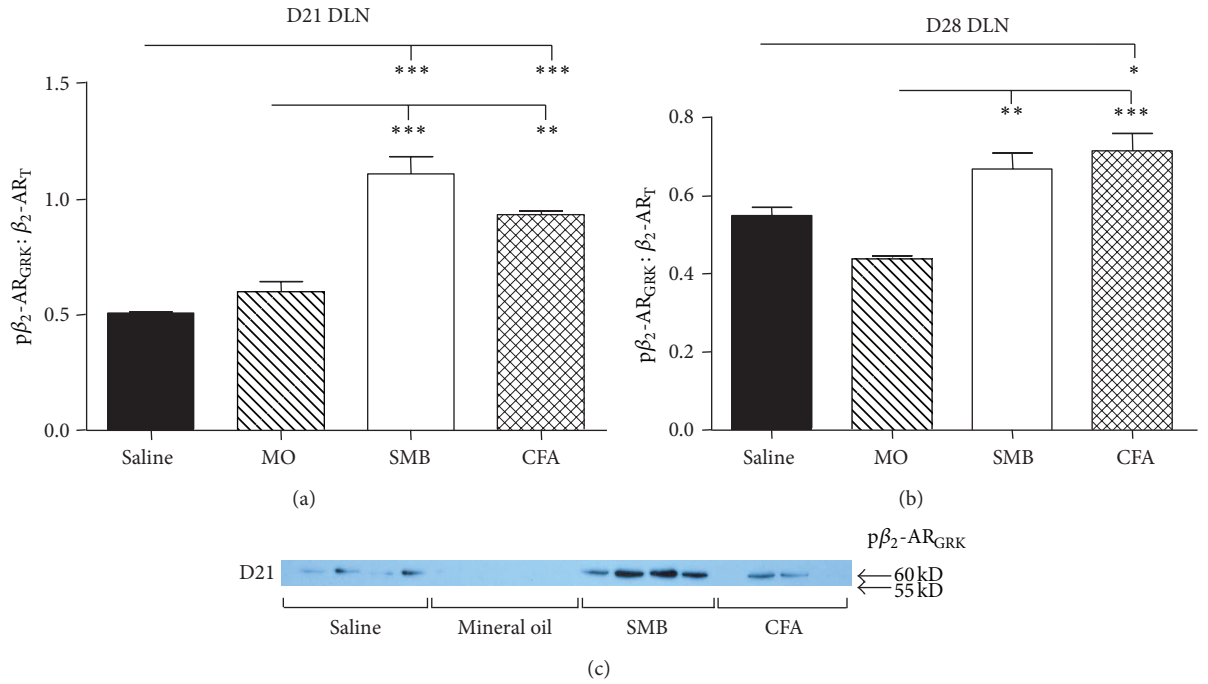

FIGURE 9: Expression of the $p\beta_2\text{-AR}_{GRK}/\beta_2\text{-AR}_T$ in DLN cells from rats challenged with CFA, SMB, or MO compared with Saline-treated controls on D21 (a) and D28 (b). No difference in $p\beta_2\text{-AR}_{GRK}/\beta_2\text{-AR}_T$ was observed between the MO- and Saline-treatment groups at either time point. Expression of $p\beta_2\text{-AR}_{GRK}/\beta_2\text{-AR}_T$ was increased in CFA-, SMB- compared with MO-challenged and Saline-treated rats on D21 and D28. DLN cells were harvested, lysed, and proteins resolved by SDS-PAGE. Cellular extracts were probed with an antibody against GRK phosphorylated Ser355/Ser356 of the β_2-AR. A western blot is shown (c) that is representative of the blots seen within each treatment. The data were normalized to β-actin, and $p\beta_2\text{-AR}_{GRK}$ expression was normalized to $\beta_2\text{-AR}_T$. Data are expressed as a mean $p\beta_2\text{-AR}_{GRK}/\beta_2\text{-AR}_T \pm$ SEM with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing ($^{**}P < 0.01$; $^{***}P < 0.001$).

Western blots revealed an increase in $\beta_2\text{-AR}_T$ expression (membrane and intracellular) in splenocytes obtained from AA rats on D21, an effect also observed in SMB-treated controls. In contrast, our binding data show reduced cell surface expression of β_2-ARs, suggesting increased internalization of β_2-ARs. By D28, β_2-ARs were decreased in AA rats compared with all controls, indicating a disease-specific internalization and degradation of receptors between D21 and D28. In contrast, β_2-ARs levels returned to baseline in SMB-treated rats at D28, supporting recycling of β_2-AR to the membrane or β_2-AR synthesis keeps pace with degradation.

Others have reported reduced β-AR agonist-induced cAMP and β-AR densities in PBMC and B cells in RA patients that negatively correlate with systemic disease activity [9, 43]. In contrast, β-AR agonist-induced cAMP do not differ in CD4+ and CD8+ T cells and are increased in PBMCs in RA patients compared with healthy controls in other studies [13, 43]. These seemingly inconsistent findings may be explained, in part by, the duration of β_2-AR stimulation, use of membranes versus whole cells, and/or reported increase in the incidence of polymorphisms in the β_2-AR in RA patients. RA patients have a greater incidence of the $Arg^{16}Gly$ polymorphism in the β_2-AR compared with healthy controls, which in association with HLA-DR alleles imparts a greater risk for developing RA [44]. Further, these polymorphisms modulate the age of disease onset, with additional polymorphisms at $Gln^{27}Glu$ and $Thr^{164}Ile$ imparting an increased risk for developing arterial hypertension in RA patients [44]. Ahles et al. [45] have demonstrated that the $Arg^{16}Gly$ polymorphism imparts differential signaling of β_2-ARs. Similar to Arg^{16} encoded receptors, the polymorphisms $Arg^{16}\rightarrow Gly$ or $Gln^{27}\rightarrow Glu$ is recruited to the cell membrane to a similar extent with similar ligand binding affinities. They also have similar activation and deactivation kinetics after a single stimulation with an β-AR agonist. However, receptors encoded by Arg^{16}, $Arg^{16}Gly$, and $Gln^{27}Glu$ β_2-AR genes display different activation kinetics after repeat activation that result in differential efficacies to generate cAMP [45] reviewed in [46]. The $Arg^{16}Gly$ polymorphism result in faster, more persistent, and more effective downstream signaling via cAMP regardless of its association with Gln^{27} or Glu^{27}. In contrast, the $Arg^{16}Gln^{27}$ variant demonstrates slower activation kinetics and reduced cAMP production, an effect independent of receptor internalization, as this did not differ among the three β_2-AR variants. Enhanced activation of the $Arg^{16}GLy$ is coupled with greater phosphorylation of the receptor by GRK-2 and a more rapid recruitment of β_2-arrestin to β_2-AR, which is required for enhanced activation kinetics. Once β_2-AR is stimulated the altered activation kinetics is independent of activation by G proteins. Thus, reported inconsistencies in lymphocyte β_2-AR-induced cAMP between RA and

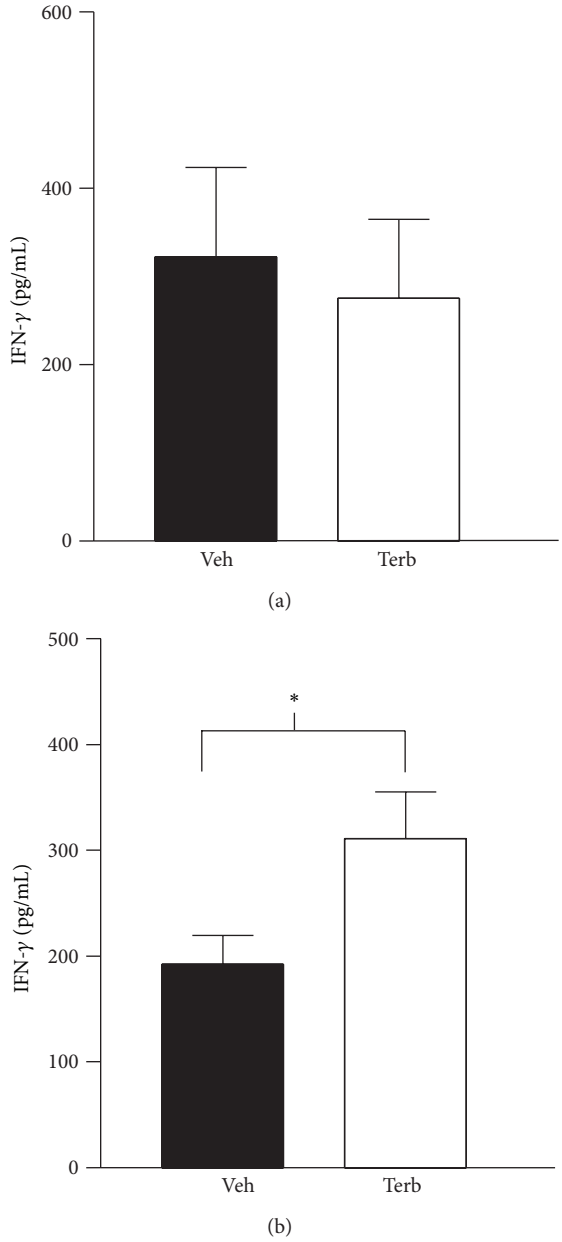

Figure 10: The effects of twice-daily treatment with terbutaline (Terb) (total of 1200 μg/day i.p.) or vehicle (Veh) in arthritic rats from D12–D28 post-CFA challenge on *ex vivo* IFN-γ secretion by splenocytes (a), and draining lymph node (DLN) cells (b). Terb had no effect on IFN-γ secretion by cultured spleen cells (a), but significantly increased ($^*P < 0.05$) IFN-γ production by DLN cells. Data are expressed as means ± SEM (n = 8/group) and analyzed using a Student's t-test.

healthy controls may result from differences in duration and repetitiveness of receptor stimulation.

It is not known whether different rodent strains also have variants in genes encoding β_2-AR. if they do, they could explain differences in strain susceptibility to induction of inflammatory arthritis. If so, these strains could provide models for understanding the contribution of β_2-AR polymorphisms to disease onset and severity in RA patients. An understanding of the physiological and clinical relevance of β_2-AR variants could substantially enhance our understanding of mechanisms for differences among experimental subjects and patients in their response to β_2-AR agonists reviewed in [47]. Collectively, our findings in AA and reported findings in RA suggest that complex site- and cell-specific changes in signal transduction and β_2-AR polymorphisms impart different catecholamine-induced effects on lymphocyte functions in RA.

Since the SNS dampens inflammation and cell-mediated responses, impaired β-AR signaling in splenocytes of AA rats is expected to promote disease processes. However, previous studies from our group [5] and others [6] have shown that β-AR agonists reduce disease severity if administered from disease onset through severe disease. Disruption of β-AR signaling in splenocytes after disease onset suggests that the attenuating effects of β-agonists on disease outcome are not due to regulation of splenocyte function via the β-AR-Gs-cAMP pathway. Alternatively, the attenuating effects of β-AR agonists could reflect signaling via cAMP in other secondary lymphoid organs and/or at the affected joints.

Loss of β-agonist-induced cAMP response and β_2-AR desensitization observed in this study is likely mediated, in part by, altered NE availability. While sympathetic activity is increased in RA (reviewed in [39]), no studies have directly assessed sympathetic nerve activity or NE turnover locally in lymphoid organs from animal models of RA; however, other data provide indirect support for this hypothesis. Loss and reorganization of sympathetic nerves in the spleen [48] are consistent with chronically high NE levels [40, 49] that subsequently auto-destroy NA nerves in target tissues [50]. Elevated plasma NE levels in AA rats at D1–5 or D10–21 after adjuvant challenge have been reported [49] and reflect spillover of NE released from sympathetic nerves in innervated tissues. Splenic and DLN NE concentrations in AA rats are reduced and elevated in chronic disease, respectively, supporting altered sympathetic nerve activity that is site specific [40]. Further, central and peripheral injection of proinflammatory cytokines, particularly, IL-1 and IL-6 (cytokines elevated in RA) increase SNS activity in the spleen [51–53].

Desensitization and downregulation of β-AR are dependent on pβ_2-AR by PKA and GRK, which occurs via site-specific phosphorylation at different serines/threonines [16–18]. Patterns of pβ_2-AR in splenocytes are disease specific. In AA rats, pβ_2-ARs at the PKA site are reduced on D21 and D28, while pβ_2-ARs at the GRK site are unchanged on D21 and elevated on D28 compared with each control group. pβ_2-AR by GRK2 uncouples β_2-AR from Gs protein and facilitates the binding of β-arrestin to the receptor causing receptor downregulation by internalization [21]. Thus, the observed changes in splenocyte pβ_2-AR by PKA and GRK provide a mechanism for β_2-AR downregulation and alternative second messenger signaling observed in splenocytes from AA rats.

Total β_2-AR protein and its pβ_2-AR patterns in splenocytes and DLN cells are strikingly different in AA rats. On D21 and D28, the β_2-AR$_T$ protein levels in DLN cells are similar in all treatment groups, indicating no effect of AA induction on β_2-AR expression. Unlike the spleen, pβ_2-AR$_{PKA}$ are

increased on D21 in DLN cells from CFA- and SMB-treated rats compared with the other control groups (MO and Saline). These findings suggest that the cell wall component of the adjuvant is responsible for increased $p\beta_2\text{-}AR_{PKA}$. By D28, $p\beta_2\text{-}AR_{PKA}$ site is reduced in CFA-, SMB-, and MO-treated rats compared with the Saline control group. In contrast, on D21 and D28, $p\beta_2\text{-}AR_{GRK}$ is greater in CFA- and SMB-treated rats than in rats treated with MO or Saline. These findings indicate a role for the mycobacterial cell wall component of CFA in driving $p\beta_2\text{-}AR_{GRK}$ and therefore implicate TLRs in regulating $p\beta_2\text{-}AR_{GRK}$, particularly TLR2 and TLR4 [54, 55]. Different GRKs (i.e., GRK2 versus GRK5/6) may be responsible for the disparate $p\beta_2\text{-}AR_{GRK}$ patterns in spleen and DLN cells [18, 27, 28]. $p\beta_2$-AR by GRK5/6 shifts signaling into the ERK 1/2 cascade in HEK-293 cells treated with high physiological concentration of β-agonists [15, 20, 26].

Proinflammatory cytokines elevated in inflammatory arthritis may also contribute to the altered $p\beta_2\text{-}AR_{GRK}$, as IFN-γ and IL-6 or TNF-α, IL-1, and IL-12 induce different expression of GRKs and $p\beta_2$-AR in PBMC from healthy controls [56–58]. Given that the SNS is crucial for maintaining homeostasis of the immune system, future research aimed at elucidating the mechanisms responsible for differential $p\beta_2$-AR by PKA and GRKs and their consequences for immune function in AA is required to understand disease-specific SNS-immune crossregulation in RA.

Of significance for RA, the ERK 1/2 signaling pathway increases IFN-γ production in AA CD4+ TH cells, cells that are prevalent in the arthritic joints and drive disease processes [59–61]. It is unclear whether stimulation of T cell β_2-AR can increase IFN-γ by activating ERK 1/2 under conditions of chronically elevated NE release. If so, then it would provide a mechanism to explain the increased IFN-γ production after β_2-AR stimulation in activated T cells [62], in splenic T cells shortly after onset of collagen-induced arthritis [7, 63] and DLN on day 28 after CFA-challenge in AA rats [41]. Many lymphocyte subsets are well known to express β_2-AR, including CD4+TH1, CD8+ T cells, NK cells and B cells. Recently, CD4+FoxP3+ T regulatory (Treg) cells have been demonstrated to express β_2-AR, and when stimulated, enhances their suppressive activity in a cAMP-PKA-dependent manner [64]. Further, the activation of β_2-ARs on Treg cells enhances the ability of Treg cells to convert from CD4+CD62L+FoxP3+ T cells to FoxP3+ inducible Treg (iTreg) cells, an effect mediated by cAMP-PKA pathways. These cells are essential for maintaining tolerance and regulating adaptive immune responses [64, 65]. A lack of Treg cells is linked to the promotion of autoimmune disease development in both mice and humans [66–70]. Consistent with a role for the SNS in regulating Treg cell functions and their importance in autoimmune diseases, chemical sympathectomy with 6-hydroxydopamine increases the number of these cells in the spleen and lymph nodes in experimental autoimmune encephalomyelitis, a model for multiple sclerosis, an effect associated with reduced disease severity [71]. Cytokine profiles in AA rats indicate multiple lymphocyte subtypes are likely to contribute to the β_2-AR changes observed in this study. Given that multiple T lymphocytes express β_2-AR and the importance of interactions between various T cell subtypes, it will be necessary to determine the T cell subtypes responsible for the altered β_2-AR expression, signaling and phosphorylation reported here. Future studies to understand the mechanism for altered β_2-AR signaling in different lymphoid compartments in specific immune cell subtypes in our model and in RA are required. An understanding of β_2-AR signaling changes that occur in RA also will be required before the potential to use β_2-AR agonists are realized for treatment of RA. Understanding the mechanisms for altered β_2-AR signaling may also lead to the improvement of current clinical use of β_2-AR agonists.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Detection of Antiphosphatidylserine/Prothrombin Antibodies and Their Potential Diagnostic Value

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Antiprothrombin antibodies, measured with phosphatidylserine/prothrombin complex (aPS/PT) ELISA, have been reported to be associated with antiphospholipid syndrome (APS). They are currently being evaluated as a potential classification criterion for this autoimmune disease, characterized by thromboses and obstetric complications. Given the present lack of clinically useful tests for the accurate diagnosis of APS, we aimed to evaluate *in-house* and commercial assays for determination of aPS/PT as a potential serological marker for APS. We screened 156 patients with systemic autoimmune diseases for antibodies against PS/PT, β_2-glycoprotein I, cardiolipin and for lupus anticoagulant activity. We demonstrated a high degree of concordance between the concentrations of aPS/PT measured with the *in-house* and commercial assays. Both assays performed comparably relating to the clinical manifestations of APS, such as arterial and venous thromboses and obstetric complications. IgG aPS/PT represented the strongest independent risk factor for the presence of obstetric complications, among all tested aPL. Both IgG and IgM aPS/PT were associated with venous thrombosis, but not with arterial thrombosis. Most importantly, the association between the presence of IgG/IgM aPS/PT and lupus anticoagulant activity was highly significant. Taken together, aPS/PT antibodies detected with the *in-house* or commercial ELISA represent a promising serological marker for APS and its subsets.

1. Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease identified by clinical manifestations of vascular thromboses and obstetric complications, together with the serology of persistently positive antiphospholipid antibodies (aPL) [1, 2]. aPL represent a heterogeneous group of immunoglobulins detected by coagulation tests, such as lupus anticoagulant activity (LA) or measured by an enzyme-linked immunosorbent assays (ELISAs) as anticardiolipin antibodies (aCL) or antibodies against β_2-glycoprotein I (anti-β_2GPI).

Antiprothrombin antibodies have not yet been included in the classification criteria of APS, although they are emerging as an increasingly important supportive marker. In recent years, their association with APS was evaluated with contradictory outcomes. Some studies failed to reveal a significant association of antiprothrombin antibodies with manifestations of APS [3–6], yet in other studies, their correlation to APS was found. The possibility of antiprothrombin antibodies becoming an additional serological classification criterion for APS emerged, especially relevant in APS patients negative for classical aPL [7–10].

Antibodies recognizing prothrombin can be detected by ELISA targeting prothrombin alone, coated onto irradiated plates (aPT), or targeting the phosphatidylserine/prothrombin complex (aPS/PT). It was demonstrated that antibodies recognized prothrombin more efficiently in aPS/PT ELISA [11] and that aPS/PT correlated better with APS and LA activity [7, 8, 12, 13] as compared to aPT. The inclusion of aPS/PT, but not aPT, to the laboratory criteria for APS has been proposed [14]. The first published aPS/PT protocol [7] was later modified in our previous study [10] in order to

increase the analytical sensitivity of the test. We have reported that our *in-house* aPS/PT ELISA was the most optimal method for the determination of all clinically relevant aPS/PT antibodies, exhibiting the highest percentage of LA activity, compared to aCL and anti-β_2GPI [10, 15]. We reported different avidity of antiprothrombin antibodies, as it is also known for several other autoimmune antibodies [16–18]. Moreover, we showed that the avidity was associated with their detection by different ELISAs.

Until recently, only some aPT commercial kits were available and they showed poor diagnostic sensitivity and specificity [6]. In 2010, the *commercial QUANTA Lite* aPS/PT IgG/IgM and LAC assays became available as an aid in the diagnosis of APS.

The lack of comparative analytical data between the various aPS/PT assays led the present investigation to compare our *in-house* aPS/PT ELISA with the commercial *QUANTA Lite* aPS/PT assay, in terms of diagnostic efficiency of aPS/PT. We aimed to determine whether the presence of aPS/PT antibodies was associated with specific clinical manifestation of APS and whether they could therefore become an additional serological marker of APS diagnosis. Additionally, our goal was to compare commercial kits enabling the detection of low avidity antiprothrombin antibodies, as was previously shown for our *in-house* aPS/PT ELISA [10].

2. Materials and Methods

2.1. Subjects. Sera from 156 of patients with systemic autoimmune diseases (34 males and 122 females, mean age 47 years, range 16–85) were analyzed in a cross-sectional study. APS, based on the revised International Consensus criteria [1], was diagnosed in 58 patients, APS associated with systemic lupus erythematosus (SLE) [19] in 38 patients. The control groups of patients were comprised of 24 patients with SLE, 25 patients with rheumatoid arthritis (RA) [20], and 11 Sjögren's syndrome patients (SS) [21]. Among all, 42 patients experienced an arterial event, 53 had a venous event, and 28 had obstetric complications (Table 1). The patients had their sera collected and analyzed when they were examined at the Department of Rheumatology (University Medical Centre, Ljubljana). This study was conducted as part of the National Research Program titled "Systemic Autoimmune Diseases" (number P3-0314). Participants signed an informed consent and the study was approved by the National Medical Ethics Committee, Ljubljana, Slovenia.

2.2. In-House aPS/PT ELISA. The levels of aPS/PT were detected according to the previously described aPS/PT ELISA protocol [10]. Medium binding plates (Costar, Cambridge, USA) were coated with phosphatidylserine in chloroform/methanol 1 : 4 and dried overnight at 4°C. Following blocking with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 5 mM $CaCl_2$ (1% BSA/TBS-Ca), 25 μL of human prothrombin (Enzyme Research Laboratories, Ltd., Swansea, UK) (20 mg/L) and 25 μL of patients' sera diluted 1 : 50 were applied to wells immediately one after the other and incubated for 1 h at room temperature. After that, alkaline phosphatase-conjugated goat anti-human IgG or IgM (ACSC, Westbury, USA) were applied in TBS/Tween (0.05% Tween) and incubated for 30 min. Following 4 washes in TBS/Tween, 100 μL/well of para-nitrophenylphosphate (Sigma Chemical Company, St. Louis, USA) in diethanolamine buffer (pH 9.8) was applied and OD_{405} was kinetically measured by a spectrometer (Tecan Sunrise Remote, Grödig, Austria).

2.3. INOVA QUANTA Lite aPS/PT ELISA. A semiquantitative ELISA for the individual detection of IgG and IgM aPS/PT was performed following the manufacturer's instruction (INOVA Diagnostics, CA, USA).

2.4. INOVA QUANTA Lite LAC ELISA. A semiquantitative ELISA for the detection of both IgG and IgM aPS/PT class antibodies was performed following the manufacturer's instructions (INOVA Diagnostics, CA, USA).

2.5. aCL ELISA and Anti-β_2GPI ELISA. IgG and IgM aCL were determined according to the previously described method [22, 23]. Anti-β_2GPI were measured with our *in-house* ELISA [24] and evaluated through the European forum for aPL [25].

2.6. Avidity Determination of IgG aPS/PT by Chaotropic aPS/PT ELISA. The chaotropic aPS/PT ELISA with increased concentrations of NaCl during the antibody binding phase was used for avidity determination [10, 15]. The presence of high avidity aPS/PT antibodies was identified when the binding of antibodies at 0.5 M NaCl remained higher than 70% of the initial binding at 0.136 M NaCl. Low avidity aPS/PT antibodies were declared when the binding decreased ≤30% of the initial binding. The remaining samples were considered to be of heterogeneous avidity.

2.7. Lupus Anticoagulant. The assay was performed in blood samples collected in tubes containing 0.109 M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2400 g for 20 min at 4°C. After filtration, aliquots were stored at −80°C until use. Clotting tests were performed using coagulation analyzer BCS Siemens according to the previous guidelines of the International Society on Thrombosis and Haemostasis ISTH [26]. Simplified Dilute Russell's Viper Venom Test (dRVVT) was performed using LA1 Screening reagent and LA2 Confirmatory reagent (Siemens) following the manufacturer's instructions [27]. A dRVVT ratio (LA1 screen/LA2 confirmation) above 1.2 was considered positive for LA activity. Activity of LA was quantified as follows: low positive (LA1/LA2 = 1.2–1.5), medium (LA1/LA2 = 1.5–2.0), and high positive (LA1/LA2 > 2.0).

2.8. Statistical Analysis. Statistical analysis was performed using the SPSS 15.0 program. Normal distribution was evaluated using descriptive statistic parameters, curve fittings, and Kolmogorov-Smirnov test. The Receiver Operating Characteristic (ROC) analysis and the area under the curve (AUC)

Table 1: Prevalence of arterial thrombosis, venous thrombosis, and obstetric complications in the groups of selected autoimmune patients.

	No. (f/m)	Arterial thrombosis (41)	Venous thrombosis (53)	Obstetric complications (28)	Total
APS	**58** (34/24)	**21** (13/8)	**33** (16/17)	**12**	**97**
APS + SLE	**38** (31/7)	**16** (14/2)	**17** (11/6)	**14**	
SLE	**24** (24/0)	**4** (4/0)	**1** (1/0)	**1**	**60**
RA	**25** (22/3)	**0**	**2** (2/0)	**0**	
SS	**11** (11/0)	**0**	**0**	**1**	

No.: number of patients, f/m: female/male, APS: antiphospholipid syndrome, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, and SS: Sjögren's syndrome.

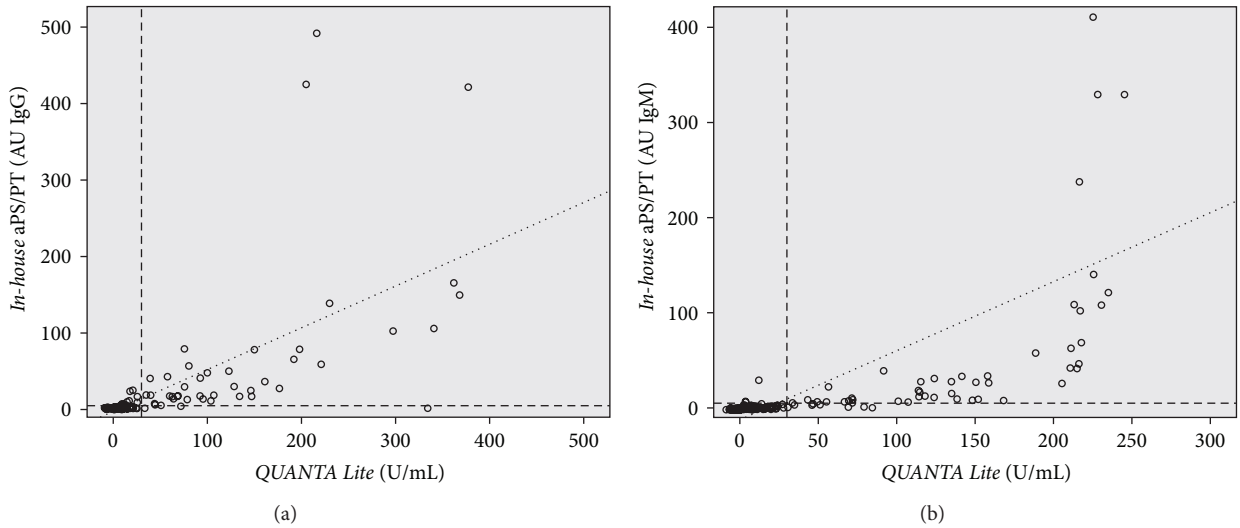

Figure 1: aPS/PT antibodies detected with the *in-house* ELISA correlated significantly with results of *QUANTA Lite* IgG (a) and IgM (b) in 156 patient sera. The dashed lines represent the cut-off value (*in-house* ELISA 5 AU, *QUANTA Lite* 30 U/mL). AU: arbitrary units.

were used to assess the diagnostic performance of the measured marker(s). The results of multivariate logistic models were approximated by odds ratio with its 95% confidence interval (OR (95%)). A 2-sided P value < 0.05 was considered statistically significant.

3. Results

3.1. Correlation between In-House and QUANTA Lite aPS/PT Assays. Both IgG and IgM aPS/PT antibodies detected with *in-house* ELISA correlated significantly with results of *QUANTA Lite* immunoassays using Spearman correlation (rho = 0.744 for IgG; rho = 0.865 for IgM) (Figures 1(a) and 1(b)) in 156 patient sera. Substantial concordance was validated also with Lin's concordance correlation coefficient (Rc = 0.625 for IgG and Rc = 0.572 for IgM), which is a reproducibility measure.

3.2. Diagnostic Applicability Comparison of Different Antiphospholipid Antibody Assays. We evaluated APS diagnostic applicability of all assays with a receiver operating characteristic curve (ROC curve) and estimated the area under the curve (Figure 2). The highest diagnostic efficiency for APS was achieved by aCL IgG (AUC = 0.88) (Figure 2(a)). Both, the *in-house* and the *QUANTA Lite, IgG aPS/PT* methods were comparable (0.73 and 0.72, resp.). All methods detecting IgM aPL (Figure 2(b)) showed a lower overall performance compared to IgG aCL.

3.3. Relationship of aPL with Thrombosis and Obstetric Manifestations. The positivity of an individual aPL test and clinical manifestations of APS were considered in a logistic regression analysis (Table 2). IgG and IgM aPS/PT measured with the *in-house* and *QUANTA Lite* ELISA presented the highest independent risk factor for obstetric complication, among all tested aPL (OR = 9.3 and OR = 6.3, resp., for IgG and IgM). Both IgG and IgM aPS/PT measured with either assay were an independent risk factor for the presence of venous thrombosis. However, the highest risk for venous thrombosis was achieved by LA (OR = 5.6). IgG aPS/PT measured with *QUANTA Lite* ELISA were also an independent risk factor for the presence of arterial thrombosis, but the association was rather weak (OR = 2.3, $P = 0.03$).

The *QUANTA Lite* LAC screen test detected all sera positive in the individual IgG or IgM aPS/PT assays. The LAC screen did not achieve the diagnostic efficiency of the established LA coagulation test (Table 2).

3.4. Relationship between LA and aCL, Anti-β_2GPI, or aPS/PT Antibodies. Out of 156 patients included in the study, 16

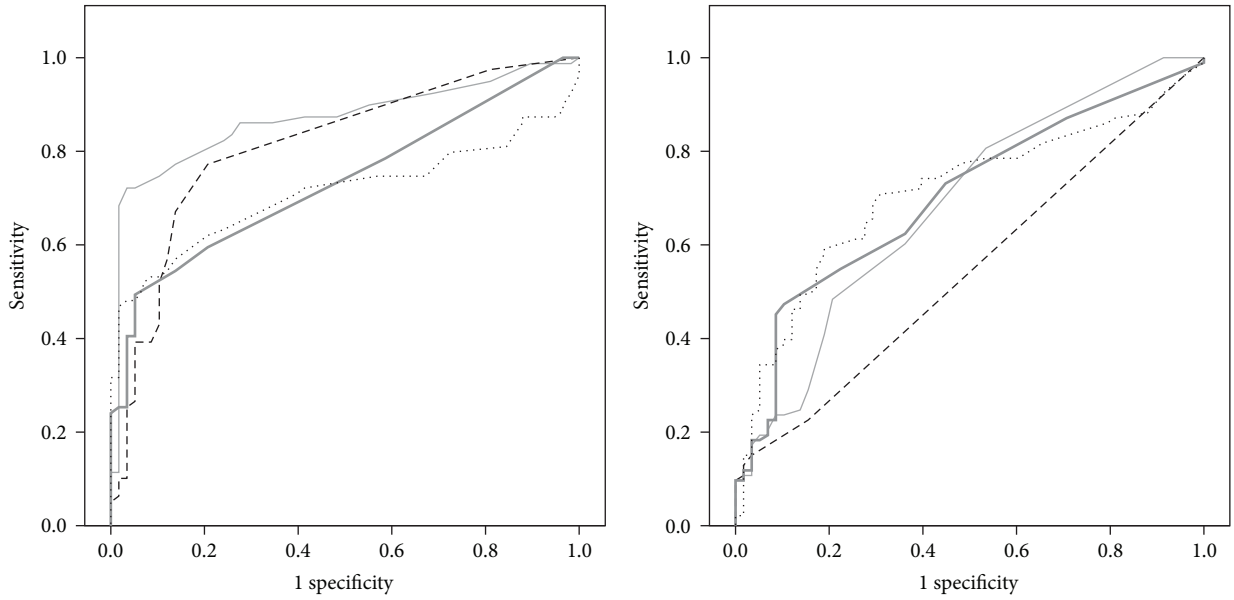

Figure 2: Receiver Operating Characteristic (ROC) curves and area under the curve (AUC) of different antiphospholipid antibody methods for APS ($n = 156$). The higher values of AUC indicate better diagnostic efficiency of the test. aCL: anticardiolipin, anti-β_2GPI: anti-$\beta_2$glycoprotein, and aPS/PT antiphosphatidylserine/prothrombin.

(10%) did not have their LA activity determined due to their anticoagulant treatment. Seven patients (3 APS, 3 RA, and 1 SS) were solely positive for LA, three of them had low, and four medium LA activity. Among all the aCL positive patients, 51% had LA; among the anti-β_2GPI positive patients, 55% had LA, while among the aPS/PT positive patients, 66% had LA activity. aPS/PT, measured with either *in-house* or commercial assay, were much higher independent risk factors for the presence of LA activity (OR > 15.3 for IgG and OR > 12.9 for IgM) as compared to either aCL or anti-β_2GPI (OR < 9.0 for IgG and OR < 4.6 for IgM, resp.) (Table 3).

3.5. Avidity of aPS/PT. Avidity of IgG aPS/PT was determined using a chaotropic IgG aPS/PT ELISA in aPS/PT positive patients detected by the *in-house* IgG aPS/PT ELISA, regardless of the antibody level. Antibodies were detected of predominantly low, heterogeneous, and predominantly high avidity ($n = 9, 33, 9$ out of 51, resp.). Both the *QUANTA Lite* IgG aPS/PT ELISA and LAC screen assays detected more than 40% of sera with low avidity antibodies and more than 85% of those with heterogeneous or high avidity aPS/PT (Table 4).

Among nine patients with low avidity aPS/PT, seven were diagnosed with APS; three of which experienced arterial thrombosis, five venous thrombosis, and one had obstetric complications. Two out of 97 APS patients included in the study were positive in the *QUANTA Lite* IgG aPS/PT ELISA, but negative in the *in-house* aPS/PT ELISA and their avidity was not determined.

4. Discussion

A comprehensive comparative study of anti-prothrombin antibodies (on two *in-house* and three commercial aPT tests) conducted in 2007 by Tincani et al. reported issues with reproducibility and interpretation of results and advised against their routine use [6]. Antibodies against PS/PT were first described by Matsuda et al. in patients with LA in 1996 [28], while one year later, Galli et al. [11] reported that the aPS/PT assay was more sensitive than the aPT test. In 2000, Atsumi et al. pointed out that aPS/PT can be used not only to confirm the presence of LA, but also to serve (in addition to aCL and anti-β_2GPI) as one of the markers of APS and also thrombotic events in patients with autoimmune diseases [7]. Since 2000, aPS/PT antibody detection stood the test of time and was proven as a useful tool for the diagnosis of APS [29]. Comparative studies gave additional indication of their diagnostic relevance and confirmed their closer correlation with APS and LA activity as compared to aPT [7, 8, 13, 30]. The protocol by Matsuda et al. [12] used a higher concentration of phosphatidylserine (65 μg/mL) and prothrombin (20 μg/mL), while the protocols by Atsumi et al. [7], Tincani et al. [6], and Žigon et al.

Table 2: Antiphospholipid antibodies and LA in a relationship to arterial thrombosis (AT), venous thrombosis (VT), and obstetric complications (OC).

Antibody		No.	*P* value	Odds ratio (95% Cl)	Sensitivity %	Specificity %
				Arterial thrombosis (41)		
LA		17	0.43	1.4 (0.6–2.9)	49	59
aCL	IgG	34	<0.001	**5.5 (2.3–13.4)**	83	53
	IgM	6	0.67	1.2 (0.4–3.5)	15	88
Anti-β_2GPI	IgG	26	0.02	2.4 (1.2–5.1)	63	58
	IgM	5	0.99	1.0 (0.3–2.9)	12	88
In-house aPS/PT	IgG	18	0.08	1.9 (0.9–4.1)	44	71
	IgM	16	0.36	1.4 (0.6–2.9)	39	69
QUANTA Lite aPS/PT	IgG	17	0.03	2.3 (1.0–4.9)	41	77
	IgM	17	0.38	1.4 (0.7–2.9)	41	66
	LAC	26	0.50	1.3 (0.6–2.7)	62	43
				Venous thrombosis (53)		
LA		31	<0.001	**5.6 (2.6–12.2)**	70	70
aCL	IgG	39	<0.005	3.0 (1.5–6.2)	74	52
	IgM	12	0.010	3.5 (1.3–9.2)	23	92
Anti-β_2GPI	IgG	35	<0.001	3.2 (1.6–6.5)	66	63
	IgM	10	0.070	2.5 (0.9–6.5)	19	91
In-house aPS/PT	IgG	27	<0.001	3.5 (1.7–7.0)	51	77
	IgM	24	0.021	2.2 (1.1–4.5)	45	73
QUANTA Lite aPS/PT	IgG	25	<0.001	4.0 (1.9–8.3)	47	82
	IgM	26	0.013	2.4 (1.2–4.7)	49	71
	LAC	37	0.043	2.1 (1.0–4.2)	70	47
				Obstetric complications (28)		
LA		13	<0.005	4.3 (1.6–11.9)	62	73
aCL	IgG	22	<0.001	5.8 (2.1–15.9)	79	61
	IgM	6	0.130	2.5 (0.8–7.8)	21	90
Anti-β_2GPI	IgG	19	0.002	4.1 (1.7–10.4)	68	66
	IgM	5	0.278	2.0 (0.6–6.6)	18	90
In-house aPS/PT	IgG	18	<0.001	**9.3 (3.5–24.6)**	64	84
	IgM	15	<0.005	4.0 (1.6–9.9)	54	78
QUANTA Lite aPS/PT	IgG	14	<0.001	6.3 (2.4–16.7)	50	86
	IgM	16	<0.005	4.3 (1.7–10.6)	57	76
	LAC	21	0.042	2.7 (1.0–9.1)	75	48

aCL: anticardiolipin, anti-β_2GPI: anti-$\beta_2$glycoprotein, aPS/PT: anti-phosphatidylserine/prothrombin, LA: lupus anticoagulant, OR: odds ratio, and CI: confidence interval.

[10] all used lower concentrations of phosphatidylserine (50 μg/mL) and prothrombin (10 μg/mL). Additional major modifications between Matsuda et al. and later protocols are different times and temperatures of phosphatidylserine and prothrombin incubation. Recently, we have reported that our modified *in-house* aPS/PT ELISA (with increased analytical sensitivity) detects both presumably different populations of antibodies and low avidity antibodies, as well as it enables the identification of patients negative for other antiphospholipid antibodies [10]. The modification (by means of the concomitant antigen and antibody incubation) resulted in increased prothrombin concentration on phospholipid surface and possible exposure of additional epitopes on prothrombin, enabling a higher intensity of antiprothrombin antibody binding.

The current report shows that aPS/PT antibodies were the strongest independent risk factor for obstetric complications in our population of patients. Two previous studies on females with obstetric disorders found that aPS/PT antibodies were not frequent in patients with unexplained recurrent miscarriages without APS [5, 31]. However, in a recent study, comprising 163 women negative for the classical repertoire of aPL, antiprothrombin antibodies appeared to be associated with previous adverse pregnancy outcome [32]; however, the author did not support the potential use of these antibodies in clinical practice. In our study, among 28 female patients (26 were diagnosed with APS), IgG aPS/PT antibodies (measured by either *in-house* or commercial assay) showed the strongest correlation with obstetric complications, among all aPL antibodies. Further studies are warranted on a larger population

Table 3: Association between the presence of antiphospholipid antibodies and LA activity.

Antibody		Lupus anticoagulant activity	
		P value	Odds ratio (95% Cl)
aCL	IgG	<0.001	5.0 (2.4–10.6)
	IgM	<0.001	4.6 (1.6–13.7)
Anti-β_2GPI	IgG	<0.001	9.0 (4.2–19.4)
	IgM	<0.001	1.2 (1.0–1.4)
In-house aPS/PT	IgG	<0.001	**21.6 (8.1–57.7)**
	IgM	<0.001	12.9 (5.4–30.6)
QUANTA Lite aPS/PT	IgG	<0.001	15.3 (5.8–40.5)
	IgM	<0.001	13.2 (5.6–30.8)
	LAC	<0.001	10.2 (4.4–23.6)

aCL: anticardiolipin, anti-β_2GPI: anti-$\beta_2$glycoprotein, and aPS/PT: antiphosphatidylserine/prothrombin.

Table 4: Association of aPS/PT avidity with clinical features of autoimmune patients.

aPS/PT avidity	Low ($n = 9$)	Heterogeneous ($n = 33$)	High ($n = 9$)
APS	7 (77%)	32 (97%)	9 (100%)
SLE	2	1	0
RA	0	0	0
SS	0	0	0
Thrombosis	8	26	8
Arterial	3	11	4
Venous	5	10	5
Obstetric disorder	1	14	4
aPS/PT positivity			
In-house IgG aPS/PT	9	33	9
QUANTA Lite IgG aPS/PT	4	29	8
QUANTA Lite LAC	7	32	9

APS: antiphospholipid syndrome, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, SS: Sjögren's syndrome, and aPS/PT: antiphosphatidylserine/prothrombin antibodies.

of obstetric patients. Taking into account the logistic regression results, IgG/IgM aPS/PT were also independent risk factors for the presence of venous thrombosis (OR = 3.5 and OR = 2.2, resp.). The highest OR for venous thrombosis is presented by LA (OR = 5.6), while all of the measured aPL showed statistically significantly correlation. On the other hand, aPS/PT antibodies were not strong independent risk factor for arterial thrombosis. Similarly, Vlagea et al. found no association between the presence of aPS/PT and arterial thrombosis [33]. Two previous studies [7, 8] have shown anti-prothrombin antibodies as an independent risk factor for arterial thrombosis, but other reports [6, 9, 34] presented their data without differentiating between arterial or venous thromboembolic events. In general, all IgM antibody subtypes of aPL demonstrated a lower diagnostic efficiency for thrombosis as compared to IgG aPL. These data are in line with the results of a systematic review [35] and a later study [36] which reported IgM aCL, anti-β_2GPI, and anti-prothrombin antibodies to be less often associated with clinical events of APS than IgG.

Correlation between aPS/PT and LA activity has been reported previously [7, 13, 33, 37] and our current study confirmed a strong correlation between aPS/PT and LA activity. IgG/IgM aPS/PT were the highest independent risk factors for LA activity with an OR > 12.9 as compared to aCL and anti-β_2GPI with an OR < 9.0. Despite internationally accepted guidelines and many efforts to improve the standardization of LA activity assays, accurate detection and intralaboratory reproducibility are still not fully achieved. LA determination is a sequential series of analyses, which requires careful treatment of plasma specimens obtained from patients who are not receiving any anticoagulant therapy. So, the aPS/PT assay could represent a solid additional test performed using sera samples of patients regardless of anticoagulant therapy.

Very few studies have reported on the avidity of anti-prothrombin antibodies. Avidity was shown to importantly influence positivity in different antiprothrombin ELISAs given that none of the low avidity antibodies were positive in the aPT ELISA [15]. On the contrary, aPS/PT assay enables the detection of low avidity antibodies, as evidenced in the current report. Our *in-house* IgG aPS/PT detected 9 positive patients with low avidity; however, *QUANTA Lite* IgG aPS/PT assay detected only 40% of low avidity anti-prothrombin antibodies. (Table 4). Our group has previously shown that avidity of anti-β_2GPI importantly correlated with the clinical onset; therefore, it appeared reasonable to assume the same for the avidity of antiprothrombin antibodies [16]. However, we could not draw the same conclusion, but found that out of nine patients with low avidity aPS/PT antibodies, seven had APS. Therefore, a method enabling the detection of low avidity aPS/PT is also essential for possible inclusion of aPS/PT in the classification criteria for APS.

In conclusion, the present study is in line with the recommendations advising confirmation of previous data for "noncriteria aPL," such as aPS/PT [38]. The only commercially available aPS/PT assay was evaluated in comparison to our *in-house* aPS/PT. aPS/PT detected with either *in-house* aPS/PT ELISA or with *QUANTA Lite* aPS/PT ELISA showed very high specificity for APS that could serve as an additional serological diagnostic marker for venous thrombosis and obstetric complications. The association of aPS/PT with LA activity was the highest among all aPL tested and therefore can be a useful feature of these antibodies. In summary, aPS/PT, measured with either *in-house* or commercial assay, in addition to aCL and anti-β_2GPI antibodies, could represent an additional marker in patients with clinical manifestations of APS.

Abbreviations

Anti-β_2GPI: Antibodies against β_2-glycoprotein I
aCL: Anti-cardiolipin antibodies
APS: Antiphospholipid syndrome
aPS/PT: Phosphatidylserine-dependent anti-prothrombin antibodies
aPT: Antibodies against prothrombin alone

AUC: Area under the curve
CI: Confidence interval,
LA: Lupus anticoagulant
OR: Odds ratio
PS: Phosphatidylserine
RA: Rheumatoid arthritis
ROC: Receiver operating characteristic
SLE: Systemic lupus erythematosus
SS: Sjögren's syndrome.

Conflict of Interests

The authors state that there is no conflict of interests.

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Clinical Study

A Study on Clinical and Pathologic Features in Lupus Nephritis with Mainly IgA Deposits and a Literature Review

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Objective. To study the clinical and pathologic features of systemic lupus erythematosus (SLE) that has atypical lupus nephritis (LN) with mainly IgA deposits. *Methods*. We searched the SLE patients who had nephritis with mainly IgA deposits in our hospital and selected the information including clinical manifestations, laboratory tests, treatments, and prognosis. *Results*. From January 2009 to June 2012, 5 patients were definitely diagnosed as SLE according to both 1982 and 2009 ACR classification criteria. But renal biopsy showed that all cases had mainly IgA deposits and were free of IgG, C1q, and fibrinogen-related antigen deposits under immunofluorescent microscopy, which did not match with typical LN. There were 2 males and 3 females, aging from 31 to 64 years and with an average of (42.20 ± 13.59) years. The 5 cases had multiple-system involvements, mainly the renal system. Compared to primary IgAN, the atypical LN showed some differences: older than primary IgAN, more women than men, no previous infection history, lower incidence of serum IgA elevation, and ACL positive rate as high as 100%. *Conclusion*. Nephritis with mainly IgAN deposits, as an atypical LN, may be a special subtype of SLE.

1. Introduction

In clinic, LN is divided into six types, which are minimal mesangial LN (class I), mesangial proliferative LN (class II), focal proliferative LN (class III), diffuse proliferative LN (class IV), membranous LN (class V), and advanced sclerosing LN (class VI) [1]. The previous typical LN are characterized by the so-called "Full House" stain under immunofluorescent microscopy, staining positively for IgG, IgA, IgM, C3, and C1q [2]. Besides the typical changes, some SLE patients were reported to have IgAN established by renal biopsy [3–8], staining positively for mainly IgA. Here, we report 5 cases of SLE patients who had nephritis with mainly IgA deposits.

2. Information and Methods

2.1. Patients and Information Collecting. Select the SLE patients who had nephritis with IgA-predominant deposits in our hospital from January 2009 to June 2012. The information collected included sex, age, duration, clinical and pathological manifestations, laboratory tests, treatments, and prognosis. The detailed tests were as follows. (1) Blood routine: blood leukocyte, platelet, and hemoglobin. (2) Urine routine: urine leukocyte, erythrocyte, cast. (3) 24-hour urine protein and bacteria culture of clean midstream urine. (4) Auto-antibodies: antinuclear antibody (ANA), anti-Sm antibody, anti-dsDNA antibody, anticardiolipin antibody (ACL), and anti-β2-glycoprotein-1 antibody (anti-β2-GP-1). (5) Renal biopsies examinations: HE staining, MASSON staining, PAS staining, PASM staining, and immunofluorescent staining. (6) Others: serum IgG, IgA, IgM, C3, and C4 levels and SLEDAI scores for disease activity assessment.

2.2. Main Reagents and Detection Methods. ANA and anti-dsDNA were both detected by indirect immunofluorescence assay, and the reagents were from German EU. Both anti-β2-GP-1 and ACL were tested by way of ELISA, and the ELISA kits were from German Human and German Orgenpec, respectively. Anti-Sm antibody was detected by linear immunoassay, and the reagent was from Germany Human.

Serums IgA, IgG, and IgM, complements C3 and C4 were all tested by rate nephelometry, and the reagents were from the American Beckman.

2.3. Diagnostic Criteria. SLE was diagnosed according to both 1982 and 2009 ACR classification criteria [9, 10].

2.4. Renal Biopsy Examination. The histological changes of renal biopsies were observed by HE staining, MASSON staining, PAS staining, and PASM staining. Use the method of direct immunofluorescence to detect accumulation of IgA, IgG, IgM, complement C3, C1q, and fibrinogen-related antigen (FRA) in renal tissues, and determine the fluorescence intensity under a fluorescence microscope: "–" indicated no or weak fluorescence; "+" indicated only clearly visible fluorescence; "++" indicated bright fluorescence; "+++" indicated dazzling fluorescent.

3. Results

3.1. General Information. From January 2009 to June 2012, 5 SLE patients were established to have mainly IgA deposits by renal biopsy. In the 5 cases, 2 cases were males, and 3 were females (M/F ratio 2 : 3). The age ranged from 31 to 64 years, with an average of (42.20 ± 13.59) years. The duration lasted from 1.0 to 108 months, with an average of (29.40 ± 44.91) months.

3.2. Clinical Manifestations and Laboratory Tests. In clinic manifestations, the 5 cases had multiple-system involvements, mainly renal system (5/5), manifesting in hematuria (gross hematuria 2 cases, microhematuria 3 cases), proteinuria (5/5), pyuria (2/5), cylindruria (4/5), renal dysfunction (2/5), and edema (2/5). Besides, the patients also presented hematologic involvement (3/5), serositis (2/5), and joint synovitis (1/5). In laboratory tests, anti-ANA was positive in all cases (5/5), anti-dsDNA in 3 cases (3/5), anti-Sm in 2 cases (2/5), anti-β2-GP-1 in 1 case (1/5), and ACL in all cases (5/5). Besides, complement C3 or C4 decreased in 4 cases (4/5), immunoglobin increased in 2 cases (2/5), and IgA increased in only 1 case (1/5).

In diagnosis, all cases fulfilled both 1982 and 2009 ACR classification criteria for SLE. According to 1982 criteria for SLE, cases 1 and 5 satisfied five of the criteria, and the rest cases satisfied four. According to 2009 criteria for SLE, case 1, 2, and 4 satisfied six of the criteria, case 3 satisfied five, and case 5 satisfied eight of the criteria, including at least 2 clinical criteria and 3 immunologic criteria. SLEDAI score ranged from 12 to 25, with an average of (19.2 ± 5.12). The details were as follows.

Case 1 showed gross hematuria, edema of lower limbs, pericardial effusion, and pleural effusion under ultrasound examination. The patient had no fever, cutaneous lupus, photosensitivity, oral/nasal ulcers, alopecia, inflammatory synovitis, or neurologic symptoms. Electroencephalogram was normal. Blood routine showed anemia (hemoglobin 96 g/L, normal range is 113~151 g/L) and thrombocytopenia (63×10^9/L, normal $101\sim320 \times 10^9$/L). Urine test showed hematuria, pyuria, cylindruria, and proteinuria (24-hour urine protein 2393 mg, normal 50~150 mg). Renal function was normal. ANA was positive, with the titer S1:320. ACL IgM was positive, while anti-Sm, anti-dsDNA, anti-β2-GP-1, ACL IgA, and IgG were negative. Complement C3 decreased (67.70 mg/dL, normal range is 79.00~152.00 mg/dL), while C4 was normal. SLEDAI score was 23.

Case 2 had no fever, cutaneous lupus, photosensitivity, oral/nasal ulcers, alopecia, serous cavity effusion, inflammatory synovitis, or neurologic symptoms. Blood routine showed anemia (hemoglobin 98 g/L) and thrombocytopenia (81×10^9/L). Urine test showed hematuria, cylindruria, and proteinuria (24-hour urine protein 2497 mg), without pyuria. Renal function was abnormal (serum creatinine was 163.00 umol/L, normal range 53.00~115.00 umol/L; serum urea nitrogen 11.10 mmol/L, normal range 2.85~7.14 mmol/L). ANA titer was S1:320, and anti-dsDNA titer was 1 : 10. ACL IgM, and IgG were positive, while anti-Sm, anti-β2GP-1, and ACL IgA were negative. Both C3 and C4 decreased (resp., 77.40 mg/dL and 11.00 mg/dL; normal range of C4 is 12.00~36.00 mg/dL). SLEDAI score was 17.

Case 3 had repeated oral ulcers but no fever, cutaneous lupus, photosensitivity, alopecia, serous cavity effusion, inflammatory synovitis, or neurologic symptoms. Urine test showed hematuria and proteinuria (24-hour urine protein 651 mg), without pyuria or cylindruria. Blood routine, renal function, C3, and C4 were all normal. ANA titer was S1:320, and anti-dsDNA titer was 1 : 10. Anti-β2GP-1, ACL IgM, and IgA were positive, while anti-Sm and ACL IgG were negative. SLEDAI score was 12.

Case 4 had hands Raynaud's phenomenon and edema of lower limbs, without fever, cutaneous lupus, photosensitivity, oral/nasal ulcers, alopecia, serous cavity effusion, inflammatory synovitis, or neurologic symptoms. Blood routine showed leukopenia (white blood cell 2.72×10^9/L, normal $3.69\sim9.16 \times 10^9$/L). Urine test showed hematuria, pyuria, cylindruria, and proteinuria (24-hour urine protein 3619 mg). Renal function was abnormal (serum creatinine 221.70 umol/L; serum urea nitrogen 28.78 mmol/L). ANA was positive, with a titer of S1:3200. Anti-Sm and ACL IgM were positive, while anti-dsDNA, anti-β2GP-1, ACL IgA, and IgG were negative. C3 decreased (53.10 mg/dL), while C4 was normal. SLEDAI score was 19.

Case 5 presented fever, arthritis, pericardial effusion, and pleural effusion under lung HRCT. He had no infection, cutaneous lupus, photosensitivity, oral/nasal ulcers, alopecia, or neurologic symptoms. Blood routine and renal function were normal. Urine test showed hematuria, cylindruria, and proteinuria (24-hour urine protein 4692 mg), without pyuria. ANA titer was S1:3200, and anti-dsDNA titer was 1 : 1000. Anti-Sm and ACL IgG were positive, while anti-β2GP-1, ACL IgA, and IgM were negative. Both C3 and C4 decreased (resp., 37.40 mg/dL and 4.50 mg/dL). SLEDAI score was 25.

Besides, all the cases had no allergic purpura, gastrointestinal, or urinary tract irritation symptoms. Bacteria cultures of clean midstream urine in all patients were negative. 3 of the 5 patients had hypertension (3/5), and 1 case had slightly abnormal coagulation (1/5; prothrombin time was 9.1 seconds, while normal value is 9.6 to 13.0 seconds; activated partial prothrombin time was 16.2 seconds, while normal

Table 1: Laboratory test results of SLE that has nephritis with mainly IgA deposits.

Case	HGB (g/L)	WBC ($\times 10^9$/L)	PLT ($\times 10^9$/L)	ANA	dsDNA	Sm	C3 (mg/dL)	C4 (mg/dL)	Anti-β2 GP-1	ACL	SLEDAI score
1	96▲	5.22	63▲	1 : 320	–	–	67.70▲	16.30	–	IgM(+)	23
2	98▲	6.41	81▲	1 : 320	1 : 10	–	77.40▲	11.00▲	–	IgM, G(+)	17
3	124	5.35	279	1 : 320	1 : 10	–	104.00	14.70	+	IgM, A(+)	10
4	145	2.72▲	237	1 : 3200	–	+	53.10▲	13.10	–	IgM(+)	19
5	124	9.82	214	1 : 3200	1 : 1000	+	37.40▲	4.50▲	–	IgG(+)	25

▲Indicates lower than normal; + indicates positive; – indicates negative. Normal range: (hemoglobin) HGB 113~151 g/L; (white blood cells) WBC 3.69~9.16 $\times$ 10^9/L; (platelet) PLT 101~320 $\times$ 10^9/L; C3 79~152 mg/dL; C4 12.0~36 mg/dL.

Table 2: Renal involvement of SLE that has nephritis with mainly IgA deposits.

Case	Sex age	Red cells (per HPF)	White cells (per HPF)	Casts (per HPF)	Protein (g/24 h)	Serum creatinine (umol/L)	Serum urea nitrogen (mmol/L)
1	F/64	All view	All view	8–10 granular	2393▲	74.60	4.49
2	M/45	All view	—	1-2 granular	2497▲	163.00▲	11.10▲
3	F/31	30–35	—	—	651▲	60.60	4.00
4	F/31	24–26	20-21	1–3 granular	3619▲	221.70▲	28.78▲
5	M/40	15–20	1-2	1–3 granular	4692▲	86.10	5.22

▲Indicates higher than normal range. Red cells, white cells, cast, and protein were all tested in urine. Normal range: urine protein (g/24 h): 50~150 mg; serum creatinine 53~115 umol/L; serum urea nitrogen 2.85~7.14 mmol/L.

value is 21 to 34 seconds; thrombin time was normal). Serum immunoglobulin was abnormal in 2 cases (2/5), and IgA increased in only 1 case (1/5). In case 1, IgG was 2340 mg/dL (normal range was 751~1560 mg/dL), IgA 1050 mg/dL (normal range was 45~382 mg/dL), and IgM 349 mg/dL (normal range was 46 to 304 mg/dL). In case 4, only IgG slightly increased (1690 mg/dL), while IgA and IgM were normal.

Most of the previous laboratory results were listed in Tables 1 and 2.

3.3. Renal Biopsy Findings. All cases performed light and immunofluorescent microscopy. The result showed that all cases had mainly IgA deposits and did not match with LN. Under light microscope (Figure 1), all cases showed mild diffuse hyperplasia of glomerular mesangium and matrix, with focal and segmental aggravation. The renal tubular epithelial cell showed vacuolar degeneration, granular degeneration, and spotty or flake atrophy, while the renal interstitial showed fibrosis and infiltration of lymphocytes and monocytes. In case 2, glomerular sclerosis can be clearly seen. Immune complex deposits were seen in glomerular mesangium under immunofluorescent microscope. All cases had IgA deposits (Figure 1) and were free of IgG, C1q, and FRA deposits. In addition, as well as IgA deposit, 1 case had C3 deposit, and the other 4 cases had IgM and C3 deposits.

3.4. Treatment and Prognosis. All cases were given prednisone at a dose of 1 mg/(kg·d) after percutaneous renopuncture, and cases 1, 2, and 5 also received intravenous cyclophosphamide treatment. All the cases achieved remission after therapy, for example, clinical symptoms got relief (such as arthritis, edema, orrhomeningitis, Raynaud's phenomenon, and oral ulcers), blood routine, urine tests, and immunological tests improved, including reduction of protein, red blood cells, white blood cells, and casts in urine, decrease of SLEDAI score, as well as increase of white blood cells, platelet, C3, and C4.

4. Discussion

4.1. Diagnosis of SLE. In 1982 ACR classification criteria for SLE, if the patient satisfies four or more than four of the criteria, we can classify the patient as having SLE. According to that, cases 1 and 5 satisfied five of the criteria, and the rest cases satisfied four. So they can be definitely diagnosed as SLE. In 2009 ACR classification criteria for SLE, if (1) the patient has biopsy-proven LN with ANA or anti-dsDNA or (2) the patient satisfied four of the criteria, including at least one clinical and one immunologic criterion, we classify the patient as having SLE. The 5 patients in our study were in the second case. They satisfied 5 to 8 criteria, including at least 2 clinical criteria and 3 immunologic criteria. Even if we exclude renal injury, the patients still satisfied 4 to 7 criteria and can be diagnosed as SLE. So, whichever criteria we choose or whether we include renal injury, the five cases can be diagnosed as SLE.

Typical LN are characterized by "Full House" stain under immunofluorescent microscopy, staining positively for IgG, IgA, IgM, C3, and C1q. However, the five SLE patients showed mainly IgA deposits and free of IgG and C1q deposits, which did not match with typical LN. It is unusual in a clinic for SLE patients to have nephritis with mainly IgA deposits, so we made a review to get a further understanding of the problem.

4.2. Relationship between SLE and Nephritis with Mainly IgA Deposits. In the recent 3.5 years, as many as 5 SLE

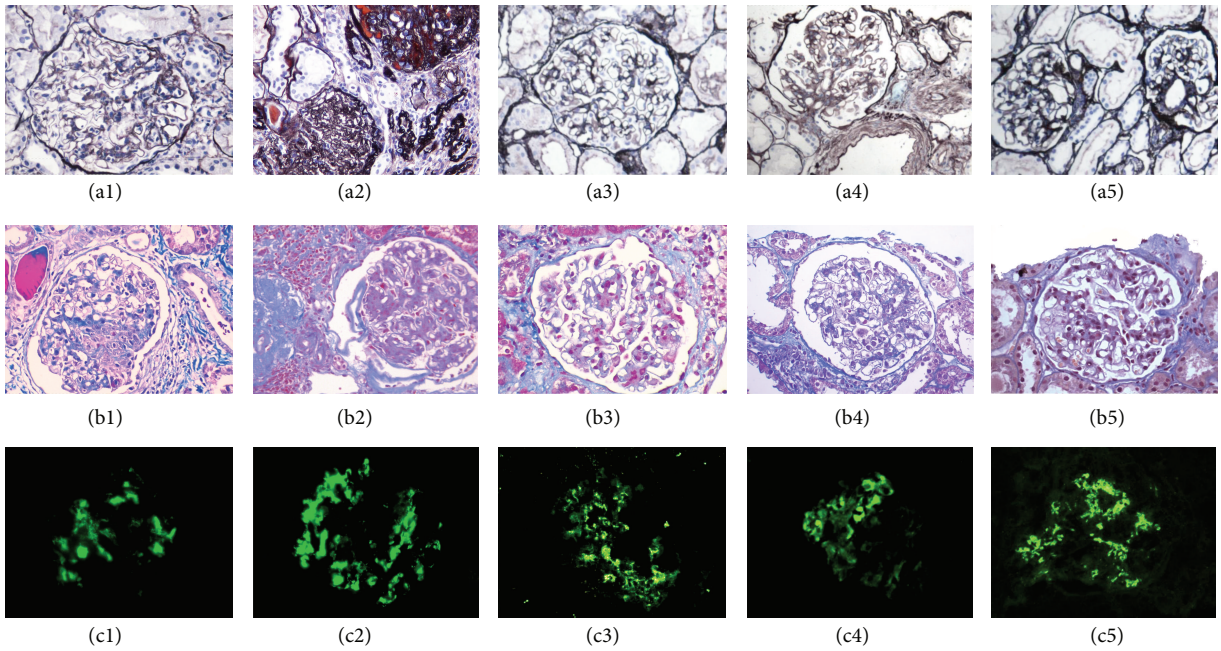

Figure 1: Light and immunofluorescent microscope findings of SLE that has nephritis with mainly IgA deposits. (a1), (a2), (a3), (a4), and (a5), respectively, indicate PASM staining of cases 1~5 under light microscope (×400). (b1), (b2), (b3), (b4), and (b5), respectively, indicate MASSON staining of cases 1~5 under light microscope (×400). Hyperplasia of glomerular mesangium and matrix can be seen in all cases. In addition, case 2 has clear glomerular sclerosis. (c1), (c2), (c3), (c4), and (c5), respectively, indicate IgA deposits of cases 1~5 under immunofluorescence microscope and all cases are positive (++~+++).

patients in our study were found to have mainly IgA deposits by renal biopsy. Retrieved from the Pubmed and Chinese National Knowledge Infrastructure (CKNI) since 1995, 8 pieces of literature reported a total of 10 SLE patients who have nephritis with mainly IgA deposits, all of whom were regarded as IgAN [3–8]. In these pieces of literature, the relationship between SLE and IgAN was discussed, and the result is still controversial. Most scholars believe that the typical LN includes the previous 6 types but not IgAN. They regard IgAN as a complication of SLE when the two diseases coexist [3, 4, 7], which means that the patient is affected by SLE and IgAN simultaneously. In typical LN, the complements C1q, C3, and C4 and the IgG-predominant accumulation of polyclonal immune complexes can be seen in the capillary basal membrane. In IgAN, however, IgA is the major deposit. At the same time, 22–60% of IgAN patients combine with IgM deposit, and 80% combine with C3 deposit. The previous features of IgAN do not match with the typical LN.

Some other scholars believe that IgAN may be a special clinical subtype of SLE. In 2010, the Japanese scholar Horino et al. [6] reported a case of a male SLE patient whose renal biopsy was established as class II LN. He was given the second renal biopsy because of repeated proteinuria, and the result suggested IgAN. The authors proposed that IgAN may be a special clinical subtype of SLE. As there is a mutual transition among the types of typical LN, typical LN may also convert into IgAN.

In our study, we found some differences in clinical characteristics between atypical LN with mainly IgA deposits and primary IgAN. The primary IgAN mainly occurred in young men, mostly 20 to 30 years old. Before the onset of IgAN, the patients can affect upper respiratory or gastrointestinal infection. Primary IgAN is characterized by gross hematuria or asymptomatic microscopic. They may also have edema, hypertension, renal dysfunction, and other clinical manifestations. Besides, serum IgA level is higher than normal in about 50% patients. The five cases in our report, similar to primary IgAN, showed gross hematuria or microscopic hematuria, edema, hypertension, renal dysfunction, and so on. However, compared with primary IgAN, the five patients have some different characteristics: older than primary IgAN, more women than men, no previous infection history before disease onset, and low incidence of serum IgA elevation. Some SLE patients are ACL positive, and the rates reported are different but not more than 60% [11]. However, the ACL positive rate in this report is as high as 100%. ACL can interfere with blood clotting mechanism, resulting in generation and aggravation of hypercoagulable state. It can also cause coagulation in the glomerular capillary and aggravate kidney damage. This may be one of the reasons why the five cases of SLE patients have much more serious kidney damage. However, it is still unclear why the ACL positive rate increases in SLE that had nephritis with mainly IgA deposits.

We speculate that nephritis with mainly IgA deposits may be a special clinical subtype of SLE. In other words, nephritis with mainly IgA deposits, as an atypical LN, may be another nephropathy of SLE in addition to the typical LN. The supporting points are as follows. (1) The cases of nephritis

with mainly IgA deposits are not rare. As many as 10 cases have already been reported before, and other 5 cases are found here in only 3.5 years. Also, it is possible that some similar cases were not reported because of neglect. (2) There was no significant difference between SLE with typical LN and SLE with atypical LN in clinical manifestations or laboratory tests. (3) Compared with primary IgAN, nephritis with mainly IgA deposits observed in SLE has its own clinical characteristics as mentioned previously: older than primary IgAN, more women than men, low incidence of serum IgA elevation, and ACL positive rate as high as 100%. To conclude, nephritis with mainly IgA deposits, as an atypical LN, may be a special clinical subtype of SLE, although it still needs a lot of further research.

4.3. Pathogenesis. SLE is also an immune-complex-mediated disease. Although C1q, C3, C4, and IgG depositions [2] are more common in typical LN, IgA deposition can also be seen. We speculated that, when some uncertain mechanisms lead to a majority of IgA deposition, the renal biopsy finding may present atypical LN with mainly IgA deposits.

4.4. Prognosis. In this research, five patients got relief after treatment of glucocorticoids and immunosuppressive drugs. The relief indicates good effect of glucocorticoids and immunosuppressive drugs on SLE that has atypical LN with maily IgA deposits. But there are also some complex and ineffective cases reported. Lai et al. [8] reported one patient who died of systemic infection. This alerts clinicians that they should give early diagnosis and treatment to such patients and be aware of the appearance of complications, especially infection.

In summary, nephritis with maily IgAN deposits is not rare in SLE and has its own clinical characteristics which are different from those of primary IgAN. We speculated that nephritis with mainly IgAN deposits, as an atypical LN, may be a special subtype of SLE. It may be another nephropathy of SLE in addition to the typical LN and has a relative good prognosis. However, the speculation still needs further clinical observation and research.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Comparison of Characteristics of Connective Tissue Disease-Associated Interstitial Lung Diseases, Undifferentiated Connective Tissue Disease-Associated Interstitial Lung Diseases, and Idiopathic Pulmonary Fibrosis in Chinese Han Population: A Retrospective Study

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Our study compared the prevalence and characteristics of patients with connective tissue disease-associated interstitial lung disease (CTD-ILD), undifferentiated connective tissue disease-associated interstitial lung disease (UCTD-ILD), or idiopathic pulmonary fibrosis (IPF) between January 2009 and December 2012 in West China Hospital, western China. Patients who met the criteria for ILD were included and were assigned to CTD-ILD, UCTD-ILD, or IPF group when they met the criteria for CTD, UCTD, or IPF, respectively. Clinical characteristics, laboratory tests, and high-resolution CT images were analyzed and compared among three groups. 203 patients were included, and all were Han nationality. CTD-ILD was identified in 31%, UCTD-ILD in 32%, and IPF in 37%. Gender and age differed among groups. Pulmonary symptoms were more common in IPF, while extrapulmonary symptoms were more common in CTD-ILD and UCTD-ILD group. Patients with CTD-ILD had more abnormal antibody tests than those of UCTD-ILD and IPF. Little significance was seen in HRCT images among three groups. A systematic evaluation of symptoms and serologic tests in patients with ILD can identify CTD-ILD, UCTD-ILD, and IPF.

1. Introduction

Interstitial lung disease (ILD) is a heterogeneous group of parenchymal lung disorders that result from variable etiologies but share common radiologic, pathologic, and clinical manifestations [1]. The prevalence of ILD is high and varies from 10.7/100,000 to 27.14/100,000 in different countries [2–5]. Several rheumatologic conditions are associated with the development of ILD [6]. These diseases include systemic sclerosis (SSc), rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM), Sjogren's syndrome, systemic lupus erythematosus (SLE), and mixed connective tissue disease(CTD) [1]. Connective tissue disease-associated ILD (CTD-ILD) refers to patients who are diagnosed as ILD and met the diagnosis criteria for a defined CTD simultaneously. The prevalence of CTD-ILD occupied 19%~34% of ILD [6, 7]. Recently, a large number of ILD patients who have one or several features of systemic autoimmune disease but do not fulfill American College of Rheumatology (ACR) classification criteria for defined CTD have been classified [8]. These patients are considered to have undifferentiated connective tissue disease (UCTD) and take up as many as 25% of ILD patients as reported [8].

Recent studies have shown that CTD-ILD, UCTD-ILD, and IPF were three distinct subgroups of diseases, which differ from prognosis and treatment. Patients with IPF were found to have much worse outcome compared with patients with CTD-ILD and UCTD-ILD [6, 9, 10]. Patients with a diagnosis of CTD-ILD or UCTD-ILD may lead to additional immunosuppressive therapy, whereas a diagnosis of IPF may

lead to different therapies other than immunosuppressive therapy to prevent substantial treatment-related side effects. Thus, it is necessary to identify patients of CTD-ILD and UCTD-ILD from IPF. At present, most studies were conducted in USA and European countries, and little knowledge is known in Asia, especially in China. In this study, we retrospectively studied 203 cases of Chinese ILD patients. The prevalence and clinical features of CTD-ILD, UCTD-ILD, and IPF were analyzed.

2. Patients and Methods

2.1. Study Population. Patients with a diagnosis of ILD in West China Hospital from January 2009 to December 2012 were selected in this study. ILDs were diagnosed according to the ATS/ERS consensus classification [11]. Patients with environmental exposures and other known causes of ILD were excluded. Patients were classified into three groups (CTD-ILD, UCTD-ILD, and IPF) based on the presence of CTD or UCTD. Study subjects who met the American College of Rheumatology (ACR) criteria for CTD were defined as CTD-ILD group [12–17]. ILD patients who did not meet ACR criteria for connective tissue diseases were defined as UCTD-ILD group if they had at least one sign or symptom suggestive of a connective tissue disease and at least one serologic test supportive of an autoimmune process, as listed in Table 1 [6, 8]. IPF group was defined using the ATS criteria for IPF [11]. Serologic tests were considered positive if the results were above the reference value. Anti-nuclear antibody was considered abnormal only when its titer was higher than 1 : 160.

2.2. Data Collection. Clinical data including detailed patients history, clinical manifestations, laboratory findings, and HRCT findings were obtained from patients' medical records from the first encounter.

2.3. Patient History. Clinical manifestations including (1) symptoms related to ILD such as cough, sputum production, and chest distress and (2) symptoms related to CTD such as skin rash, arthralgia, Raynaud's phenomenon, and fever.

2.4. HRCT Findings. All high-resolution CT (HRCT) scans were reviewed by two independent doctors from Department of Radiology without knowledge of this study. Signs including consolidation, ground glass opacities, traction bronchiectasis, irregular linear opacities, subpleural curvilinear shadows, and honeycombing were evaluated.

2.5. Statistical Analysis. Patient characteristics, clinical symptoms, HRCT findings, and serologic test results were reported as mean ± SEM or as frequency counts and percentages. The prevalence of clinical findings, serologic tests for antibodies, and radiographic patterns among the three groups was compared using chi-square test or analysis of variance. The serologic tests were calculated using analysis of variance. A $P < 0.05$ was considered significant. All data were analyzed using SPSS 19.0 software.

3. Results

3.1. Study Population. 207 patients were diagnosed as ILD during the study period, and all were Han patients. 4 patients with environmental exposures and other known causes of ILD were excluded. 203 patients were included in our study. 63 patients met the criteria for CTD-ILD, 65 patients met the criteria for UCTD-ILD, and 75 patients met the criteria for IPF. The prevalence of CTD-ILD, UCTD-ILD, and IPF were 31.0%, 32.0%, and 36.9%.

3.2. Clinical Features. Clinical features of patients are shown in Table 2. The mean age of CTD-ILD was 57.24 ± 1.55 years, younger than patients with UCTD-ILD and those with IPF ($P < 0.05$). The percentage of male patients was 31.7% in the CTD-ILD group, significantly lower than the UCTD-ILD (63.1%) and IPF group (69.3%), and the percentage of ever smoker was significantly lower in CTD-ILD group than in the UCTD-ILD and IPF group, which indicate that young female ILD patients were more prone to be CTD-ILD patients, while older male ILD patients with smoking history were more prone to be UCTD-ILD and IPF patients.

Cough, sputum production, dyspnea, and fatigue were common in all three groups but were less common in CTD-ILD group compared with CTD-ILD and IPF group. Symptoms of hemoptysis, chest discomfort, and chest discomfort were not seen in CTD-ILD patients. Symptoms suggestive of a connective tissue disease including arthralgia, dry eyes/dry mouth, Raynaud's phenomenon, proximal muscle weakness, and muscle pain were common in CTD-ILD patients and UCTD-ILD patients except proximal muscle weakness and muscle pain, which seemed to be specific to CTD-ILD groups. All of these symptoms were seldom seen in IPF patients. Face swelling and oral ulceration were only seen in few patients in the CTD-ILD group. The symptoms with significant difference between the CTD-ILD group and UCTD-ILD group are cough ($P = 0.007$), sputum production ($P = 0.002$), dyspnea ($P = 0.018$), chest discomfort ($P = 0.004$), chest pain ($P = 0.001$), proximal muscle weakness ($P = 0.008$), and muscle pain ($P = 0.000$). The symptoms with significant difference between the CTD-ILD group and IPF group are cough ($P = 0.007$), dyspnea ($P = 0.11$), chest discomfort ($P = 0.000$), chest pain ($P = 0.011$), skin rash ($P = 0.000$), arthralgia ($P = 0.000$), dry eyes/dry mouth ($P = 0.043$), Raynaud's phenomenon ($P = 0.002$), proximal muscle weakness ($P = 0.004$), and muscle pain ($P = 0.000$). The symptoms with significant difference between UCTD-ILD and IPF are cough ($P = 0.000$), skin rash ($P = 0.000$), arthralgia ($P = 0.000$), dry eyes/dry mouth ($P = 0.012$), and Raynaud's phenomenon ($P = 0.001$). These data suggest that ILD patients with symptoms of hemoptysis, chest discomfort, or chest pain were less likely to be CTD-ILD patients. ILD patients with symptoms suggestive of a connective tissue disease including arthralgia, dry eyes/dry mouth, Raynaud's phenomenon, proximal muscle weakness, and muscle pain were not likely to be IPF patients. Thus, a scan for evidence of CTD or UCTD is necessary in these patients.

3.3. Laboratory Findings. Serologic test results are shown in Tables 3 and 4. Autoantibodies were commonly seen in

Table 1: Diagnostic criteria for patients with undifferentiated connective tissue disease (UCTD).

Diagnostic criteria	Presence of
Symptoms (at least one symptom)	(1) Skin rash
	(2) Arthralgia
	(3) Dry eyes/Dry mouth
	(4) Raynaud's phenomenon
	(5) proximal muscle weakness
	(6) Leg/foot swelling
	(7) Face swelling
	(8) Oral ulceration
	(9) Hand ulcers
	(10) Mouth ulcers
	(11) Raynaud's phenomenon
	(12) Morning stiffness
	(13) Recurrent unexplained fever
Serologic test (at least one test positive)	(1) Antinuclear antibody titer ≥1 : 160
	(2) Rheumatoid factor
	(3) Antidouble-stranded DNA
	(4) Anti-ribonucleoprotein antibody
	(5) Anti-Smith antibody
	(6) Anti-Sjoren syndrome A antibody
	(7) Anti-Sjoren syndrome B antibody
	(8) Anti-Scl-70
	(9) Anti-neutrophil cytoplasmic antibody
	(10) Anti-cyclic citrullinated peptide antibody
	(11) Anti-Jo-1
	(12) AKA

CTD-ILD groups; only ANA, RF, anti-SSB, and anti-Scl-70 were seen in UCTD-ILD patients, while only ANA and RF were found in IPF patients. 71% of CTD-ILD patients and 52% of UCTD-ILD patients had a positive ANA, higher than that of IPF (21%). Presence of positive RF was more common in CTD-ILD group than UCTD-ILD and IPF group, while there was no significant difference between UCTD-ILD and IPF group. Fifteen patients with CTD-ILD (24%) had one, 15 (24%) had two, and 22 (35%) had three or more abnormal serologic tests for autoantibodies. 35 patients with UCTD-ILD (54%) had one and 4 (6%) had two abnormal serologic tests for autoantibodies. Only 21 patients with IPF (28%) had one abnormal serologic test for autoantibodies. These data indicate that ANA and RF can be found in IPF patients, but if other autoantibodies were found in ILD patients, a diagnosis of CTD-ILD and UCTD-ILD should be considered.

Significant difference in other serologic tests between CTD-ILD groups and IPF group was seen in hemoglobin, platelet, ALT, AST, ALB, LDH, HBDH, IgM, C3, and C4. Significant difference was also found in ALB, LDH, and HBDH between CTD-ILD and UCTD-ILD group. Significant difference was only found in C3 between UCTD-ILD and IPF group.

3.4. HRCT Findings. HRCT image characteristics of these patients are shown in Table 5. All individuals showed UIP on HRCT scan. Almost all these patients showed irregular linear opacities in HRCT images. The most common images in all three groups were ground glass opacities, honeycombing, and consolidation. Subpleural curvilinear shadows were less common in all groups, 3% in CTD-ILD, 2% in UCTD-ILD, and 4% in IPF. The percentage of presence of consolidation in CTD-ILD patients was lower than UCTD-ILD patients ($P = 0.034$) and IPF patients ($P = 0.023$), while the presence of ground glass opacities in CTD-ILD patients was more common than UCTD-ILD patients ($P = 0.002$) and IPF patients ($P = 0.006$). However, there was no significant difference between UCTD-ILD group and IPF group in the image characteristics.

4. Discussion

Our study showed that patients with CTD-ILD, UCTD-ILD, and IPF were three distinct subgroups of diseases which differ from clinical features and serologic tests, and a systematic evaluation of symptoms and serologic tests in patients with ILD can identify these three subgroups. To date, this is the largest study to systematically evaluate patients with ILD to analyze the characteristics of CTD-ILD, UCTD-ILD, and IPF patients.

By retrospectively studied 203 cases of Chinese ILD patients, we found that CTD-ILD occupied about one-third of these patients. These results of our study were consistent with those reported by previous studies, with the prevalence

Table 2: Comparison of clinical characteristics among CTD-ILD, UCTD-ILD, and IPF patients.

	CTD-ILD	UCTD-ILD	IPF	P1	P2	P3
Subject (*n*)	63	65	75			
Age (years)	57.24 ± 1.55	63.58 ± 1.53	64.7 ± 1.68	**<0.05**	**<0.05**	0.614
Sex (M/F)	20/43	41/24	52/23	**0.000**	**0.000**	0.434
Ever smoker *n* (%)	15 (24)	35 (54)	35 (47)	**0.000**	**0.005**	0.397
Symptoms *n* (%)						
Cough	45 (71)	65 (100)	67 (89)	**0.000**	**0.007**	**0.007**
Sputum	33 (52)	51 (78)	48 (64)	**0.002**	0.167	0.061
Dyspnea	35 (56)	49 (75)	57 (76)	**0.018**	**0.011**	0.933
Fatigue	21 (33)	27 (42)	27 (36)	0.338	0.743	0.502
Hemoptysis	0 (0)	5 (8)	4 (5)	0.074	0.177	0.824
Chest discomfort	0 (0)	8 (12)	16 (21)	**0.004**	**0.000**	0.158
Chest pain	0 (0)	11 (17)	13 (17)	**0.001**	**0.001**	0.949
Skin rash	14 (22)	16 (25)	0 (0)	0.749	**0.000**	**0.000**
Arthralgia	19 (30)	14 (19)	1 (1)	0.265	**0.000**	**0.000**
Dry eyes/dry mouth	5 (8)	7 (11)	0 (0)	0.583	**0.043**	**0.012**
Raynaud's phenomenon	9 (14)	10 (15)	0 (0)	0.861	**0.002**	**0.001**
Proximal muscle weakness	8 (13)	0 (0)	0 (0)	**0.008**	**0.004**	—
Muscle pain	13 (21)	0 (0)	0 (0)	**0.000**	**0.000**	—
Recurrent unexplained fever	4 (6)	9 (14)	7 (9)	0.160	0.519	0.403
Leg/foot swelling	4 (6)	6 (9)	10 (13)	0.781	0.176	0.447
Face swelling	2 (3)	0 (0)	0 (0)	0.462	0.401	—
Oral ulceration	2 (3)	0 (0)	0 (0)	0.462	0.401	—

P1: Possibility when comparing CTD-ILD group and UCTD-ILD group. P2: Possibility when comparing CTD-ILD group and IPF group. P3: Possibility when comparing UCTD-ILD group and IPF group.

Table 3: Comparison of presence of autoantibodies among CTD-ILD, UCTD-ILD, and IPF patients.

	CTD-ILD	UCTD-ILD	IPF	P1	P2	P3
Subject (*n*)	63	65	75			
ANA *n* (%)	45 (71)	34 (52)	16 (21)	**0.026**	**0.000**	**0.000**
RF *n* (%)	26 (42)	9 (14)	7 (9)	**0.001**	**0.000**	**0.403**
Anti-ds-DNA *n* (%)	0	0	0	—	—	—
RNP *n* (%)	6 (10)	0	0	**0.011**	**0.006**	—
Anti-Smith *n* (%)	2 (3)	0	0	0.148	0.102	—
Anti-SSA *n* (%)	18 (28)	0	0	**0.000**	**0.000**	—
Anti-SSB *n* (%)	5 (8)	5 (8)	0	0.959	**0.011**	**0.014**
Anti-Scl-70 *n* (%)	4 (6)	4 (6)	0	0.964	**0.027**	**0.029**
ANCA *n* (%)	0	0	0	—	—	—
ACA *n* (%)	3 (5)	0	0	0.075	0.056	—
Anti-CCP *n* (%)	8 (13)	0	0	**0.003**	**0.001**	—
Anti-Jo-1 *n* (%)	2 (3)	0	0	0.148	0.120	—
AKA *n* (%)	6 (3)	0	0	**0.011**	**0.001**	—

P1: Possibility when comparing CTD-ILD group and UCTD-ILD group. P2: Possibility when comparing CTD-ILD group and IPF group. P3: Possibility when comparing UCTD-ILD group and IPF group.

of CTD-ILD varying from 12.4% to 34% [3, 7, 18, 19]. The UCTD-ILD as a nearly defined new group of ILD was also common in ILD patients, with prevalence of 32.0%, which was higher than CTD-ILD, but a little lower than IPF (36.9%). However, CTD-ILD patients and UCTD-ILD patients occupied about two-thirds of ILD patients, which meant that most ILD patients could be found to be autoimmune related; these patients may have a better prognosis, and immunomodulatory therapy should be considered.

We found that patients with CTD-ILD were more likely to be younger women and nonsmokers, with more antibody abnormalities and presentation of skin and muscle damage, as reported previously [9, 20, 21]. Symptoms of hemoptysis, chest discomfort, and chest pain were less likely to be

Table 4: Comparison of laboratory findings among CTD-ILD, UCTD-ILD, and IPF patients.

	CTD-ILD	UCTD-ILD	IPF	P1	P2	P3
Subject (*n*)	63	65	75			
Erythrocyte ($\times 10^{12}$/L, mean ± SEM)	4.07 ± 0.100	4.39 ± 0.100	4.41 ± 0.080	0.075	**0.028**	0.998
Hemoglobin (mg/L, mean ± SEM)	120.08 ± 3.186	130.31 ± 3.159	134.53 ± 2.244	0.071	**0.001**	0.645
Platelet ($\times 10^{9}$/L, mean ± SEM)	207.84 ± 12.643	189.72 ± 10.102	168.69 ± 8.821	0.603	**0.037**	0.317
Hematokrit (1, mean ± SEM)	0.37 ± 0.011	0.40 ± 0.009	0.41 ± 0.007	0.129	**0.008**	0.695
Alanine aminotransferase ALT (IU/L, mean ± SEM)	38.58 ± 4.636	29.80 ± 4.871	25.96 ± 2.129	0.476	**0.045**	0.853
Aspartate aminotransferase AST (IU/L, mean ± SEM)	43.59 ± 4.500	30.43 ± 3.671	27.25 ± 1.630	0.074	**0.003**	0.815
Serum albumin ALB (mg/L, mean ± SEM)	32.33 ± 0.741	35.33 ± 0.571	36.75 ± 0.738	**0.005**	**0.000**	0.272
Lactate dehydrogenase LDH (IU/L, mean ± SEM)	331.81 ± 18.282	256.10 ± 12.395	260.56 ± 15.083	**0.003**	**0.010**	0.994
Hydroxybutyric dehydrogenase HBDH (IU/L, mean ± SEM)	273.28 ± 16.568	210.62 ± 11.161	215.16 ± 13.132	**0.007**	**0.021**	0.991
Immunoglobulin G (g/L, mean ± SEM)	15.57 ± 1.046	14.36 ± 0.493	13.10 ± 0.646	0.655	0.137	0.311
Immunoglobulin A (mg/dL, mean ± SEM)	2847.26 ± 187.573	3051.06 ± 192.602	2781.23 ± 204.235	0.834	0.993	0.710
Immunoglobulin M (mg/dL, mean ± SEM)	1671.00 ± 152.988	1462.94 ± 202.364	1110.78 ± 73.626	0.799	**0.004**	0.285
Immunoglobulin E (mg/dL, mean ± SEM)	189.19 ± 44.544	166.33 ± 31.581	183.73 ± 47.300	0.966	1.000	0.986
C3 (g/L, mean ± SEM)	0.85 ± 0.033	0.92 ± 0.024	1.01 ± 0.026	0.306	**0.001**	**0.034**
C4 (g/L, mean ± SEM)	0.18 ± 0.010	0.33 ± 0.140	0.21 ± 0.008	0.624	**0.022**	0.785

P1: Possibility when comparing CTD-ILD group and UCTD-ILD group. P2: Possibility when comparing CTD-ILD group and IPF group. P3: Possibility when comparing UCTD-ILD group and IPF group.

Table 5: HRCT findings of ILD patients.

	CTD-ILD	UCTD-ILD	IPF	P1	P2	P3
Subject (*n*)	63	65	75			
Consolidation *n* (%)	11 (17)	22 (34)	26 (35)	**0.034**	**0.023**	0.919
Ground glass opacities *n* (%)	42 (67)	34 (52)	42 (56)	**0.002**	**0.006**	0.662
Irregular linear opacities *n* (%)	59 (94)	62 (95)	71 (95)	0.666	1.000	1.000
Traction bronchiectasis *n* (%)	6 (10)	11 (17)	13 (17)	0.217	0.185	0.949
Honeycombing *n* (%)	28 (44)	32 (49)	31 (41)	0.587	0.713	0.349
Subpleural curvilinear shadows *n* (%)	2 (3)	1 (2)	3 (4)	0.716	0.740	1.000

P1: Possibility when comparing CTD-ILD group and UCTD-ILD group. P2: Possibility when comparing CTD-ILD group and IPF group. P3: Possibility when comparing UCTD-ILD group and IPF group.

presented in CTD-ILD patients. Patients with CTD-ILD also had lower levels of erythrocyte, hemoglobin, and hematocrit but highest levels of platelet and IgM in their serologic tests, but most of these parameters were within normal references. However, anemia was reported previously common in CTD, resulting from autoimmune hemolysis in most conditions [22–25].

IPF patients were more likely to be older male ILD patients with smoking history. ILD patients with symptoms suggestive of a connective tissue disease including arthralgia, dry eyes/dry mouth, Raynaud's phenomenon, proximal muscle weakness, and muscle pain were not likely to be IPF patients. Thus, a scan for evidence of CTD or UCTD is necessary in these patients. Only ANA and RF could be found in IPF patients, while other autoantibodies could be seldom found in these patients.

We also found that patients with UCTD-ILD were a distinct entity in patients with ILD, with their own clinical and serologic characteristics. The characteristics of UCTD-ILD patients seemed to lie between CTD-ILD and IPF, and the differentiation between UCTD-ILD and IPF appeared to be a little difficult. Unlike previous studies, patients with UCTD-ILD in our study were more likely to be men, and the mean age of them had no significance with that of IPF [6, 8]. Patients with UCTD-ILD had more extrapulmonary presentations and more antibody abnormalities than those of IPF. Skin rash, arthralgia, and Reynaud's phenomenon were common in UCTD-ILD [26], while not common in IPF. Autoantibodies of anti-SSB and anti-Scl-70 could be found in UCTD-ILD patients, while these two autoantibodies were not likely to be found in IPF patients.

All individuals showed usual interstitial pneumonia on HRCT scan, and it was hard to distinguish IPF from CTD-ILD or UCTD-ILD based on HRCT scan itself. With no evidence for lung biopsy, a typical UIP pattern on HRCT scan did not exclude CTD-ILD or UCTD-ILD from IPF [6, 27]. Most characteristics of HRCT images of CTD-ILD were not different from those of other two groups, except for consolidation and ground glass opacities. Signs of consolidation were less common, and ground glass opacities were more commonly seen in HRCT images of CTD-ILD patients. However, images of consolidation and ground glass

opacities were nonspecific, because even infection could display these features on HRCT scan [28]. There was no significant difference of these signs on HRCT scan between UCTD-ILD and IPF patients.

This study has the following limitations, Firstly, it is a retrospective study conducted in only one institute. Secondly, almost all of these patients refused to do lung biopsy for the possibility that lung biopsy is an invasive test and gives little contribution to treatment.

5. Conclusion

CTD-ILD and UCTD-ILD patients occupied the most part of ILD patients, and patients with CTD-ILD, UCTD-ILD, and IPF differed in clinical features and laboratory findings. A systematic evaluation of symptoms and serologic tests in patients with ILD can identify CTD-ILD, UCTD-ILD, and IPF. In addition, there is much to be learned about the underlying pathogenesis of CTD-ILD and UCTD-ILD or IPF, and appropriate intervention trials should be conducted to learn about the treatment of these diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Lin Pan and Yuan Liu contributed equally to this study.

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Review Article

Recent Advances in the Pathogenesis of Autoimmune Hair Loss Disease Alopecia Areata

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Alopecia areata is considered to be a cell-mediated autoimmune disease, in which autoreactive cytotoxic T cells recognize melanocyte-associated proteins such as tyrosinase. This review discusses recent advances in the understanding of the pathogenesis of alopecia areata, focusing on immunobiology and hormonal aspects of hair follicles (HFs). The HF is a unique "miniorgan" with its own immune and hormonal microenvironment. The immunosuppressive milieu of the anagen hair bulb modulated by immunosuppressive factors is known as "hair follicle immune privilege." The collapse of the hair follicle immune privilege leads to autoimmune reactions against hair follicle autoantigens. Alopecia areata is sometimes triggered by viral infections such as influenza that causes excess production of interferons (IFN). IFN-γ is one of the key factors that lead to the collapse of immune privilege. This paper reviews the interactions between the endocrine and immune systems and hair follicles in the pathogenesis of alopecia areata.

1. Introduction

1.1. Clinical Features. Alopecia areata (AA) is a relatively common disease encountered by dermatologists. Patients with AA represent approximately 0.7% to 3.8% of all the patients attending dermatology clinics [1, 2]. Males and females are affected equally [3]. Disease onset before the 4th decade was reported to be 85.5% in the Asian population [2].

Based on the extent of hair loss, the hair loss pattern of AA can be described as single delimited patches; patchy AA, in which there is a partial loss of scalp hair; alopecia totalis (AT), in which 100% of scalp hair is lost; or alopecia universalis (AU), in which there is a 100% loss of all scalp and body hair [4]. Less common types of AA include reticular patches of hair loss; ophiasis type, a band-like hair loss in the parieto-temporo-occipital area; ophiasis inversus (sisaipho), a rare band-like hair loss in the frontal parietotemporal scalp; and a diffuse thinning of a part or all of the scalp [4]. Another variant named "acute diffuse and total alopecia" is characterized by acute scalp hair loss, extensive involvement with infiltration of eosinophils around hair follicles (HFs), and a favorable prognosis. It was first described by Sato-Kawamura et al. and was thought to be limited to females [5], but Lew et al. described that is affected male patients in their case series [6].

1.2. Association with Other Autoimmune Diseases. AA may be associated with other autoimmune diseases, particularly thyroid autoimmune disease such as Hashimoto's thyroiditis and Basedow's disease. The prevalence of thyroid disease in patients with AA ranges from 8% to 28% [7]. The presence of thyroid autoantibodies does not correlate with AA severity [8], and treatment is not warranted. Vitiligo, an autoimmune skin disease affecting melanocytes, is also associated with AA. The prevalence of vitiligo in AA patients is 3% to 8% compared with 1% in the United States population [9]. These disease associations suggest a relationship between autoimmunity and AA.

1.3. Psychiatric Morbidity. AA is associated with psychiatric morbidity, especially anxiety and depression [10]. In a study of 31 AA patients, Colón et al. reported that 74% were

given one or more lifetime psychiatric diagnoses based on structured psychiatric interviews [11]. The lifetime prevalence rates of major depression (39%) and generalized anxiety disorder (39%) were particularly high [11]. These studies indicate that patients with AA are at an increased risk for developing anxiety and depression, and psychiatric referral may be warranted in AA patients.

1.4. Histopathology. The accumulation of mononuclear cells in and around hair bulbs—so-called "swarm of bees"—is the most characteristic histopathological change in AA [12]. This is observable particularly in the acute stage of the disease, and it is composed of both CD4+ and CD8+ cells with a high CD4+/CD8+ ratio in clinically active disease [13]. In the chronic stage, there is a marked HF miniaturization and cell accumulation decreases, but accumulation of CD8+ T cells is still observable [14].

2. Pathogenesis

2.1. Genetics. Genetics play an important role in the pathogenesis of AA. For example, monozygotic twins who suffered from AA after mumps infection had a similar disease onset and hair loss patterns [15]. Specific alleles such as DQB1*03 and DRB1*1104 have been reported as markers of susceptibility to AA [16–20]. The HLA alleles DRB1*1104 (HLA-DR11) and DQB1*0301 (HLA-DQ7) may be associated with AT/AU [17]. These findings suggest that the onset and progression of AA is associated with specific HLA class II alleles [18–21]. Recently, Petukhova et al. [22] performed a genome-wide association study (GWAS) to determine the genetic architecture of AA in a sample of 1,054 AA cases and 3,278 controls using a combination of Illumina 610 K and 550 K arrays. The GWAS revealed 139 single nucleotide polymorphisms (SNPs) that are significantly associated with AA ($P \leq 5 \times 10^{-7}$). Several susceptibility loci for AA were identified, most of which were clustered in eight genomic regions and fell within discrete linkage disequilibrium blocks. These include loci on chromosome 2q33.2 containing *CTLA4*, chromosome 4q27 containing *IL-2/IL-21*, chromosome 6p21.32 containing the *HLA*, chromosome 6q25.1 harboring the *ULBP* genes, chromosome 10p15.1 containing *IL-2RA (CD25)*, and chromosome 12q13 containing *Eos (IKZF4)* and *ERBB3*. One SNP resides on chromosome 9q31.1 within syntaxin 17 (*STX17*), and one resides on chromosome 11q13, upstream from peroxiredoxin 5 (*PRDX5*). The *ULBP* genes encoding the ligands of the natural killer cell receptor NKG2D reside in a 180-kilobase MHC class I-related cluster on human chromosome 6q25.1. NKG2D is expressed on natural killer (NK) and CD8+ T cells, and is activated by ULBP. The role of the NK cell activating receptor, NKG2D, in the pathogenesis AA will be described later in this review.

2.2. Collapse of Hair Follicle Immune Privilege. One of the most intriguing features of hair biology is the immune privilege of the anagen HF [23, 24] that is characterized by an immunosuppressive milieu around the hair bulb. Apart from the anagen HF, other sites of immune privilege include the anterior eye chamber, parts of testis and ovaries, adrenal cortex, parts of the central nervous system enclosed by the blood-brain barrier, the placenta, and the hamster cheek pouch [23, 24]. The unique microenvironment of these immune-privileged sites protects the organs from deleterious immune reactions and loss of function. For example, severe inflammation of the anterior chamber of the eye could lead to blindness, and an immune reaction in the central nervous system could cause serious brain damage. Although scalp and trunk hair are not necessary for human survival, significant hair loss could be deadly for mammals such as polar bears, seals, and reindeer [25].

The HF immune privilege (IP) is maintained by several factors, including the lack of major histocompatibility complex (MHC) class I in the proximal outer root sheath (ORS) and matrix cells (Table 1). HFIP is present during anagen but is lost during the resting (telogen) and regression (catagen) phases of the hair cycle. Collapse of HFIP is thought to contribute to the development of AA (Figure 1), in which pigment-producing anagen hair bulbs are attacked by inflammatory cells [23]. MHC class I is strongly expressed in AA lesions, raising the possibility that CD8+ T cells react to autoantigens by binding to MHC class I molecules. Interferon (IFN)-γ, a key cytokine implicated in the pathogenesis of AA, upregulates MHC class I expression in cultured HFs in vitro [26]. Virus infections can increase the production of IFN-γ in vivo, and it has been reported that the swine flu virus can trigger or exacerbate AA [27].

2.3. Autoantigens. Several studies of AA suggest that melanogenesis-associated peptides expressed by melanin-producing anagen HFs are the key autoantigens targeted by autoreactive cytotoxic T cells [28, 29]. Possible involvement of melanogenesis-associated autoantigens in AA was suggested by the following observations: sparing of white/greying HFs in AA; regrowing hair shafts are usually white followed by repigmentation, association with vitiligo, and the sudden onset of fulminant AA affecting mostly pigmented HFs (overnight greying). Follicular melanocytes are possible targets in AA. Indeed, follicular melanocytes in AA show both histological and ultrastructural abnormalities [30]. Using the human scalp explant/SCID mouse transfer model, Gilhar et al. demonstrated that melanocyte-associated T cell epitopes are capable of functioning as autoantigens and result in AA in human scalp grafts [29]. Melanocyte HLA-A2-restricted peptides can activate T cells for the transfer of AA to autologous scalp skin grafts on SCID mice, indicating that melanocyte-associated autoantigens can be pathogenic.

2.4. "Swarm of Bees" Linked to Th1 Cytokines, Chemokines, and Chemotaxis. A unique histopathological feature in the acute stage of AA is the dense accumulation of lymphocytes around the hair bulbs—so-called "swarm of bees"—that is a result of the collapse of HFIP with exposure of autoantigens, leading to an accumulation of autoreactive T cells [12]. The mononuclear cells that accumulate in and around the lesional hair bulb consist of 60–80% CD4+ T cells, 20–40% CD8+ T cells, and NK cells [13, 31]. IFN-γ, a representative Th1 cytokines,

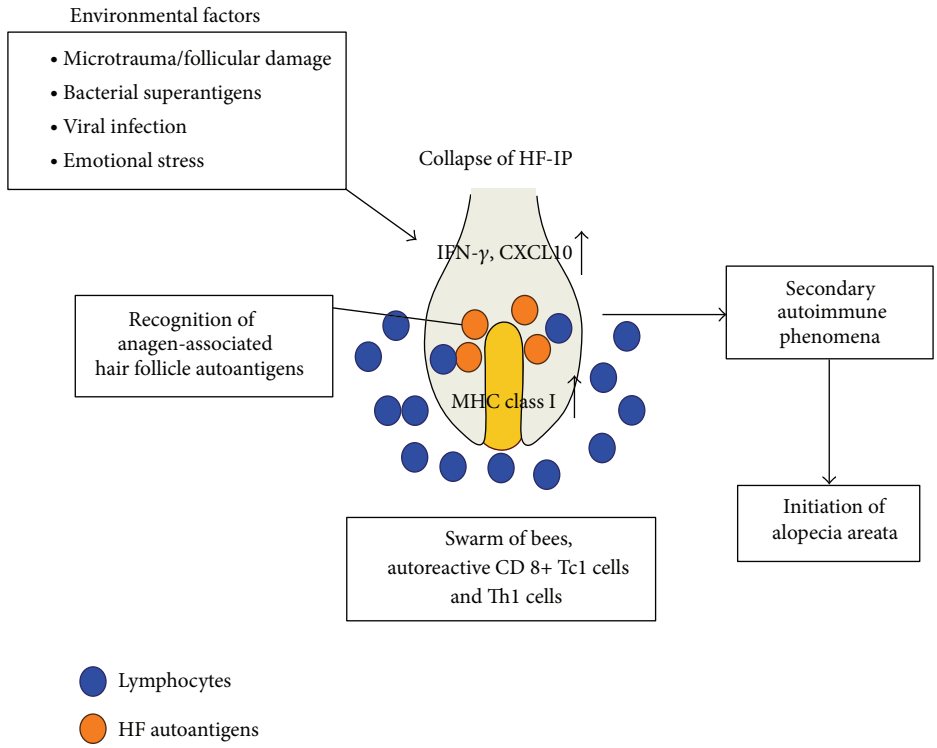

Figure 1: The pathogenesis of alopecia areata and treatment strategies. Environmental factors such as viral infections and bacterial superantigens may induce IFN-γ and CXCL10 expressions in the hair bulbs. Subsequently, autoreactive Th1 and Tc1 cells (blue circles) accumulate in and around hair bulbs—the so-called "swarm of bees." Anagen-associated hair follicle (HF) autoantigens (orange circles) are recognized by Th1 and Tc1 cells, which lead to a secondary autoimmune phenomenon and resultant hair loss.

Table 1: The mechanism of HF immune privilege.

(i) Absent or barely detectable expression of MHC class I.
(ii) Hair follicular melanocytes of the human anagen scalp are MHC class I-negative.
(iii) Downregulation of the MHC class I pathway-related molecules β2-microglobulin and transportation of antigen processing-2 (TAP-2).
(iv) Downregulation of interferon regulatory factor-1 expression.
(v) Upregulation of immunosuppressive factors such as TGF-β1 and TGF-β2, ACTH, and α-MSH.
(vi) Absence of MHC class II+ or NLDC-145+ Langerhans cells.
(vii) Sparse distribution of NK cells and CD4+ and CD8+ T cells.
(viii) Absence of lymphatics.

is prominently expressed in AA lesions and may induce the collapse of HFIP by upregulating MHC class I expression [32]. Autoimmune hair loss similar to AA can be reproduced in C3H/HeJ mice by injecting IFN-γ, which induces the follicular expression of MHC classes I and II [33].

Several studies have examined the expression of chemokines and their receptors in AA. For example, the expression of Th1 chemokines CXCL9/MIG and CXCL10/IP-10 is increased in AA lesions [34, 35]. Serum CXCL9 is elevated in AA patients, and its level is correlated with disease activity [36]. Transcriptional profiling revealed that CXCL10 is highly upregulated in AA lesions compared with nonlesional skin [37]. Our study showed that the proportions of CXCR3+ CD4+ Th1 cells and CXCR3+ CD8+ Tc1 cells are significantly increased in the PBMCs of AA patients [11]. CD8+ T cells can be differentiated into two effector phenotypes, Tc1 and Tc2, which secrete different cytokines [38–40]. CD8+ T cells that secrete IFN-γ but not IL-4 and IL-5 are known as type I CD8+ cytotoxic T (Tc1) cells, while those that secrete IL-4 and IL-5 but not IFN-γ are type II CD8+ cytotoxic T (Tc2) cells. Tc1 cells kill tumor targets by either perforin or Fas-mediated mechanisms, whereas Tc2 cells mainly use the perforin pathway [41]. Tc1 cells are implicated in the development of autoimmune diseases such as experimental autoimmune thyroiditis [42]. In the acute phase AA, the proportion of MAGE-A3 is specific, IFN-γ-producing T cells in PBMCs is increased [11]. Therefore, the increased numbers of CXCR3+ CD8+ Tc1 cells may contribute to the cell-mediated autoimmune reactions in AA.

In addition, we demonstrated that freshly isolated CD4+ and CD8+ T cells from AA patients displayed strong chemotactic activity towards CXCL10 using a real-time chemotaxis assay [14]. The T cell chemotactic activity may be a result of activation. Considering the accumulation of lymphocytes in the acute phase of AA, our T cell chemotaxis results are consistent with the histopathological findings of AA in the acute phase [14].

2.5. NKG2D in Alopecia Areata. NKG2D is expressed not only in NK cells but also in CD8+ and gamma delta T cells [43, 44]. NKG2D recognizes MHC class I chain-related proteins MICA and MICB on target cells. NKG2D also recognizes surface glycoproteins that bind human cytomegalovirus UL16 proteins (ULBPs), from ULBP1 to ULBP6 (total of eight human ligands), which stimulates immune cells to attack target cells [45].

NK cells have become a recent focus of AA research. The absent or low expression of MHC class I in HFs raises the question of how self/nonself-discrimination and self-tolerance are maintained [46]. As NK cells recognize and eliminate cells with an absent or low expression of MHC class I [27, 47–49], it is remarkable that very few NK cells gather around the MHC class I negative human anagen HFs [50]. Just like other healthy tissues, human anagen HFs in situ lack MICA expression [31, 51–53], which may explain why normal HFs are not subject to NK cell attack. However, in AA lesions, infiltrating CD56+ NK cells and CD8+ T cells prominently express NKG2D, and proximal ORS strongly expresses MICA, thus infiltrating NKG2D+ cells attack MICA-positive HFs [31]. Compared with normal controls and patients with other chronic inflammatory skin diseases such as atopic dermatitis, CD56+ NK cells and CD8+ T cells of AA patients have increased NKG2D expression. In addition, the percentage of NK cells that do not express NK cell-inhibitory KIR2DL2 and KIR2DL3 is significantly increased in AA patients compared with healthy controls [31]. A recent study of 20 families with AA from the United States and Israel has identified genes that may be associated with AA and other autoimmune diseases, such as *ULBP* genes that encode ligands for activating NKG2D [43].

In summary, immune-privileged, MHC class I-negative HFs are protected from NK cell attack by MICA-negative ORS, low expression of NKG2D on NK cells, and inhibitory KIRs. However, collapse of HFIP leads to NKG2D+ NK cell attack, and autoreactive NKG2D+ CD8+ T cells recognize autoantigens that result in apoptotic responses and hair loss in AA.

2.6. Stress Hormone and Alopecia Areata. To maintain homeostasis, the skin must respond to stressors such as ultraviolet light, mechanical injury, and chemical and biological insults. Emotional stress may also perturb skin homeostasis. Components of the hypothalamic-pituitary-adrenal (HPA) axis are present in the skin and are involved in the local response to stress [54]. As part of the HPA axis, corticotropin-releasing hormone (CRH) is a key stress-induced hormone that is present in the human HF. Specifically, CRH and CRH-1 receptors (CRH-R1) are expressed throughout the ORS [55], and CRH and CRH-R1 gene transcriptions occur in the human hair bulb. The human HF has a fully functional peripheral equivalent of the HPA axis, which may be involved in the stress response of the skin [56]. Organ culture human HFs secrete cortisol in response to CRH and possess feedback systems [56].

Using in situ hybridization, a clinical report examined CRH receptor expression in three AA patients who had experienced a significant emotional stress prior to hair loss. Skin from the affected scalp areas of all three patients showed an intense signal for CRH-2β. Samples from unaffected scalp areas of the same patients or from healthy controls showed only a weak background signal for the receptor [57]. The expression of CRH, ACTH, and α-MSH was significantly increased in the epidermis, HFs, and sebaceous glands of samples from AA patients compared with those from healthy controls. These results suggest the presence of an active neurogenic system and local HPA activity in AA lesions [57]. Immune activation not only affects CRH expression but also HPA activity [58–64].

Central and peripheral HPA activity under basal and stressful conditions were investigated in normal and AA-affected C3H/HeJ mice [65]. In response to psychological stress, normal mice showed marked plasma corticosterone elevation, whereas AA-affected mice showed no significant changes in corticosterone levels, suggesting that AA-affected mice have a blunted response to acute physiological stress.

In conclusion, emotional stress may affect AA patients because of a blunted corticosterone response to hormones and immunological damage, and altered HPA activity may occur as a consequence of the immune response associated with AA.

3. Conclusion

The HF is a dynamic "miniorgan" with unique immune and hormone microenvironments. Immune privilege is the most intriguing feature of HF immunology that is characterized by the downregulation of MHC class I. The collapse of HFIP is induced by certain environmental factors such as virus infections, and it involves the production of the Th1/Tc1 chemokine, CXCL10, in HFs that attracts Th1 and Tc1 cells towards the hair bulbs. Consequently, HF autoantigens are recognized by autoreactive cytotoxic T cells. HPA activity in the HF links the immune and hormonal aspects of the HF. Environmental stress may influence both the immune and hormonal microenvironments of the HF and result in the development of AA. Patients with AA may have a blunted response to an acute physiological stressor, resulting in a reduced expression of glucocorticoids.

Recent advances in the understanding of the pathomechanism of AA may lead to the development of novel treatments for AA in the future.

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Research Article

Upregulated MicroRNA-155 Expression in Peripheral Blood Mononuclear Cells and Fibroblast-Like Synoviocytes in Rheumatoid Arthritis

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Objective. This study was to screen for the miRNAs differently expressed in peripheral blood mononuclear cells (PBMC) of RA, to further identify the expression of miR-155 in RA PBMC and fibroblast-like synoviocytes (FLS), and to evaluate the function of miR-155 in RA-FLS. *Methods*. Microarray was used to screen for differentially expressed miRNAs in RA PBMC. miR-155 expression in PBMC and FLS of RA were identified by real-time PCR. Enforced overexpression and downexpression of miR-155 were used to investigate the function of miR-155 in RA-FLS. Expression of IKBKE which was previously identified as the actual target of miR-155 was examined by Western blot and real-time PCR in RA-FLS. *Results*. miR-155 levels were increased in both PBMC and FLS of RA and could be induced by TNF-α. Upregulation of miR-155 decreased MMP-3 levels and suppressed proliferation and invasion of RA-FLS. Inverse relationship between the expressions of miR-155 and the MMPs production-related protein IKBKE was found. *Conclusion*. An inflammatory milieu may alter miRNA expression profiles in rheumatoid arthritis. miR-155 is upregulated in RA-FLS, and it may be a protective factor against the inflammatory effect in part by attenuating expression of IKBKE.

1. Introduction

Rheumatoid arthritis (RA) is characterized as a systemic autoimmune inflammatory disease which predominantly affects multiple peripheral joints. Accumulating evidence suggests that the participation of inflammation-associated cells as well as the production of proinflammatory mediators by them plays a key role in pathogenesis of RA. Besides, resident fibroblast-like synoviocytes (FLS) contribute significantly to the perpetuation of the disease, and they may even play a role in its initiation. The local production of cytokines and chemokines by these cells accounts for many of the pathologic and clinical manifestations of RA. However, the exact mechanisms are yet unknown.

MicroRNAs (miRNAs) are a large family of highly conserved noncoding genes that regulate gene expression by translational repression or mRNA degradation. To date, hundreds of miRNAs have been identified in the human genome [1, 2], and up to 30% of all protein-encoding genes are estimated to be regulated by them [3]. miRNAs are transcribed in larger precursors and after processing are transported into the cytoplasm. The ~22 nt mature miRNAs control gene expression at the posttranscriptional level by binding to target messenger RNAs (mRNAs) and initiating either their cleavage or a reduction in the translational efficiency [4–7].

Increasing evidence has linked miRNA regulatory activities with human diseases, most notably cancer. More recently, biochemical and genetic studies have begun to reveal the physiological functions of individual miRNAs in immunity. Several articles which support an important role for these tiny RNAs in immune modulation have revealed the role of miRNAs involved in RA pathogenesis [8–11]. To provide

more evidence of miRNAs involvement in RA, we compared the levels of miRNAs expression in RA patients with normal controls via microarray profile and then chose miR-155 which had been shown to influence the immune system for further study. We explored the functional role of miR-155 in RA-FLS and the involved mechanisms. Our findings suggest that miR-155 plays a key role in regulating MMP-3 production, as well as the proliferation and invasion of RA FLS.

2. Methods

2.1. Patients and Controls. Blood samples were obtained from 26 patients admitted to the Department of Rheumatology and Immunology, People's Hospital, Peking University, between October 2007 and January 2008 (24 women, 2 men; median age 56.2 years, range 21–61), who fulfilled the American College of Rheumatology (ACR) criteria for RA. The median disease duration of RA was 9.2 ± 5.2 years. Twenty-three blood donors were included as normal controls.

For RA patients, anti-CCP antibody was tested using the second-generation ELISA kit (Euroimmun, Germany) and values >1.0 were considered positive. Erythrocyte sedimentation rate (ESR) was measured by the Westergren method, and values ≤15 mm/h for males and ≤20 mm/h for females were considered normal. Serum C-reactive protein (CRP) and rheumatoid factor (RF) were examined by an immunonephelometry method. Values >7.9 mg/L for CRP and >20 IU/mL were considered positive for RF.

2.2. Isolation and Culture of FLS of RA and OA Patients. Synovial tissues were obtained from patients with RA (n = 10, females, ages 30 to 60 years) and osteoarthritis (OA, n = 8, females, ages 40 to 70 years) at the time of knee replacement surgery. To isolate synovial fibroblasts, synovial tissue specimens were minced and digested with dispase at 37°C for 60 minutes. After washing, cells were grown in RPMI 1640 (Gibco-Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) and 50 IU/mL penicillin/streptomycin. Cultures of RA-FLS and OA-FLS were maintained at 37°C in a humidified atmosphere of 5% CO_2. All synovial fibroblasts between passages 4 and 6 were subjected to experimental procedures. The procedure was approved by the ethical committee of the Peking University People's Hospital. All patients gave written informed consent.

2.3. Microarray Experiments. Small RNA was isolated from peripheral blood mononuclear cells (PBMC) of 5 RA patients and 5 normal controls, using the miRNeasy Mini Kit (Qiagen Sciences, MD, USA) according to the manufacturer's protocol, and 0.4 μg/sample of small RNA of the two groups was mixed, respectively. Small RNA mixture of RA and normal controls were labeled Cy3 and Cy5, respectively. Then these mixtures were hybridized to a MiRCURY locked nucleic acid (LNA) microarray (LC Sciences, USA) containing 454 LNA-modified oligonucleotide probes for human, as annotated in the miRBase release 10.1 (http://microrna.sanger.ac.uk/sequences/). All microarray data are based on six probe replicates for each miRNA prediction.

The fluorescence signals were collected and converted to digital signals: the difference between RA and normal group was demonstrated by log2 = log2 (signal Cy3/ signal Cy5). Positive value of log2 means upregulation, and negative value of log2 means downregulation.

2.4. Cell Stimulation. FLS from 3 RA patients and PBMC from a separate group of 6 RA patients were transferred to 6-well (2×10^5 cells in 3 mL RPMI 1640 medium with 10% fetal bovine serum/well) cluster plates separately. They were treated with recombinant human TNF-α (20 ng/mL, R&D Systems, USA) and then incubated for 48 hours under an atmosphere of 5% CO_2. Cells were washed twice with cold PBS prior to analysis. All experiments in our study including the following study were performed independently at least three times for each point described.

2.5. Quantitative Real-Time PCR (qRT-PCR). miRNA qRT-PCR was performed using the SYBR Green miRNA assay (Hairpin-it miRNAs Real-Time PCR Quantitation Kit, GenePharma Ltd., China) to detect only the mature form of the miRNA under the following conditions: degeneration at 95°C for 3 min, 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. U6 snRNA was used as an endogenous control for data normalization. The 20 μL reaction system contains 2× real-time PCR master mix 10 μL, primers (100 nM) 1 μL, cDNA 2 μL, and Taq DNA polymerase (5 U/μL) 0.2 μL.

IKBKE mRNA qRT-PCR with SYBR Green was performed using an Applied Biosystems 7300 Sequence Detection System in a 20 μL PCR mixture containing 10 μL of 2× real-time PCR master mix, 1.0 μL of primers (100 nM), 0.2 μL of Taq DNA polymerase (5 U/μL), and 2 μL of RT product. The reaction condition was 3 min at 95°C for degeneration and 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The primers are IKBKE forward 5′-TGTCTTCAGCAAACGGCAT-3′, reverse 5′-GGTCGCCAGGTCTCAGG-3′ and GAPDH forward 5′-TGGTATCGTGGAAGGACTCA-3′, reverse 5′-GTAGAGGCAGGGATGATGTTC-3′.

The relative miRNA or mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Cell Transfection with miRNA Mimic or Inhibitor. RA-FLS were transfected in 12-well plates (5×10^4 cells/well) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol, with 100 nM (final concentration) of synthetic mature miR-155 molecule (miR-155 mimic), antagomir antisense to mature miR-155 (miR-155 inhibitor), or a scrambled control serving as a negative control (NC) (GenePharma Ltd., China), which was carried out 24 hours prior to stimulation with TNF-α (20 ng/mL). Twenty-four hours after stimulation, expression levels of the MMPs and TGF-β were measured. In separate experiments, RA-FLS were transfected with miR-155 mimic, miR-155 inhibitor, or scrambled control. Forty-eight hours after transfection and twenty-four hours after stimulation, apoptotic status and invasive behavior of RA-FLS were assayed separately.

For proliferation assays, RA-FLS were transfected in 96-well plates (5×10^3 cells/well) with 100 nM (final concentration) of synthetic mature miR-155 molecule (miR-155

mimic), miR-155 Inhibitor, or a scrambled control under the stimulation of TNF-α (20 ng/mL). The group in which RA-FLS were cultured alone served as negative controls.

2.7. Apoptosis Detection. Apoptosis of RA-FLS was measured after transfection with miR-155 mimic, miR-155 inhibitor, or scrambled control for 48 hours at 37°C. Apoptosis was measured using flow cytometric detection of annexin V binding and propidium iodide (PI) staining (annexin V-FITC) according to the manufacturer's instructions.

2.8. Proliferation Assay. RA-FLS were performed in triplicate in 96-well flat-bottom microtitre plates (Corning, NY) in a total volume of 0.2 mL in RPMI 1640 supplemented with 10% FCS. After transfection with miR-155 mimic, miR-155 inhibitor, or scramble control, the RA-FLS were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 48 h. Eighteen hours before the termination of culture, 1 μCi of ^{3}H-thymidine (GE Healthcare, Amersham, UK) was added to each well. Cells were harvested onto nitrocellulose, and the radioactivity incorporated was counted in a scintillation counter. The FLS proliferation was represented as the incorporated radioactivity in c.p.m, and the results were expressed as c.p.m. ± S.D. of the mean. All experiments in our study including the following study were performed independently at least three times for each point described.

2.9. Transwell Culture. FLS were cultivated in the inner chamber of a 6.5 mm diameter Transwell plate with an 0.4 μm pore size membrane (Corning) in RPMI 1640 without FCS, while medium in the lower chamber was RPMI 1640 supplemented with 10% FCS. After 48 h, the invasive behavior of FLS was assayed using the Cytoselect 24-Well Cell Migration and Invasion Assay (Cell Biolabs Inc., San Diego) according to the manufacturer's instructions. Briefly, FLS would be distributed to wells containing FCS. Forty-eight hours later, the inserts were stained with the cell stain solution and the OD 560 nm was measured by a plate reader.

2.10. Western Blot Analysis. Equal amounts of protein samples (20 μg) extracted from the cells were separated by SDS-PAGE and transferred onto nitrocellulose membrane. After blotting, membrane was probed with primary antibodies against IKBKE (R&D Systems, USA) and β-actin (R&D Systems, USA) and subsequently incubated with secondary antibody. The membrane was then washed and visualized by ECL detection system. In this experiment, β-actin expression was shown as protein loading control.

2.11. Statistical Analysis. Data were analyzed with SPSS 13.0 for Windows. Results were expressed as mean ± S.D. Statistical analysis was done with Student's t-test for comparison of two groups and ANOVA for multiple comparisons. Spearman correlation coefficients were used to assess the correlation between miRNA expression and laboratory data. In all the cases, differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Higher Expression Level of miR-155 in RA PBMC Identified by Microarray Experiments. MicroRNA microchip experiments revealed that RA patients and normal controls show significantly characteristic differences in microRNA expression pattern. Forty-six differently expressed miRNAs were identified ($P < 0.05$, data not shown), and 14 of them were significant in expression level between RA patients and healthy controls ($P < 0.05$, value of log2 >1 or <–1, Table 1). Among these miRNAs, miR-155 significantly increased in PBMC of RA.

3.2. Trend of Increased miR-155 Expression in RA PBMC Tested by qRT-PCR. Recent studies have shown that miRNA-155 was involved in RA inflammation [7–9], and our microarray results also showed increased miR-155 expression in PBMC of RA. Based on these data, expression of miRNA-155 in RA PBMC was chosen for further identification by qRT-PCR. Increased miR-155 expression was observed in RA PBMC compared with normal controls ($n = 26, 23$, resp.), though the difference between them did not reach statistical significance ($P = 0.053$, Figure 1(a)).

3.3. Association between miR-155 and Laboratory Features in RA. To determine the effect of miR-155 expression in RA, the associations between miR-155 and laboratory features in RA patients were analyzed. A positive correlation was found between miR-155 and serum CRP level ($r = 0.56$, $P < 0.05$, Figure 1(b)). However, there is no correlation between miR-155 and other laboratory features such as RF, anti-CCP, and ESR.

3.4. Upregulated Expression of miR-155 by Stimulation of TNF-α in RA PBMC. To evaluate the stimulatory effect of proinflammatory mediators on miR-155, we stimulated RA PBMC with TNF-α, which were known to be critically involved in the development of inflammation and destruction of RA. Expression of miR-155 was significantly upregulated in RA PBMC after TNF-α stimulation. Such enhanced miR-155 expression was observed after 12 hours when cultured with stimulant, peaking at 24 hours and decreasing at 48 h (Figure 2).

3.5. Increased miR-155 Expression and Trend of Upregulation by TNF-α on RA-FLS. Since RA-FLS contribute significantly to the pathogenesis of RA, we assessed the expression of miR-155 in cultured RA-FLS ($n = 10$) and found 16.27-fold of overexpression compared with OA-FLS ($n = 8$, $P < 0.05$, Figure 3(a)). Considering that TNF-α is the most important cytokine that essentially triggers inflammation and joint destruction in RA synovium, we next investigated the regulation of miR-155 by TNF-α. Although no statistical significance was reached, we observed an obvious trend of upregulation of miR-155 after treatment of RA-FLS with TNF-α. Such increased expression was found when stimulated for 24 h, appearing to peak at 48 h and to decrease slightly at 72 h (Figure 3(b)).

Table 1: List of significantly changed miRNAs of PBMC in RA identified by miRNA microarray.

Probe	Average signal intensity of HC group	Average signal intensity of RA group	log2 (RA group/HC group)*
hsa-miR-29b	24.26	174.27	3.16
hsa-miR-374b	28.45	108.92	1.91
hsa-miR-155	848.69	1,508.07	1.78
hsa-miR-574-5p	613.87	1,688.69	1.43
hsa-miR-483-5p	211.94	525.55	1.24
hsa-miR-625	146.11	284.96	1.09
hsa-miR-149	820.87	1,876.82	1.09
hsa-miR-765	101.13	202.72	1.03
hsa-miR-612	101.76	207.68	1.03
hsa-miR-181c	561.97	140.56	−2.00
hsa-miR-148b	511.93	143.95	−1.75
hsa-miR-181a	5,411.03	2,394.80	−1.19
hsa-miR-146a	1,871.37	863.76	−1.12
hsa-miR-221	3,852.63	1,858.00	−1.08

RA: rheumatoid arthritis, HC: healthy controls; log2 (RA group/HC group) >1 or <−1 indicated the difference was significant; $^{*}P < 0.05$.

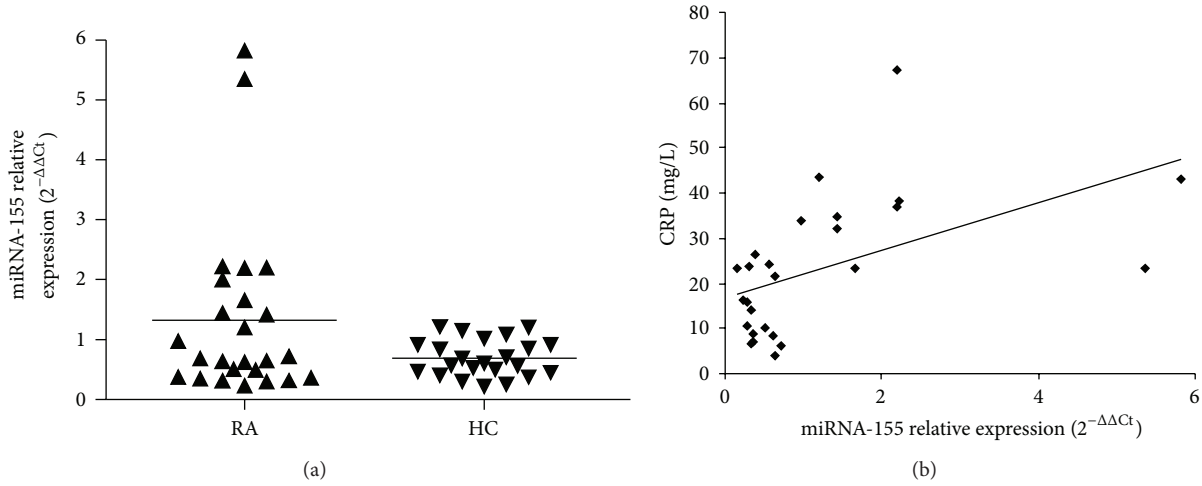

Figure 1: Validation of miR-155 expressions using qRT-PCR and correlation assessment between miR-155 and CRP. (a) Trend of miR-155 overexpression was found in RA PBMC compared to normal controls (1.29 ± 1.42, 0.69 ± 0.31, resp., $P = 0.053$). Triplicate assays were done for each RNA sample and the relative amount of each miRNA was normalized to U6 snRNA. (b) A positive correlation was found between miR-155 expression in PBMC and CRP level of RA patients ($r = 0.56$, $P < 0.05$). RA: rheumatoid arthritis, HC: healthy controls.

3.6. Effect of miR-155 on the Secretion of MMP-3, MMP-9, and TGF-β from RA-FLS. To measure the effect of miR-155 on the cytokine secretion of RA-FLS, we transfected RA-FLS with miR-155 mimic and miR-155 inhibitor separately, which are known to increase/decrease the cellular levels of mature miR-155. Following SYBR Green, qRT-PCR was carried out to confirm the effective upregulation/downregulation of miR-155 in RA-FLS on the level of mature miR-155 (Figure 4). Expression of MMP-3, MMP-9, and TGF-β as markers of the destructive and inflammatory properties of RA-FLS was detected. With up-regulated miR-155, MMP-3 levels decreased by 71.3%, while MMP-9 and TGF-β levels were not significantly changed (Figure 5). When the endogenous expression of miR-155 in RA-FLS was silenced, we found that MMP-3 levels were significantly enhanced, while MMP-9 and TGF-β levels were not influenced (Figure 5).

3.7. Suppression of RA-FLS Proliferation by miR-155. To assess the possible role of miR-155 in controlling proliferation, we transfected miR-155 mimic or miR-155 inhibitor into RA-FLS. Cell proliferation and viability were determined using the ^{3}H-thymidine incorporation assay. As shown in Figure 6(a), reducing the endogenous miR-155 brings about enhancement of RA-FLS proliferation by about 1.91-fold, and inhibition of cell proliferation to about 84.40% of control level was found when miR-155 was up-regulated.

3.8. Suppression of RA-FLS Invasion by miR-155. The invasive behavior of RAFSs was assayed using the Cytoselect 24-Well Cell Migration and Invasion Assay. RA-FLS showed a diminished invasion when transfected with miR-155 mimic. Trend of enhanced invasion was observed when endogenous expression of miR-155 was silenced ($P > 0.05$, Figure 6(b)).

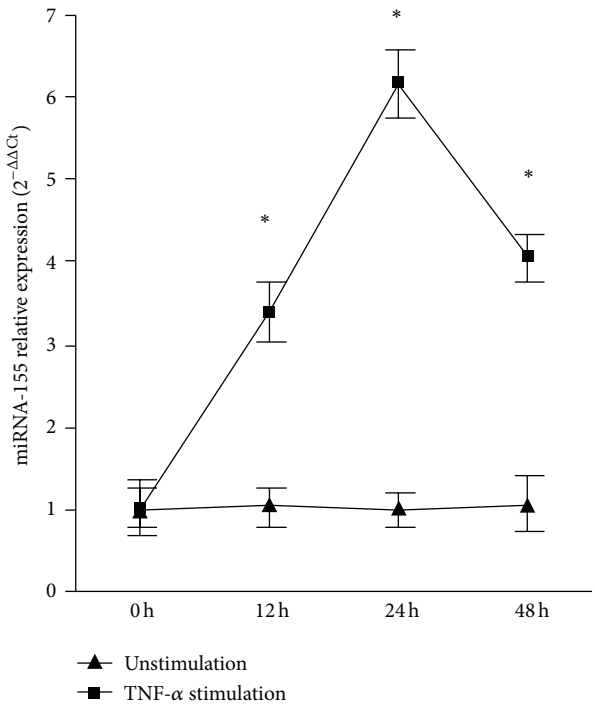

Figure 2: Induction of miR-155 expression in RA PBMC by TNF-α. The upregulation of miR-155 upon stimulation of RA PBMC, as compared with that in unstimulated control cultures ($n = 6$), was about 3.31-fold with TNF-α stimulation after 12 hours, and 6.12-fold after 24 hours, and 3.79-fold after 48 hours. $^*P < 0.05$.

3.9. No Effect of miR-155 on RA-FLS Apoptosis. To test whether or not the inhibitory effect of miR-155 on RA-FLS proliferation was associated with RA-FLS apoptosis, the percentage of apoptotic cells was evaluated by using the annexin V/PI staining. The percentage of PI and annexin V+ cells after transfection of miR-155 mimic was 0.20 ± 0.03%, which was similar to that when transfected with miR-155 inhibitor or NC control ($P > 0.05$, Figure 7).

3.10. Identification of IKBKE as Direct Targets of miR-155 in RA-FLS. IKBKE transcripts were previously identified as actual targets of miR-155 using a luciferase assay [12]. IKBKE was recently reported to play a key role in MMP gene expression and subsequent joint destruction in arthritis [13], so we investigated whether IKBKE was a target mRNA of miR-155 in RA-FLS. The effects of miR-155 overexpression/downexpression on IKBKE protein expression level were determined by Western blot. We found that miR-155 over-expression decreased IKBKE protein level compared to miRNA negative controls (Figure 8(a)). Additionally, the miR-155 inhibitor increased IKBKE protein level (Figure 8(a)). Most miRNAs function as inhibitors of target protein translation, but in a few cases, they can also induce mRNA cleavage. Therefore, we additionally determined the effects of miR-155 on IKBKE mRNA level by qRT-PCR. In comparison with the NC control, there was a significant trend of decrease in IKBKE mRNA levels in cells transfected with miR-155 and increase in IKBKE mRNA level when miR-155 was inhibited (Figure 8(b)), although no statistical significance was reached. These results showed that miR-155 down-regulated IKBKE mainly at protein level.

4. Discussion

Despite an increased understanding of the posttranscriptional regulation of gene expression via miRNA-mediated RNA interference (RNAi), the importance of miRNAs in the immune system development and response has only recently become evident. Several miRNAs involved in innate and/or adaptive immunity have been identified. Of these, evidence of miR-155 function in normal immune response has been reported.

Recent reports have shown that miR-155 is required for normal immune function. miR-155-null mice have fewer class-switched antibodies after immunization due to a failure to select high affinity plasma B cells [14, 15], indicating that miR-155 is required for B cell response. As with B cells, it seems that miR-155 is involved in T cell differentiation. miR-155-null mice showed reduced IL-2 and IFN-γ production [16], as well as an increased propensity of T cells to differentiate into Th2 rather than Th1 cells. References [14, 16] suggest miR-155 is critical in T cell differentiation.

Considering the involvement of miR-155 in immune response, studies focused on its role in autoimmunity diseases, such as RA, have emerged recently. Stanczyk et al. reported increased miR-155 expression in RA synovial fibroblasts compared to osteoarthritis synovial fibroblasts, which is the first study about the involvement of miR-155 in RA [9]. Pauley et al. observed that RA PBMC exhibited increased miR-155 compared to normal controls [8]. Here we investigated the expression of miR-155 in larger populations and showed a 1.9-fold increase in the average relative expression of miR-155 in RA PBMC compared to normal controls and 16.27-fold increase in RA-FLS compared to OA-FLS, which is similar to published reports. These findings indicate that the constitutive expression of miR-155 is increased in RA inflammatory cells.

Since TNF-α is an important proinflammatory cytokine shown to be a causative factor in RA and highly elevated in both serum and synovial fluid from patients with RA, we analyzed the expression levels of miR-155 in RA PBMC and RA-FLS upon stimulation with TNF-α. Both of them showed significantly up-regulated miRNA-155 expression upon TNF-α stimulation. These results suggested that increased level of miR-155 may be correlated with the abundant inflammatory cytokines of RA.

Our study also showed a positive correlation between miR-155 and CRP in RA patients. As CRP level is associated with high RA activity, it is reasonable to speculate that the expression of miR-155 is a potential marker indicating RA disease activity. Further studies involving a larger patient cohort are needed to fully determine whether miRNA expression can serve as a marker for disease activity; also required is an evaluation of the correlation between miRNA expression level and parameters reflecting disease activity, such as DAS28. Dynamic observations of miRNA expression are also needed to further validate the value of miRNA in disease activity assessment.

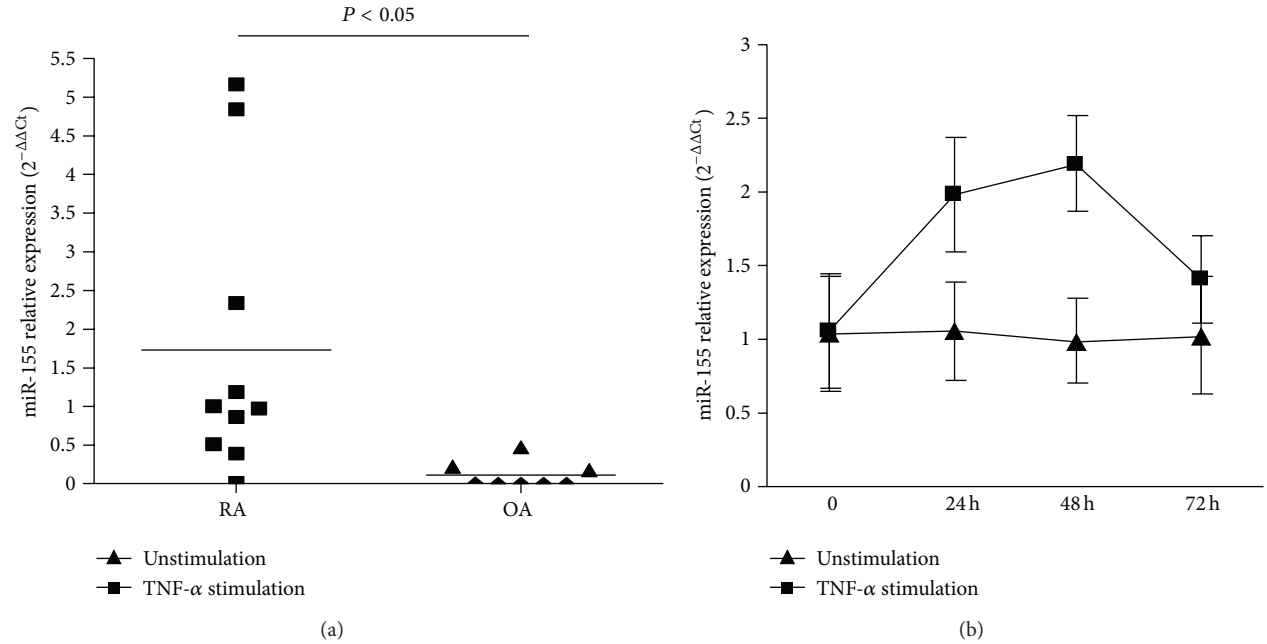

Figure 3: Constitutive expression and inductive expression of miR-155 in RA-FLS. (a) Overexpression of miR-155 in cultured RA-FLS ($n = 10$) compared to OA-FLS ($n = 8$, $P < 0.05$, 1.79 ± 1.94, 0.11 ± 0.17, resp.). (b) Trend of up-regulation by TNF-α on RA-FLS ($n = 3$). The up-regulation of miR-155 upon stimulation of RA-FLS, as compared with that in unstimulated control cultures, was about 1.87-fold with TNF-α stimulation, after 24 hours ($P = 0.068$); 2.22-fold after 48 hours ($P = 0.060$); and 1.38-fold after 72 hours ($P = 0.080$).

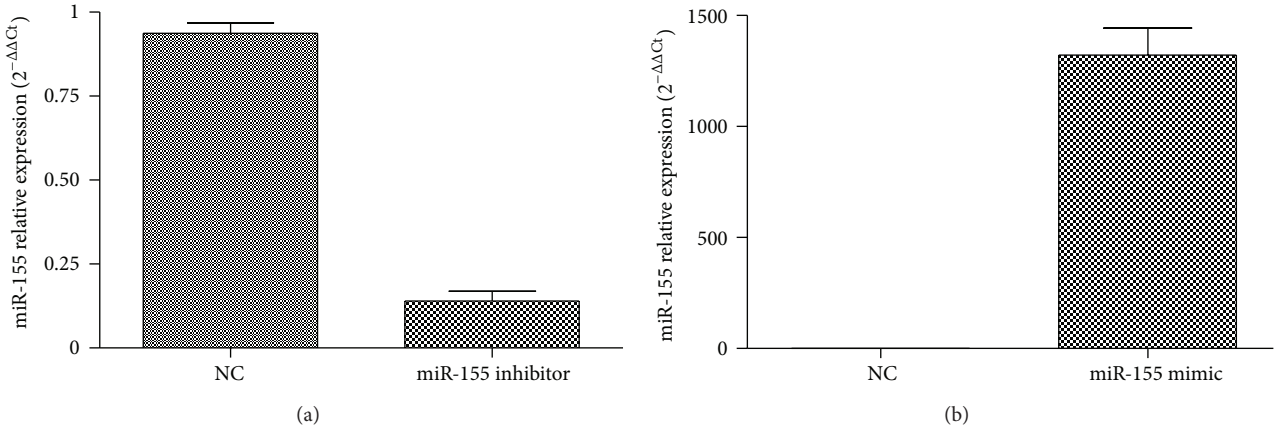

Figure 4: Altered expression of miR-155 by transfection of miR-155 inhibitor and mimic separately. (a) Transfection of miR-155 inhibitor (100 nM) for 48 h significantly decreased miR-155 level by at least 80% in RA-FLS. (b) Transfection of miR-155 mimic (100 nM) for 48 h induced at least 1000-fold increase in miR-155. miR-155 level was determined using qRT-PCR. Three independent experiments were carried out, and each experiment was done in three replicates.

To further investigate the possible role of miR-155 in RA pathophysiology, we analyzed the effect of miR-155 on RA-FLS function. RA-FLS are now considered as active players in the complex intercellular network of RA, by producing a variety of cytokines/angiogenic factors and matrix degrading enzymes as well as having aggressive and invasive behaviours. We found that silencing expression of miR-155 can significantly promote MMP-3 production and enhance proliferation of RA-FLS, which suggested that endogenous expression of miR-155 plays a key role in regulation of MMP-3 production and proliferation of RA-FLS. When transfected with miR-155 mimic, RA-FLS not only presented less MMP-3 secretion and proliferation, but also showed less aggressive behavior. Such findings revealed a possible therapeutic significance for miR-155. In addition, annexin V/PI staining showed that miR-155 induced no significant apoptosis of RA-FLS, which suggested that the effect of miR-155 on RA-FLS proliferation had no association with cell apoptosis.

Recently Stanczyk et al. reported that enforced expression of miR-155 in RA-FLS repressed the levels of MMP-3 [9], which is in line with our study. They raised the possibility of an indirect action of miR-155 on the control of expression

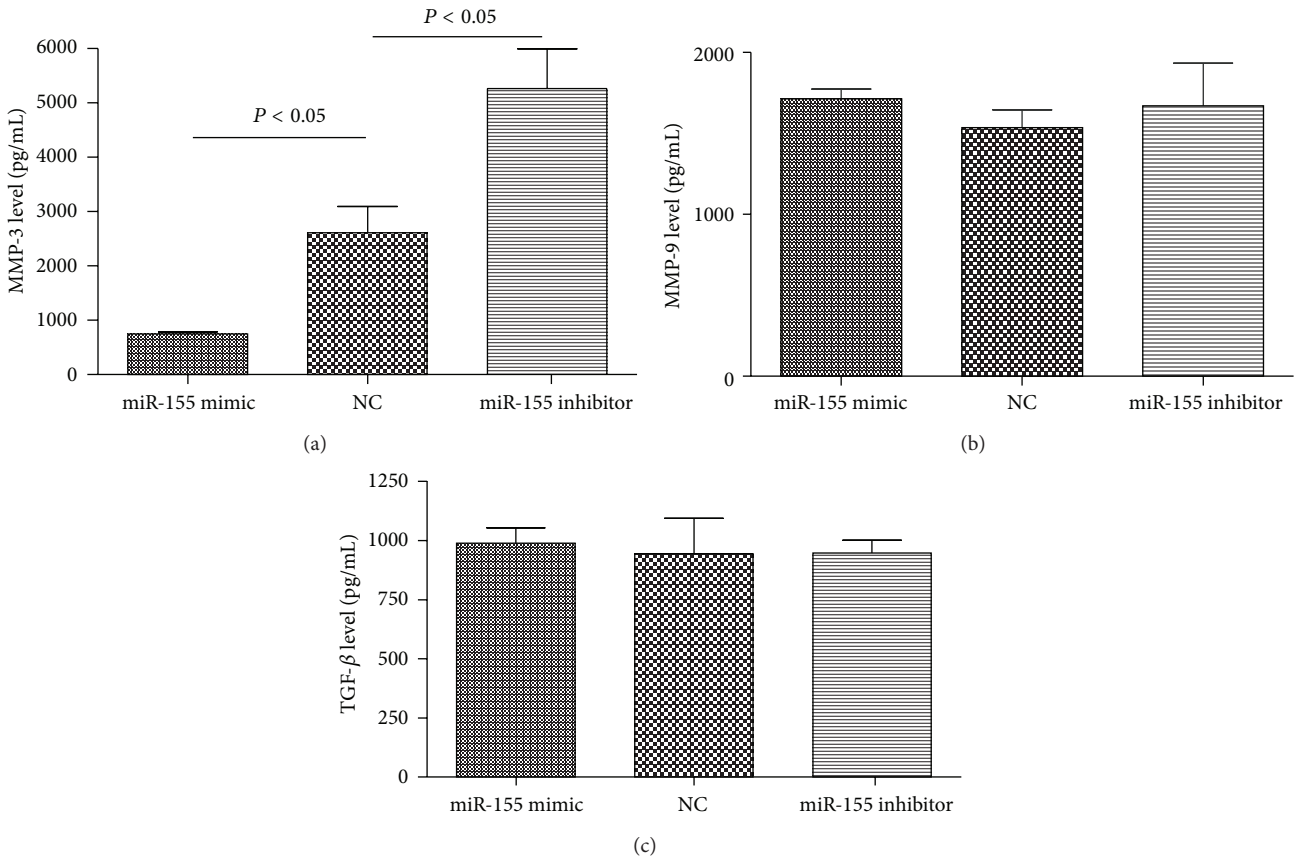

Figure 5: Effect of miR-155 on cytokines secretion of RA-FLS. (a) Increased level of miR-155 suppressed MMP-3 expression ($n = 3$, $P < 0.05$, 747.61 ± 72.19), while decreased level of miR-155 enhanced MMP-3 expression compared to NC in RA-FLS ($n = 3$, $P < 0.05$, 5263.57 ± 1260.16, 2605.23 ± 843.58, resp.). (b) Secretion of MMP-9 was not affected whether miR-155 expression was increased or decreased in RA-FLS compared to NC ($n = 3$, $P > 0.05$, 1712.28 ± 106.41, 1671.08 ± 452.25, 1535.36 ± 192.23, resp.). (c) Secretion of TGF-β was not affected whether with increased or decreased expression of miR-155 in RA-FLS compared to NC ($n = 3$, $P > 0.05$, 988.22 ± 113.55, 945.88 ± 94.56, 943.86 ± 258.72, resp.). The cells were transfected with 100 nM miR-155 mimic or miR-155 inhibitor for 48 h under 20 ng/mL TNF-α stimulation. Each experiment was in three replicates.

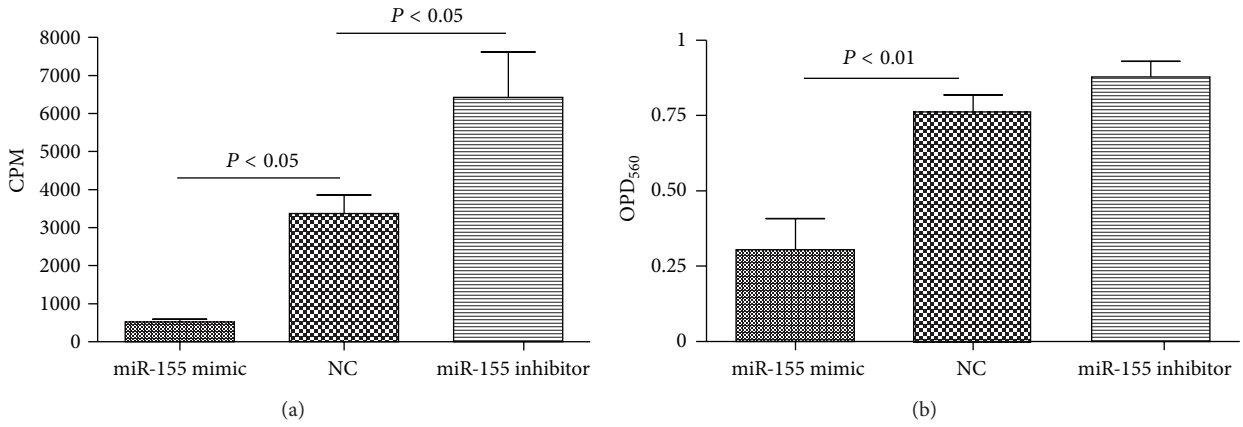

Figure 6: Suppression of RA-FLS proliferation and invasion by miR-155. (a) Cell proliferation was evaluated using ^{3}H-thymidine incorporation assay. Increased level of miR-155 reduced proliferation of RA-FLS, while decreased level of miR-155 enhanced proliferation of RA-FLS compared to NC ($n = 5$, $P < 0.05$, 525.53 ± 161.21, 6424.70 ± 2659.66, 3368.60 ± 1084.50, resp.). (b) Transwell invasive assay was used to assess the effect of miR-155 on invasion of RA-FLS. Transfection of miR-155 mimic reduced RA-FLS invasion significantly compared to NC ($n = 3$, $P < 0.01$, 0.31 ± 0.18, 0.76 ± 0.10, resp.), while transfection of miR-155 inhibitor only showed the trend of increased invasion compared to NC ($n = 3$, $P > 0.05$, 0.88 ± 0.09, 0.76 ± 0.10, resp.). Each experiment was done in three replicates.

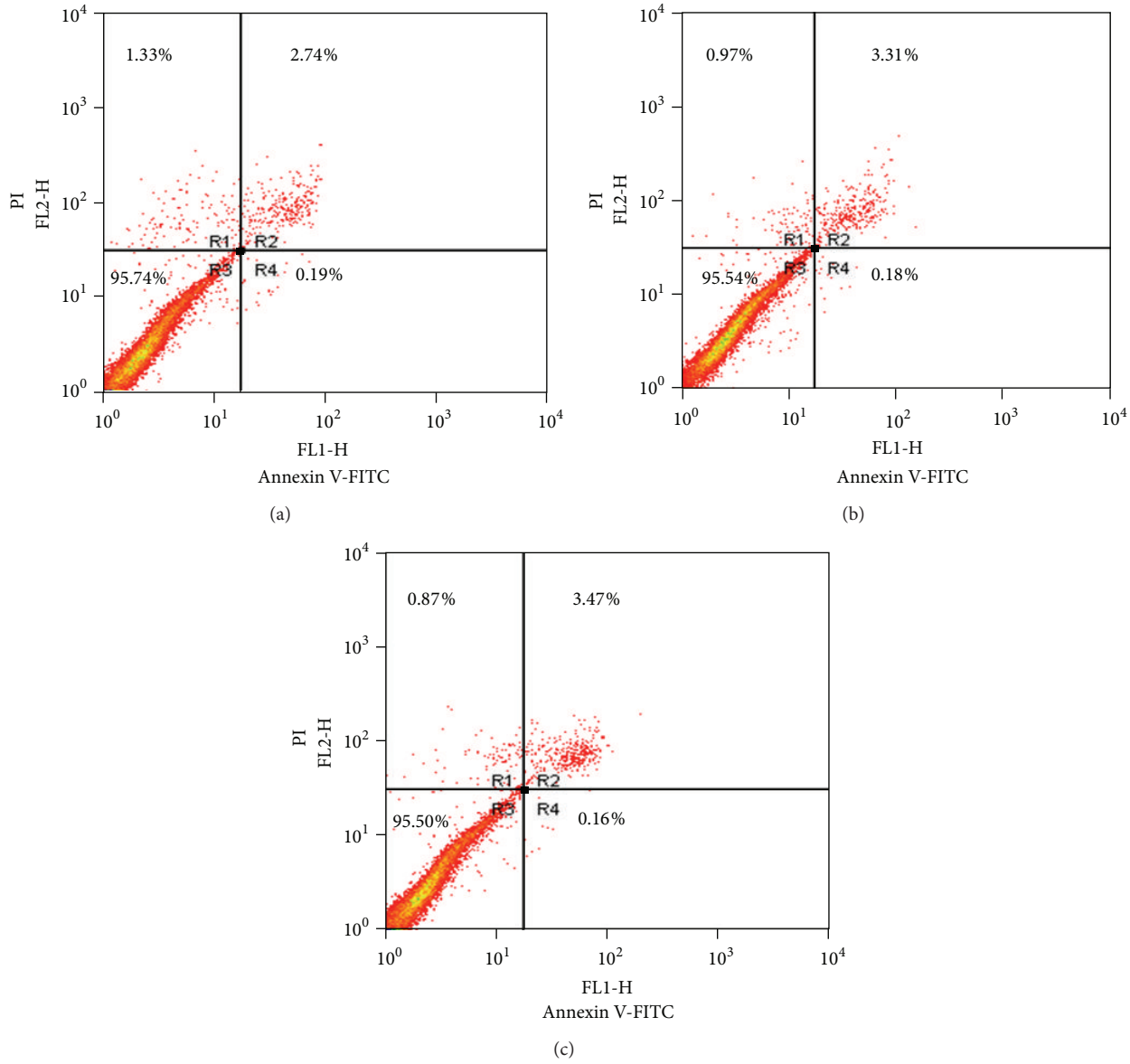

Figure 7: Effect of miR-155 on RA-FLS apoptosis. (a) Effect of miR-155 mimic on RA-FLS apoptosis. The percentage of apoptotic cells (annexin V-positive/PI-negative) was 0.20 ± 0.03%. (b) Effect of miR-155 inhibitor on RA-FLS apoptosis. The percentage of apoptotic cells was 0.20 ± 0.02%. (c) Effect of scrambled control on RA-FLS apoptosis. The percentage of apoptotic cells was 0.17 ± 0.02%. Representative results are shown. There was no statistical significance among these groups ($n = 4$, $P > 0.05$).

of MMP-3 by synergistic down-regulation of several signal-transducing proteins because MMP-3 has not been predicted (by miRGen analysis) to be a direct target of miR-155. In our study, we further investigated the possible mechanism involved. We did not find that MMP-3 transcript was a direct target of miR-155, using computational prediction provided by miRBase online searching program. However, we noticed that among reported, experimentally validated direct targets of miR-155 by luciferase report assay, after transfection with the relevant 3′UTR portion of mRNA [17], IKBKE (IKKε) was confirmed to directly increase the cytokine-mediated production of MMP-3 and MMP-13 by RA-FLS through phosphorylating c-Jun [13]. It prompted us to explore whether miR-155 regulated MMP-3 expression via targeting IKBKE in RA-FLS. We identified that protein levels of IKBKE were suppressed/enhanced when miR-155 was overexpressed/downregulated in RA-FLS. These results strongly suggested that IKBKE was the direct target of miR-155 in RA-FLS. It was tempting to speculate that miR-155 at least in part suppressed the cytokines-mediated production of MMP-3 through the down-regulation of IKBKE in RA-FLS.

Besides attenuating secretion of MMP-3, miR-155 was also shown to have potent antiproliferative and anti-invasive effects in RA-FLS. To date, there is no evidence showing that miR-155 directly targets key regulators of cellular

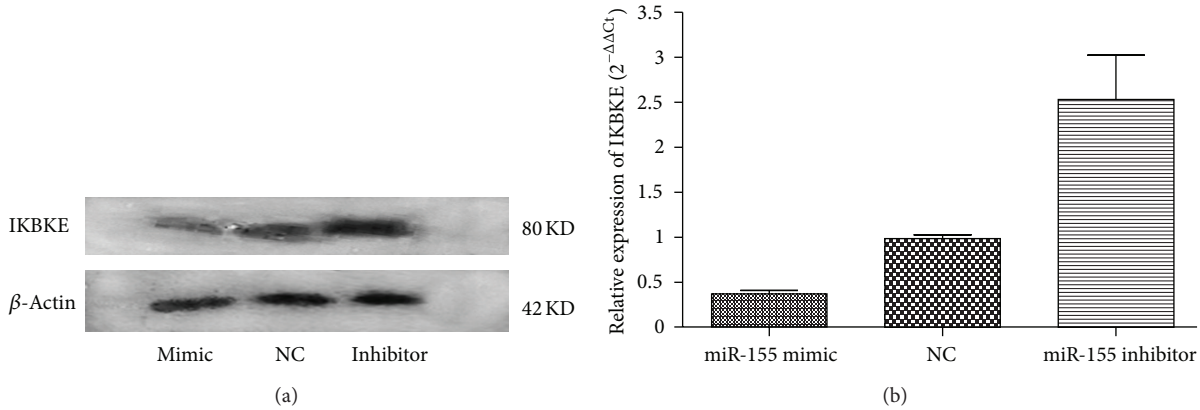

Figure 8: Regulation of miR-155 on IKBKE expression in RA-FLS. (a) Western blot analysis of IKBKE protein expression in miR-155 mimic and miR-155 inhibitor-treated RA-FLS. A representative result of 3 independent experiments is shown. Increased level of miR-155 reduced expression of IKBKE protein, while decreased level of miR-155 enhanced expression of IKBKE protein. β-Actin was used as an internal control. (b) Quantitative real-time PCR identified IKBKE mRNA levels in miR-155 mimic and miR-155 inhibitor-treated RA-FLS. Trend of reduced expression of IKBKE mRNA by increased level of miR-155 and enhanced expression of IKBKE mRNA by decreased level of miR-155 compared to NC ($n = 5$, $P > 0.05$, 0.37 ± 0.09, 2.53 ± 1.10, 0.99 ± 0.09, resp.) was observed.

proliferation and invasion, such as c-myc, ERK, and P38. MMPs especially MMP-3 were reported to be associated with proliferation and invasion of RA-FLS [18]. Since miR-155 can downregulate production of MMP-3 on RA-FLS, it is reasonable to speculate that decreased MMP-3 induced by miR-155 at least in part contributes to the suppression of proliferation and invasion.

In summary, our observation of increased miR-155 expression in both RA PBMC and RA-FLS, as well as significant up-regulation of miR-155 induced by TNF-α, suggests that such an inflammatory mediator which is usually elevated in serum and synovial fluid may at least in part contribute to increased expression of miR-155 in RA. Further functional analyses have shown that endogenous miR-155 decreases MMP-3 production and attenuates proliferation of RA-FLS in vitro, which may be a protective factor against the inflammatory effect. Additional experiments have shown that overexpression of miR-155 also inhibits RA-FLS invasion in vitro. These findings raise the possibility of a therapeutic potential of an miRNA-based approach for treating RA.

Conflict of Interests

None of the authors had any conflict of interests.

Authors' Contribution

Li Long and Ping Yu contributed equally to this work.

Acknowledgments

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Research Article

p53/p21 Pathway Involved in Mediating Cellular Senescence of Bone Marrow-Derived Mesenchymal Stem Cells from Systemic Lupus Erythematosus Patients

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Our and other groups have found that bone marrow-derived mesenchymal stem cells (BM-MSCs) from systemic lupus erythematosus (SLE) patients exhibited senescent behavior and are involved in the pathogenesis of SLE. Numerous studies have shown that activation of the p53/p21 pathway inhibits the proliferation of BM-MSCs. The aim of this study was to determine whether p53/p21 pathway is involved in regulating the aging of BM-MSCs from SLE patients and the underlying mechanisms. We further confirmed that BM-MSCs from SLE patients showed characteristics of senescence. The expressions of p53 and p21 were significantly increased, whereas levels of Cyclin E, cyclin-dependent kinase-2, and phosphorylation of retinoblastoma protein were decreased in the BM-MSCs from SLE patients and knockdown of p21 expression reversed the senescent features of BM-MSCs from SLE patients. Our results demonstrated that p53/p21 pathway played an important role in the senescence process of BM-MSCs from SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by multiorgan involvement and a remarkable variability in clinical presentations [1]. Previous studies have found that allogeneic MSCs transplantation (MSCT) used successfully in SLE achieved good efficacy [2–7]. However, Carrion and coworkers reported that autologous MSCT had no effect on disease activity in two SLE patients [8]. There are several studies that revealed that BM-MSCs from SLE patients showed impaired capacities of proliferation [9–11]. We have also found that BM-MSCs from both untreated and treated SLE patients showed prominent features of senescence, characterized by impaired capacities of proliferation, increased SA-β-gal activity, and disordered cytoskeleton distribution [12]. These findings suggested that the senescence of BM-MSCs from SLE patients may be a contributing factor to disease pathogenesis.

It has been reported that cell cycle relation proteins such as p53/p21^{Cip1}, p16^{INK4A}/Rb, and Pten/p27^{Kip1} were involved in the cellular senescence process [12–14]. We previously observed that BM-MSCs from SLE patients showed increased expression of p16^{INK4A}, knockdown of p16^{INK4A} expression increased proliferation capacities, and decreased SA-β-gal activity; it suggested that cell cycle relation protein p16^{INK4A} was involved in the cellular senescence process of BM-MSCs from SLE patients. While after knockdown of p16^{INK4A} expression, the senescence features of BM-MSCs from SLE patients were not fully reversed [12]. That implied that there were also other cell cycle relation proteins involved in regulating cell senescence of BM-MSCs from SLE patients. Recently, studies have shown that p53/p21 pathway played an important role in regulating the cell senescence progress of MSCs [15–17]. The discovery that upregulation of the p53 pathway may have a critical role in mediating the reduction proliferation of human MSCs was also reported [17]. These

data suggested that p53/p21 pathway took a part in regulating cell senescence of BM-MSCs. However, whether p53/p21 pathway was closely associated with the senescence of BM-MSCs from SLE patients has not been explored.

In the present study, we further clarified that BM-MSCs from SLE patients showed prominent features of senescence. We also found that the expressions of p53 and p21 were significantly increased, while the levels of Cyclin E, cyclin-dependent kinase-2 (CDK2), and phosphorylation of retinoblastoma protein (p-Rb) expression were decreased in BM-MSCs from SLE patients. Furthermore, we found that the expressions of p53 and p21 were significantly increased in nucleus of BM-MSCs from SLE patients, while the expressions of Cyclin E and CDK2 were significantly decreased in nucleus of BM-MSCs from SLE patients. Knockdown of p21 expression could reverse the senescent behavior of BM-MSCs from SLE patients. In our current study, our data showed that the cell senescent of BM-MSCs in SLE patients may get through the accumulation of p53 and p21 proteins.

2. Materials and Methods

2.1. Patients. Twenty-two female SLE patients aged 14–42 years (mean 27.73 ± 8.81 years) were enrolled in the study and retrieved from the archival files of the Department of Rheumatology, Affiliated Hospital of Nantong University from 2010 to 2012. The clinical features of patients summarily are shown in Table 1. The SLE diagnosis was made based on the criteria proposed by the American College of Rheumatology. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure disease activity [18]. Using a cutoff SLEDAI score of 8; all patients were categorized as active. Twenty-two healthy subjects were included as normal controls. All patients were females, and their age distribution was similar to that of the cases. All patients and controls gave consent to the study, which was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

2.2. Isolation of BM-MSCs from Bone Marrow and Cell Culture. BM-MSCs were isolated and cultured as we have reported previously [12]. Five milliliters of BM was mixed with an equal volume of phosphate-buffered saline (PBS). Then, the resuspended cells were layered over Ficoll solution (1.077 g/mL) and centrifuged at 2,000 rpm for 20 minutes at room temperature. The mononuclear cells were collected at the interface. Next, the cells were resuspended in low-glucose Dulbecco Modified Eagle Medium (L-DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). The cell viability was determined by trypan blue exclusion. Then, the cells were counted and plated at a density of 2×10^7 cells per 25 cm^2 dish. The cultures were maintained at 37°C in a 5% CO_2 incubator, and the medium was changed after 48 hours and every three days thereafter. When the BM-MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin-EDTA. The cells were then replanted at a density of 1×10^6 cells per 25 cm^2 dish. Flow cytometric analysis showed that the cells were positive for CD29, CD44, CD105, and CD166 but negative for CD14, CD34, CD38, CD45, and HLA-DR [12]. After 3 passages (p3), cells were used for the following studies.

2.3. Proliferation Assays. Cell proliferation was measured using the commercial Cell Counting Kit (CCK)-8 assays in accordance with the manufacturer's instructions. Briefly, cells were seeded onto 96-well cell culture cluster plates (Corning Inc., Corning, NY, USA) at a concentration of 2×10^4 cells/well in volumes of 100 μL, and grown overnight. Cell Counting Kit-8 reagents (Dojindo, Kumamoto, Japan) were added to a subset of wells under different treatment and incubated for 2 h at 37°C, and absorbance was quantified using an automated plate reader.

2.4. Assay for Colony Forming Unit (CFU). BM-MSCs were plated at densities of 1000, 500, 250, 100, 50, and 25 cells/cm^2 in 24-well dishes. Cells were cultured for fifteen (15) days before they were fixed and stained with 1% crystal violet in methanol. Colonies with diameters larger than 3 mm were considered for counting.

2.5. Cell Cycle Analyses. For cell cycle analysis, cells were fixed in 70% ethanol for 1 h at 4°C and then incubated with 1 mg/mL RNase A for 30 min at 37°C. Subsequently, cells were stained with propidium iodide (50 mg/mL; Becton Dickinson, San Jose, CA, USA) in phosphate-buffered saline (PBS), 0.5% Tween-20, and analyzed using a Becton Dickinson flow cytometer BD FACScan (San Jose, CA, USA) and Cell Quest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

2.6. SA-β-gal Assay. The SA-β-gal assay was used to detect cell senescence. The SA-β-gal activity was determined using a kit from the Chemical Company following the manufacturer's instructions. In brief, cells were cultured on slips in the 24-well plates overnight and fixed with paraformaldehyde. After incubated with SA-β-gal overnight, the slips were washed and analyzed under the microscope.

2.7. Western Blotting. To assay p53, p21, Cyclin E, CDK2, Rb, and p-Rb protein, the total cellular proteins was extracted through the following methods: BM-MSCs were washed in cold-buffered PBS and were then lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% NaDOD, 0.1% SDS, and 50 mM Tris, pH 8.0). After centrifugation (12,000 rpm, 5 min) at 4°C, the protein supernate was transferred into new tubes. The protein concentration of the samples was determined with a bicinchoninic acid protein assay (Pierce, USA). Equal amounts of protein were resolved using 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF, Millipore, USA) membranes. The membranes were blocked with 5% dried skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20). After 2 h at room temperature, the membranes were incubated overnight with polyclonal antibody. Antibodies used were as follows: anti-p53 (1 : 500; Santa Cruz Biotechnology); anti-p21 (1 : 500; Santa Cruz Biotechnology); anti-Cyclin E (1 : 500; Santa Cruz Biotechnology); anti-CDK2

Table 1: Clinical features of 22 SLE Patients.

Patients	Age (years) and sex	Disease duration	Current treated	SLEDAI
1	19 F	2 y	Pred: 20–40 mg/day; CTX: 0.4 g/2 weeks HCQ: 0.2/day; MMF: 1.5–2.0 g/day	30
2	22 F	1.5 y	Pred: 15–20 mg/day CTX: 0.4 g/2 weeks; HCQ: 0.2/day	24
3	39 F	10 y	Pred: 10 mg/day; HCQ: 0.2/day	8
4	37 F	8 y	Pred: 15–20 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	22
5	24 F	1 y	Pred: 15 mg/day	8
6	42 F	6 y	Pred: 20–30 mg/day HCQ: 0.4/day; CTX: 0.6 g/3 weeks	26
7	28 F	1 y	Pred: 10 mg/day; HCQ: 0.2/day	12
8	23 F	1 y	Pred: 5–7.5 mg/day HCQ: 0.2/day; CTX: 0.4 g/4 weeks	18
9	32 F	3 y	Pred: 5–10 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	12
10	25 F	4 y	Pred: 5–7.5 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	16
11	14 F	2 y	Pred: 5–10 mg/day HCQ: 0.2/day; MMF: 1.5–2.0 g/day	9
12	21 F	2 m	Pred: 7.5–10 mg/day MMF: 1.5–2.0 g/day; HCQ: 0.2/day	19
13	32 F	4 m	Pred: 5–7.5 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	16
14	20 F	7 m	Pred: 10–15 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	21
15	24 F	2 m	Pred: 7.5–10 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	18
16	22 F	3 m	Pred: 30–40 mg/day MMF: 1.5–2.0 g/day; HCQ: 0.4/day	26
17	15 F	1 m	Pred: 5 mg/day; HCQ: 0.2/day	8
18	33 F	4 m	Pred: 5–7.5 mg/day LEF: 0.2 g/day; HCQ: 0.2/day	12
19	36 F	3 m	Pred: 7.5 mg/day; HCQ: 0.2/day	9
20	23 F	8 m	Pred: 20–40 mg/day CTX: 0.4 g/2 weeks; HCQ: 0.2/day	17
21	20 F	4 m	Pred: 20–30 mg/day CTX: 0.4 g/4 weeks; HCQ: 0.2/day	14
22	29 F	6 m	Pred: 20–40 mg/day CTX: 0.4 g/2 weeks; HCQ: 0.2/day	20

y: years; m: mouth; S: skin; J: joints; H: hematologic; M: myositis; V: vasculitis; R: renal; Pred: prednisolone; HCQ: hydroxychloroquine; CTX: cyclophosphamide; LEF: Leflunomide; MMF: Mycophenolate Mofetil.

(1 : 1000; Santa Cruz Biotechnology); anti-Rb (1 : 500; Santa Cruz Biotechnology); and anti-p-Rb (1 : 500; Santa Cruz Biotechnology). Then, horseradish peroxidase-linked IgG was used as the secondary antibody. Immunoreactive bands were visualized by chemiluminescence (NEN Life Science Products, Boston, MA, USA). After the chemiluminescence was exposed to X-ray films, the films were scanned using a Molecular Dynamics densitometer (Imaging Technology, ON, Canada). The experiments were carried out on three separate occasions.

2.8. Immunofluorescence. Immunofluorescence was used to examine the lactation and expression of p53, p21, Cyclin E and CDK2 in BM-MSCs. At p3, the cells were seeded onto 25 mm dishes and cultured for 24 h. After washing with PBS, BM-MSCs were fixed with 4% paraformaldehyde (PFA) and the cells were blocked in 1% bovine serum albumin (Sigma-Aldrich, St. Louis) and 0.2% Triton-100 (Sigma-Aldrich) and then incubated at 37°C for 1 h with primary antibody to p53 (anti-rabbit, 1 : 100, Santa Cruz), p21 (anti-mouse, 1 : 200, Santa Cruz), CDK2 (anti-rabbit, 1 : 200, Santa Cruz), and Cyclin E (anti-mouse, 1 : 200, Santa Cruz). Then, the cells were washed and incubated in the dark for 1 h at 37°C with goat anti-rabbit- (cy3-) conjugated antibodies (1 : 300, ICN Cappel, USA) or goat anti-mouse FITC-conjugated antibodies (1 : 300, Dako, USA); the nuclei were counterstained with DAPI. After being washed and mounted, the cells were examined under a fluorescence microscope.

2.9. Separation of the Nuclei and Cytoplasm. To assay the p53, p21, Cyclin E, and CDK2 proteins, cytoplasmic and nuclear proteins from cultured cells were prepared using

NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Company, USA), respectively. β-actin and β-tubulin were used as the internal control for the cytoplasmic and nuclear proteins. Cells were lysed in ice-cold hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.625% Nonidet P-40) for 15 minutes on ice. After vortexing for 10 seconds, the lysate was centrifuged for 10 minutes at maximum speed to obtain the cytoplasmic fraction in the supernatant. The remaining pellet was incubated with hypertonic buffer (20 mM HEPES, 420 mM NaCl, 25% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.12 mM Aprotinin per mL) for 60 minutes on ice and then centrifuged to obtain the supernatant containing the nuclear fraction. The protein concentration of the samples was determined by a bicinchoninic acid protein assay (Pierce, USA). The cytoplasmic fraction, the nuclear fraction, and the whole-cell lysates were used for western blot analysis as described previously. Antibodies used were as follows: anti-β-actin (1 : 600; Santa Cruz Biotechnology); anti-β-tubulin (1 : 600; Santa Cruz Biotechnology); and p53, p21, Cyclin E, and CDK2 protein antibodies as described previously.

2.10. siRNAs and Transfection. A double-stranded RNA that targeted human p21 and a nonsilencing control siRNA were obtained from Santa Cruz Biotechnology. The transfection of the BM-MSCs with the synthetic siRNA was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were assayed after 48 h of transfection. For the mock transfection, the procedure was performed in the absence of the siRNA duplex.

2.11. RNA Preparation and RT-PCR. Total RNA of BM-MSCs cells were extracted using a Trizol extraction kit according to the manufacturer's procedure. Total RNA was reverse-transcribed using the Thermo Script RT-PCR system (Invitrogen). Primers pairs for p21 were sense, 5′-CAGAATCACAAACCCCTA-3′, and antisense, 5′-TGTTTTGAGTAGAAGAAT-3′. Cycling conditions were 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, and a total of 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control and was detected using the primers sense, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, and antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. Cycling conditions were 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a total of 28 cycles. The PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide staining. The relative differences in the expression levels were normalized using GAPDH.

2.12. Statistical Analysis. The density of bands in Western blots or RT-PCR were measured with image analysis system. All of the results were representative of three independent experiments. All data were presented as mean ± standard deviation (SD) of the replicates and were analyzed by Student's t-test with P values less than 0.05 considered statistically significant. All of the statistical analyses were performed using SPSS 11.0 software.

3. Results

3.1. BM-MSCs from SLE Patients Showing Prominent Feature of Senescence. As we have studied previously, BM-MSCs from SLE patients appeared bigger in size and flattened in appearance (Figure 1(a)). From growth curve, we found that BM-MSCs from SLE patients grew more slowly than those from the normal group (Figure 1(b), $P < 0.05$). Simultaneously, colony-forming unit (CFU) potential of BM-MSCs declined by about a quarter in SLE patients compared to normal group (Figures 1(c)-1(d), $P < 0.05$) indicating that the capability of replicating and forming colonies of BM-MSCs from SLE patients were decreased. Furthermore, we have found that the number of SA-β-gal-positive cells was notably increased in BM-MSCs from SLE patients, which was used to examine MSCs senescence. The cell count revealed that the numbers of SA-β-gal-positive cells from SLE patients were obviously higher than those of normal group (Figures 1(e)-1(f), $P < 0.05$). Beyond these, the cell cycle distribution of BM-MSCs was determined by FACS analysis following propidium iodide staining of cellular DNA showed that there were more BM-MSCs restricted in the G1 phase which were harvested from SLE patients than that of normal group (44.36 ± 2.36% versus 72.89 ± 3.21%, Figures 1(g)-1(h)). These data indicated that the BM-MSCs from SLE patients were senescent cells, which were similar to previous studies [12].

3.2. The p53/p21 Pathway Plays an Important Role in Cell Senescence of BM-MSCs from SLE Patients. It is reported that the p53/p21 pathway plays an important role in regulated BM-MSCs senescence process. In the present study, we found that the expressions of p53 and p21 were increased in the BM-MSCs from SLE patients (Figures 2(a)-2(b); $P < 0.05$, resp.), while the expressions of Cyclin E and CDK2 were markedly decreased in BM-MSCs from SLE patients (Figures 2(c)-2(d); $P < 0.05$, resp.). Moreover, a reduced phosphorylation of Rb in MSCs from SLE patients was detected (Figures 2(c) and 2(e); $P < 0.05$).

3.3. p53 and p21 Were Made Function That Depends on Mainly Localization in Nuclear Fraction of the BM-MSCs from SLE Patients. Recent studies have found that protein and its function were based on a subcellular localization. The p53 and p21 proteins which accumulated in the nucleus are necessary for cell cycle arrest. In our study, tested by immunofluorescence staining, we observed that p53 and p21 were mainly localized in the nuclei of the BM-MSCs from SLE patients, whereas lower levels were found in the cytoplasm of the BM-MSCs from SLE patients than that of the normal control (Figures 3(a) and 3(d)). To further detect the levels of these proteins' expression, we used separation of the nuclei and cytoplasm and Western blot analysis. We found that p53 and p21 were expressed more in the nuclei of the BM-MSCs from SLE patients, whereas lower levels were found in the cytoplasm of the BM-MSCs from SLE patients than that of the normal control (Figures 3(b), 3(c), 3(e), and 3(f); $P < 0.05$, resp.). In the meantime, we observed that Cyclin E and CDK2 were mainly localized in the cytoplasm and

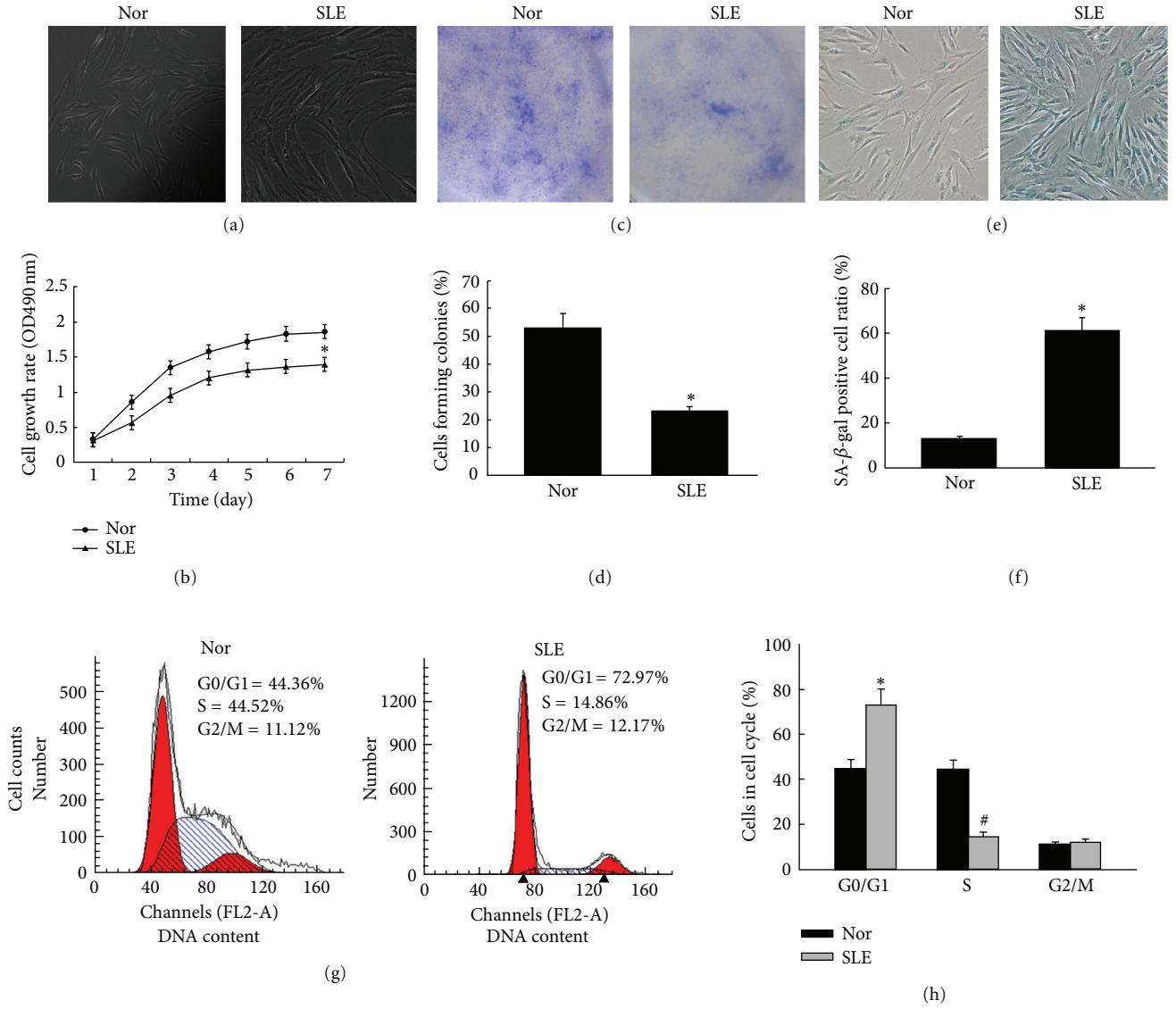

Figure 1: BM-MSCs from SLE patients are aging cells. (a) At p3, the morphology of normal BM-MSCs showed homogeneous spindle-shaped fibroblast-like growth. However, BM-MSCs from SLE patients appeared bigger in size and flattened in appearance. (b) Growth curve of BM-MSCs was tested by cell counting assay. The absorbance was shown as the proliferation rate. BM-MSCs from SLE patients grew more slowly than those from the control group. Each point represents quantities relative to the normal group at Day 1 ($^*P < 0.05$). ((c)-(d)) Counted colonies of BM-MSCs from SLE patients and normal control were plated at a density of 25 cells = cm^2 for 15 days in culture, stained with 1% crystal violet. Each bar represents quantities relative to BM-MSCs from Nor group and is mean ± SD of three experiments ($^*P < 0.05$). ((e)-(f)) SA-β-gal was used to examine BM-MSCs senescence. BM-MSCs from SLE patients and normal control were performed SA-β-Gal staining. The number of SA-β-gal-positive cells obviously increased in BM-MSCs from SLE patients compared with those of normal control. Each bar represents quantities relative to BM-MSCs from Nor group and is mean ± SD of three experiments ($^*P < 0.05$). (g) After cells culture, cells were removed from the culture dish with trypsin/EDTA at p3, fixed, stained for DNA with PI, and analyzed by flow cytometry (y-axis, cell count; x-axis, DNA content). (h) Mitotic indices of BM-MSCs from control group and SLE patients. Graphs in (h) represent DNA content indicating the percentages of cells in G0/G1, S, and G2/M phases of the cell cycle. The data are obtained from staining the DNA of ASCs from normal group and SLE patients. A decrease in the percentage of cells in S phases was seen in SLE patients, while an increase in percentage of cells in G0/G1 phase was seen in SLE patients. Each bar represents quantities relative to BM-MSCs from Nor group and is mean ± SD of three experiments ($^*P < 0.05$) ($^\#P < 0.05$). Following the cell cycle progression, these cells showed a greater fraction in quiescent of G0/G1 phase in SLE patients when compared with normal control.

lower levels were found in the nuclei of BM-MSCs from SLE patients than that of the normal control (Figures 4(a) and 4(d); resp.). Furthermore, we found that, Cyclin E and CDK2 were expressed more in the cytoplasm of the BM-MSCs from SLE patients, whereas lower levels were found in the nuclei of the BM-MSCs from SLE patients than that of the normal control (Figures 4(b), 4(c), 4(e) and 4(f); $P < 0.05$, resp.). The results suggested that p53, p21, and CyclinE-CDK2 effect on

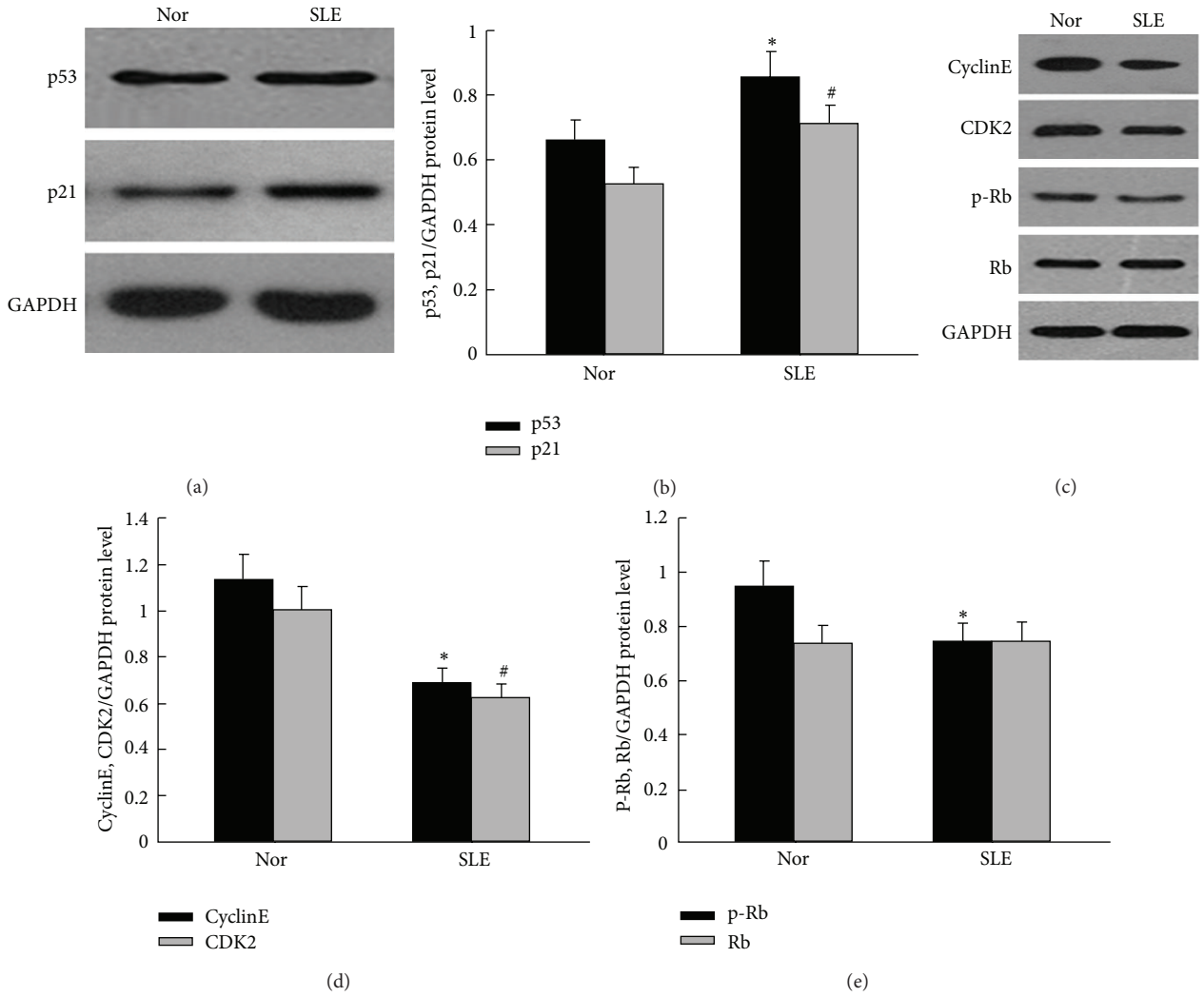

Figure 2: The expression of p53/p21 and cell cycle-related molecules in BM-MSCs from SLE patients. Western blot was used to analyze p53/p21 and their relative proteins expressions. (a) The expression of p53 and p21 was significantly increased in BM-MSCs from SLE patients. (b) Quantification of p53 and p21 protein levels: the relative levels of protein expressions were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{*}P < 0.05$; $^{\#}P < 0.05$). (c) Western blot analysis and the expressions of Cyclin E, CDK2, and p-Rb in BM-MSCs from SLE patients were decreased. (d) Quantification of Cyclin E and CDK2 protein levels: the relative levels of protein expressions were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{*}P < 0.05$; $^{\#}P < 0.05$). (e) Quantification of p-Rb and Rb protein levels: the relative levels of protein expressions were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{*}P < 0.05$). GAPDH was used as the internal control. Total p53 and p21 were significantly increased in BM-MSCs from SLE patients, while the expressions of Cyclin E, CDK2, and p-Rb were significantly decreased in BM-MSCs of SLE patients when compared with those in normal control.

BM-MSCs senescence process of SLE patients might base on them subcellular localization.

3.4. Knockdown of p21 Expression Reversed Feature Senescence of BM-MSCs from SLE Patients. To further assess the role of p53/p21 in the BM-MSCs senescence progress, we have transfected the BM-MSCs with p21 siRNA and a nonspecific siRNA. As predicted, the p21 expression was considerably decreased in the p21 siRNA-transfected BM-MSCs from SLE patients (Figure 5, $P < 0.05$). To investigate the role of p21 in BM-MSCs senescence, BM-MSCs were cultured with or without p21 siRNA. At p3, the morphology of BM-MSCs from SLE patients culture in p21 siRNA appeared a fibroblast-like morphology (Figure 6(a)); moreover, cell proliferation assay showed that the proliferation rate of p21 knockdown BM-MSCs from SLE patients was increased (Figure 6(b)) and CFU potential of BM-MSCs from SLE patients cultured with p21 siRNA increased by about a half (Figures 6(c)-6(d), $P < 0.05$). Meanwhile, compared to BM-MSCs from SLE patients cultured without p21 siRNA, SA-β-gal-positive cells were notably decreased and about a quarter of the cells were stained positive in p21-knockdown BM-MSCs from the SLE patients (Figures 6(e)-6(f), $P < 0.05$). Furthermore, for cell cycle distribution of BM-MSCs, there were more cells restricted in the S phase harvested from BM-MSCs after p21

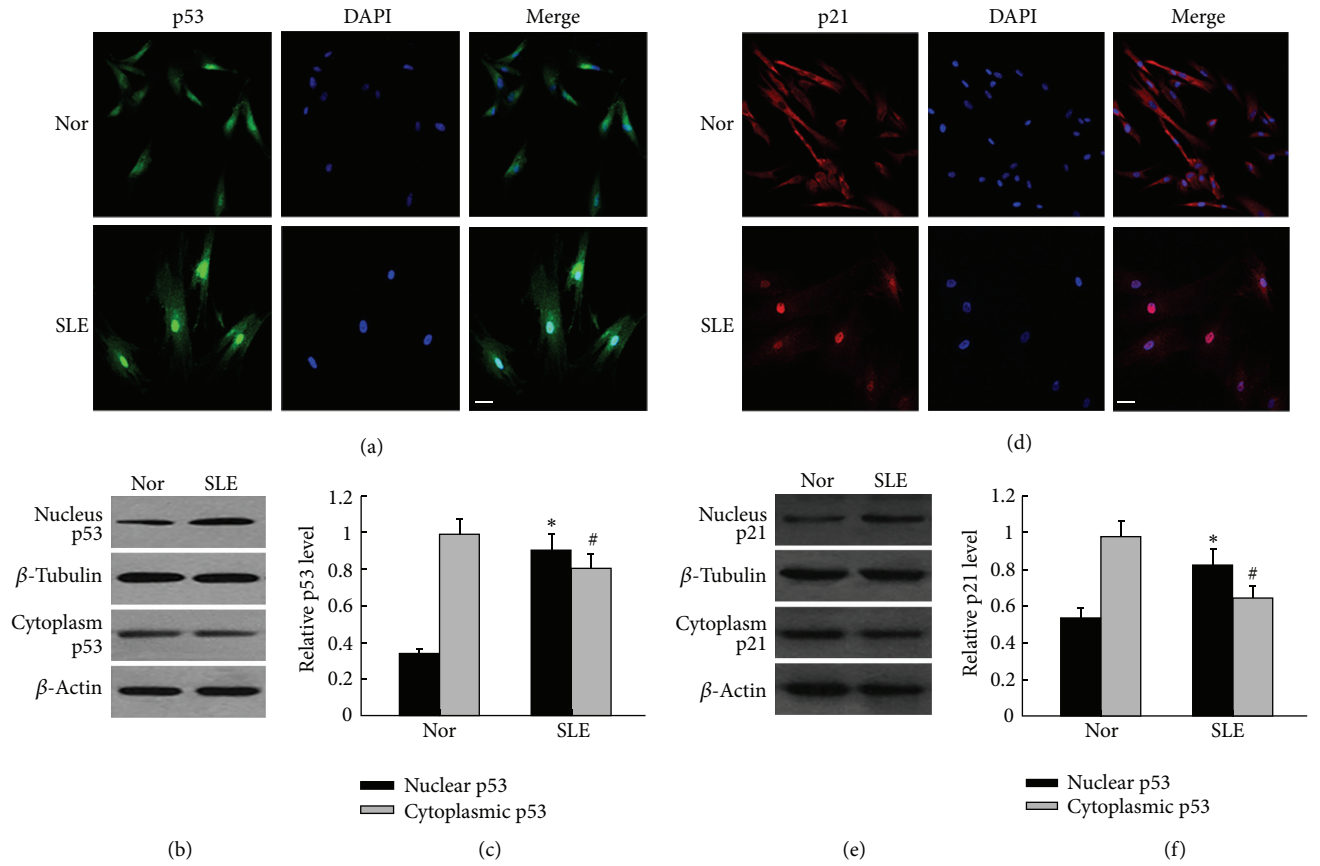

FIGURE 3: Analysis of the location and expression of p53 and p21 in the cytoplasmic and nuclear of BM-MSCs from SLE patients. ((a), (d)) Immunofluorescence staining to analyze the location of p53 and p21 in BM-MSCs. In the BM-MSCs from SLE patients, there was a clear increase in nuclears p53 and p21 expression. The scale bar is 25 mm. ((b), (e)) Western blot analysis of the cytoplasmic and nuclear p53 and p21 expressions. β-Actin was used as the internal control for the cytoplasmic proteins, whereas β-tubulin was used as the internal control for the nuclear proteins. ((c), (f)) Quantification the expressions of p53 and p21 in the nuclear and cytoplasmic. Compared with normal control, the location and expression of p53 and p21 in the nuclear of BM-MSCs were significantly higher in SLE patients. The relative levels of protein expressions were normalized to β-actin expression. Values are means ± SD of three experiments ($^*P < 0.05$).

knockdown (20.37 ± 3.25% versus 33.26 ± 3.54%, Figures 6(g)-6(h)). Meanwhile, we also detected that the expressions of Cyclin E, CDK2, and p-Rb were increased in BM-MSCs treatment with si-p21 (Figures 6(i)–6(k), $P < 0.05$). These results implied that the p53/p21 pathway plays an essential role in BM-MSCs ageing of SLE patients.

4. Discussion

In our study, we further confirmed that BM-MSCs from SLE patients showed prominent features of senescence, which were characterized by increased cell size, decreased proliferation and colony forming potential, more cells restricted in the G0/G1 phase, and increased SA-β-gal activity. We found that the expressions of p53 and p21 were significantly increased, while levels of Cyclin E, CDK2, and p-Rb expressions were decreased in BM-MSCs from SLE patients. We also found that the expressions of p53 and p21 were significantly increased, while Cyclin E and CDK2 were significantly decreased in nucleus of BM-MSCs from SLE patients. Knockdown p21 expression could reverse the senescent behavior of BM-MSCs from SLE patients.

Recently, research showed that p53/p21 pathway plays an important role in the cell senescent [19–23]. It has been reported that cell cycle progression is regulated by cyclins and CDK, such as Cyclin E and CDK2, and this regulation is negatively inhibited by tumor suppressors, such as p53 and CDK inhibitors p21, a target gene of p53 protein [24]. Elmore found that overexpression of p53 causes arrest of cell growth [25]. p21 is a key molecule in cell cycle regulation that binds to and inhibits the activity of CDK complexes, thereby that inhibits Rb phosphorylation. The phosphorylation of Rb is a well-described regulator of the cell cycle [26]. It has also been demonstrated that the p21 accumulates progressively in aging cells, binds to, and inactivates all Cyclin E-CDK2 complexes, which is responsible for the phosphorylation of the Rb results in irreversible G1 arrest [27–29]. Additionally, that overexpression of p21 arrests the cell-cycle transition from the G1- to the S-phase via inhibition of CDK2 activity, expression of p21 was greater, and simultaneously the expression of CDK2 was lower which has also been documented [30]. Further,

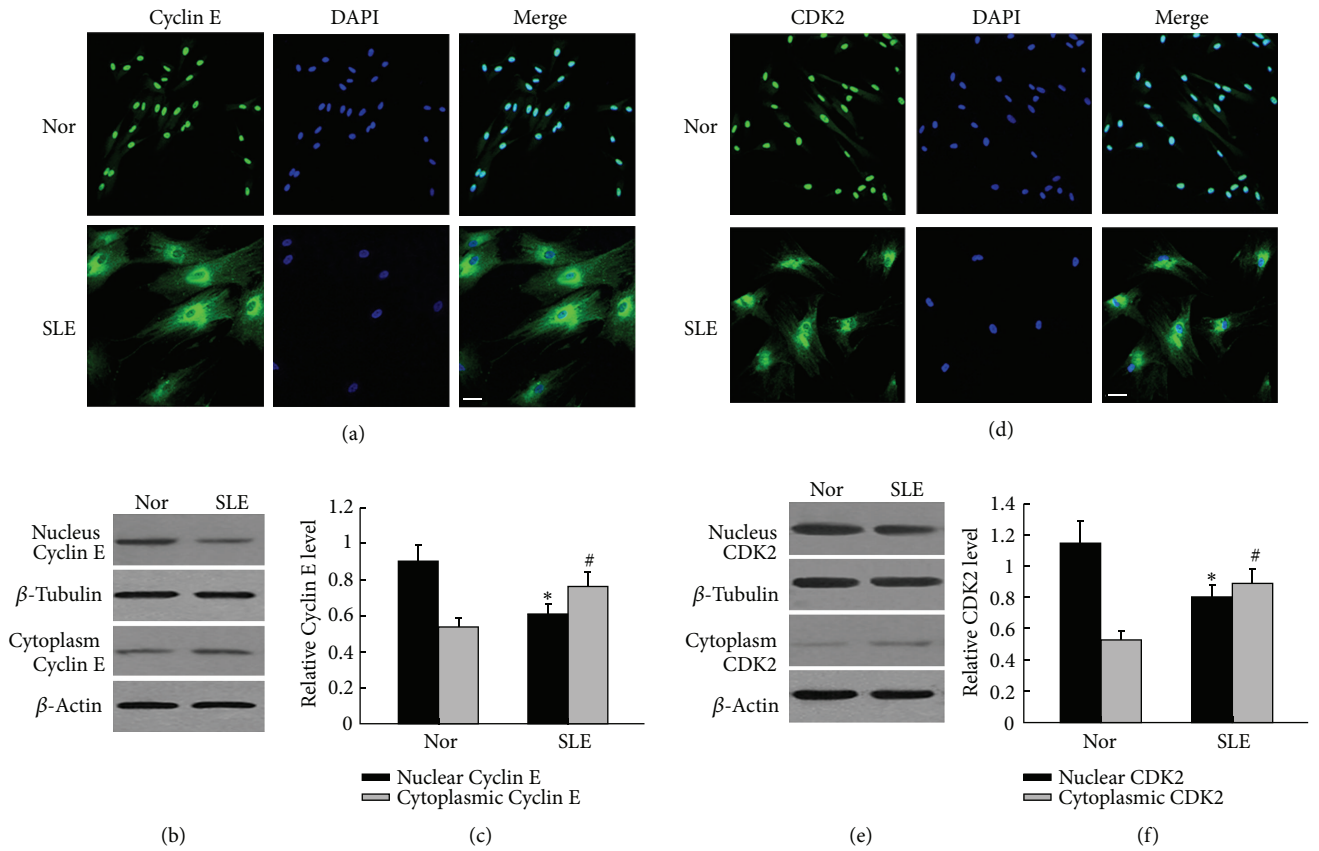

Figure 4: Analysis of the location and expression of Cyclin E and CDK2 in the cytoplasmic and nuclear of BM-MSCs from SLE patients. ((a), (d)) The locations of Cyclin E and CDK2 in BM-MSCs were tested by immunofluorescence staining. We found that the expression of Cyclin E and CDK2 was notably decreased in nuclear of BM-MSCs from SLE patients, while there were notably higher expressions in nuclears in normal control. The scale bar is 25 mm. ((b), (e)) Western blot to analyze the expressions of Cyclin E and CDK2 in cytoplasmic and nuclear. ((c), (f)) Quantification the expressions of Cyclin E and CDK2 in the nuclear and cytoplasmic. Compared with normal control BM-MSCs, Cyclin E and CDK2 in the nuclear were significantly lower in SLE patients; the relative levels of protein expressions were normalized to β-actin expression. Values are means ± SD of three experiments ($^*P < 0.05$).

it has been found that, in inhibited cell proliferation, the levels of p21 increased and the levels of Cyclin E and CDK2 decreased [31]. Others have found that virus-induced cell cycle arrest induced p53 and p21 accumulation and decreased phosphorylation of Rb [26]. Those studies suggest that the p53/p21 pathway, by upregulated p53, p21 and downregulated cyclins, CDK, and p-Rb, plays an important role in regulating the cell senescent process. Some researchs have been reported that the protein and its function were based on a subcellular localization [29]. The main role of p53 and p21 proteins that are accumulated in the nucleus is the cell cycle arrest response to genotoxic stress such as DNA damage. In the nucleus, p53 works as a transcriptional factor and regulates transactivation of several proteins, including the p21 [32]. It has been reported that the presence of p21 protein in the nucleus is necessary for cell cycle arrest. In the nucleus, p21 binds to and inhibits the activity of cyclin-dependent kinases and blocks the transition from G1 phase into S-phase [29]. Others have found that nuclear CDK2 is associated with proliferation [33]. Because of the prominent role that p53 has in the DNA damage response of differentiated cells, it is most likely that p53 has a similar function in stem cells. It has also been reported that after DNA damage, nuclear accumulation and activation of p53 were found in stem cells. Solozobova et al. have found that, in proliferating embryonic stem cells, p53 is localized predominantly in the cytoplasm. DNA damage-induced nuclear accumulation of p53 in embryonic stem cells activates transcription of the target gene p21 [32, 34]. These results suggested that p53, p21, and CDK2 exert function were based on their subcellular localization.

Numerous studies have shown that the p53/p21 is pathway involved in regulating MSCs senescence [14, 15, 17, 35, 36]. It has been found that p53 regulates the proliferation of MSCs [16]. The expressions of both p53 and p21 were significantly upregulated with diminished capacities for proliferation in old rhesus monkeys BM-MSCs compared to those from young donors [37]. Furthermore, the late-passage MSCs showed increased expression of p21 and decreased proliferation capacity has also been documented [38]. A line of evidence indicated accelerating MSCs proliferation by downregulation of p21 [38, 39]. Emerging evidence indicates that, transforming growth factor β-induced

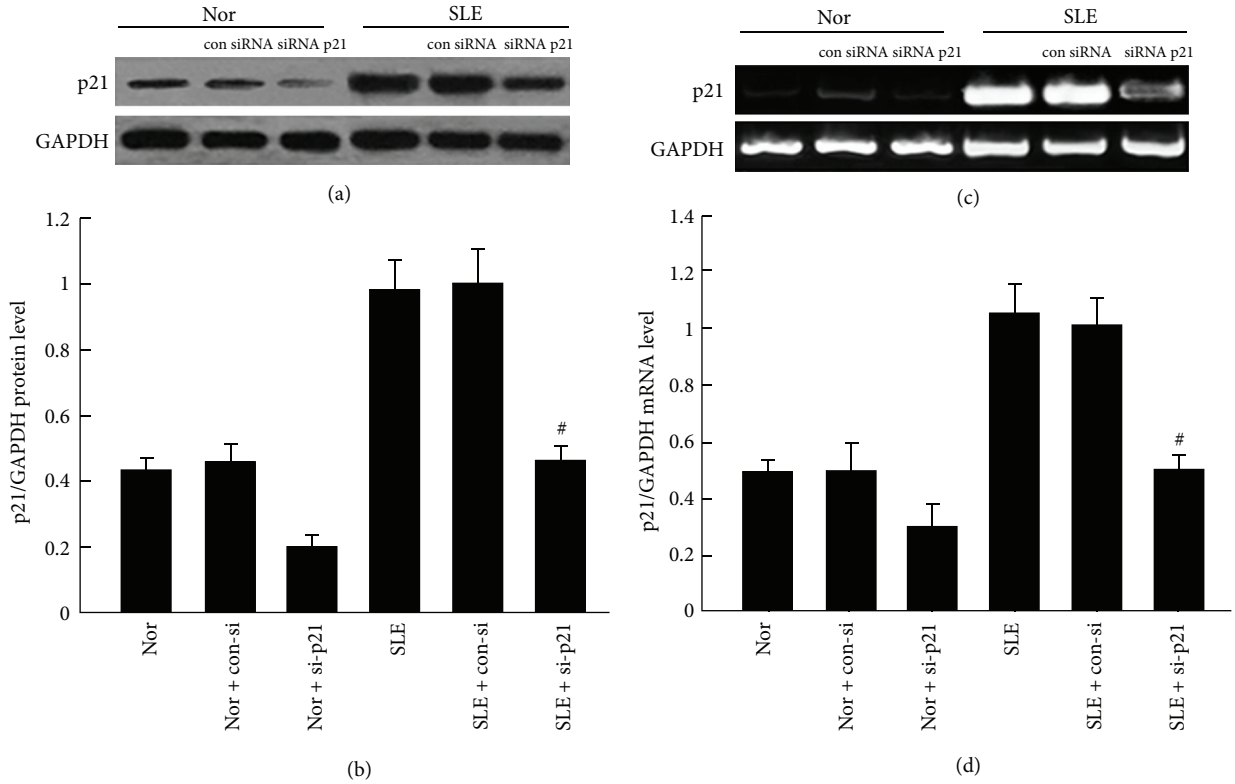

Figure 5: p21 siRNA decreased p21 expression in BM-MSCs. Cells were transfected with p21 siRNA for 24 hours. p21 expression was significantly decreased in BM-MSCs both in SLE patients and normal control by immunoblot analyses ((a)-(b)) and RT-PCR ((c)-(d)). The relative levels of protein expressions and gene expression of target mRNA were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{\#}P < 0.05$).

BM-MSCs senescence through the increased of expressions of p53 and p21, low expression of p-Rb, after treatment with cell growth factors, the cell growth arrest was suppressed through the suppression of p21 and p53 expression levels and the increase of p-Rb expression levels [40]. In addition, MSCs treated with small interfering RNA targeting p21 were demonstrated proliferation significantly faster than control cells [39], while it has been documented that knockdown p21 enhances proliferation, the expression of stemness markers, and osteogenic potential in human MSCs [38]. It has also been demonstrated that p21−/− mice had significantly less radiation damage, including 40% increased growth potential. They also have found that p21−/− MSCs had 4-fold greater proliferation rate and nearly 7-fold lower senescence as compared to control MSCs [41]. These results suggested that the p53/p21 pathway plays an important role in regulating BM-MSCs cell senescence process. In the BM-MSCs from SLE patients, we observed that the expressions of p53 and p21 were increased, while the levels of Cyclin E and CDK2 were strongly decreased, and the phosphorylation of Rb was also decreased (Figure 2). To further confirm the role of the p53/p21 pathway in regulating senescence process of BM-MSCs from SLE patients, we used MSCs transfected with p21 siRNA inhibition of p21 expression in BM-MSCs from SLE patients. We found that the morphology of BM-MSCs from SLE patients showed more spindle-shaped fibroblast-like growth, increased proliferation rate, increased CFU, less SA-β-gal-positive cells, and more cells restricted in the S-phase harvested from SLE patients' BM-MSCs after knockdown of p21 expression. We found that Cyclin E and CDK2 were increased in SLE patients' BM-MSCs culture with siRNA p21, and p-Rb also increased in the knockdown p21 of BM-MSCs (Figure 6). Interestingly, we also found that more expressions and more location of p53 and p21 were found in nuclear whereas lower levels were found in the cytoplasm of the BM-MSCs from the SLE patients than that of the normal control (Figure 3). Furthermore, Cyclin E and CDK2 were mainly localized and more expressed in the cytoplasm while lower levels were found in the nuclei of the BM-MSCs from the SLE patients than those of the normal control (Figure 4). These data indicated that the p53/p21 pathway plays an essential role in the ageing process of BM-MSCs from SLE patients.

In conclusion, in the present study, we have further determined that the BM-MSCs from the SLE patients were senescent MSCs; the abilities of proliferation and colony form were suppressed. The p53/p21 pathway plays a key role in the regulation of cell senescence process of BM-MSCs from SLE patients. These findings could explore the mechanism of cell senescence of BM-MSCs in SLE patients.

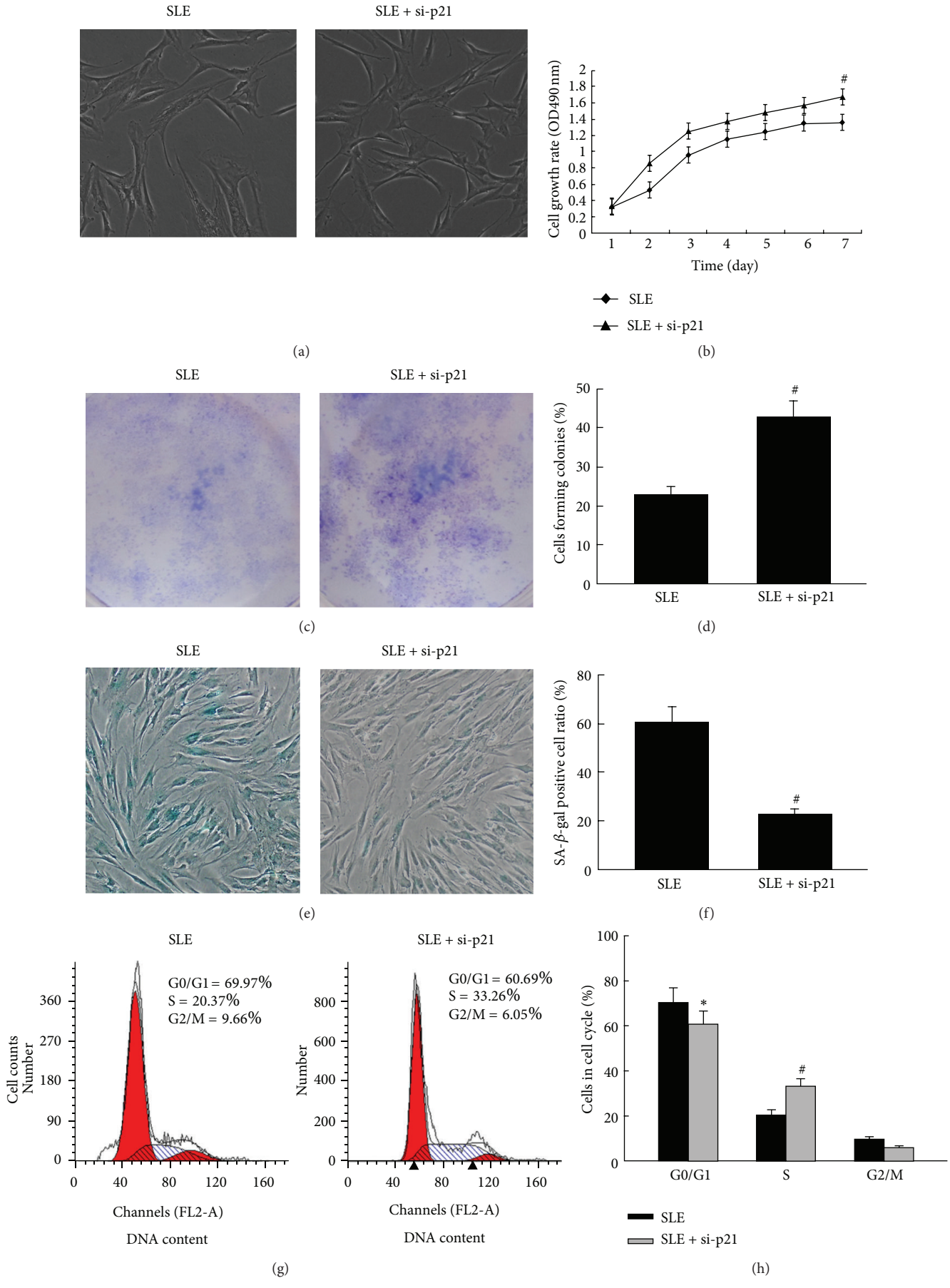

Figure 6: Continued.

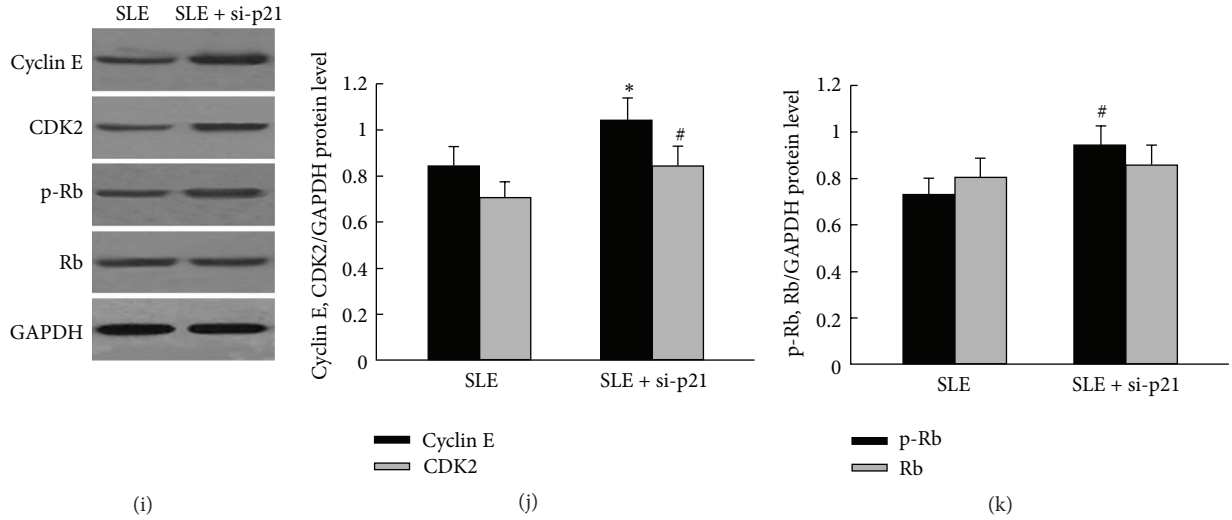

Figure 6: p21 knockdown reversed the ageing characteristics of BM-MSCs from SLE patients. (a) The morphology of BM-MSCs showed more spindle-shaped fibroblast-like growth after knockdown p21 when compared with BM-MSCs culture without si-p21. (b) Growth curve of BM-MSCs treated with and without si-p21 was tested by cell-counting assay. It has showed that, when p21 was knocked down, the cell proliferation rate was increased. Each point represents quantities relative to the BM-MSCs from SLE treated without si-p21 at Day 1 ($^{*}P < 0.05$) ($^{\#}P < 0.05$). ((c)-(d)) CFU of BM-MSCs from treated with si-p21 were increased; each bar represents quantities relative to BM-MSCs from SLE treated without si-p21 and is mean ± SD of three experiments ($^{\#}P < 0.05$). ((e)-(f)) After treated with si-p21, the number of SA-β-gal-positive cells was obviously decreased; each bar represents quantities relative to BM-MSCs from SLE treated without si-p21 and is mean ± SD of three experiments ($^{\#}P < 0.05$). (g) DNA content between BM-MSCs culture with and without si-p21 from SLE patients was compared by flow-cytometry. (h) When BM-MSCs from SLE patients treated with si-p21, the percentage of cells in G0/G1 was obviously decreased, and increase in percentage of cells in S phases was seen in SLE patients. Each bar represents quantities relative to BM-MSCs from SLE treated without si-p21 and is mean ± SD of three experiments ($^{*}P < 0.05$; $^{\#}P < 0.05$). ((i)–(k)) Expressions of cyclin E, CDK2, and p-Rb in BM-MSCs from SLE patients were tested by Western blot analyses and quantification analyses between culture with and without si-p21. The relative levels of protein expressions were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{*}P < 0.05$; $^{\#}P < 0.05$).

Conflict of Interests

There are no commercial affiliations or conflict of interests to disclose.

Authors' Contribution

Zhifeng Gu and Jinxia Jiang contributed equally to this work.

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Review Article

The Role of IL-33 in Rheumatic Diseases

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Interleukin-33 (IL-33), a novel member of IL-1 family, has been recently implicated in several inflammatory and autoimmune diseases. IL-33 can be produced by various types of tissues and cells and induce gene expression of Th2-associated cytokines via binding to the orphan receptor ST2. By promoting Th2 type immune response, IL-33 plays important roles in the allergy, whereas its function in autoimmune diseases attracts more attention. Recent studies reported the correlation of IL-33 with rheumatic diseases, and most of them found that the IL-33 expression levels were consistent with disease activity and development. Furthermore, evidence has indicated that IL-33-related treatment may ameliorate the pathogenic conditions and attenuate disease progression of those rheumatic diseases. Therefore, elucidation of the roles of IL-33 in rheumatic diseases would be beneficial to understand the pathogenesis and therapy of these diseases. In this paper, we will summarize the roles of IL-33 in the rheumatic diseases.

1. Introduction

IL-33 is a newly reported cytokine of IL-1 family, which has been demonstrated to inducing cytokine syntheses and mediating inflammatory responses through its receptor ST2 [1]. IL-33 is widely expressed in many tissues such as the liver, lung, central nervous system, and multiple types of cells including epithelial cells, endothelial cells, smooth muscle cells, macrophages, and fibroblasts [1–4]. Moreover, IL-33 mainly localizes to the nucleus, but under appropriate signal stimulation such as inflammation, IL-33 is in response processed and passively released from necrotic cells or actively secreted into the extracellular milieu [5] and functions through binding to its receptor ST2 as a proinflammatory cytokine that participates in the development and progression of many diseases, including collagen-induced arthritis [6, 7], anaphylactic shock [8], inflammatory bowel disease [9, 10], autoimmune hepatitis, and ischemia reperfusion injury [11–13]. Here, we will review the role of IL-33 in the pathogenesis of several clinical rheumatic diseases, mainly including rheumatoid arthritis, systemic lupus erythematosus, and ankylosing spondylitis.

2. IL-33 and ST2

IL-33, also named NF-HEV, IL-1F11, is a novel member of IL-1 family which was first reported by Schmitz et al. in 2005. At the protein level, IL-33 is broadly expressed in multiple tissues and organs especially enriched in the central nervous system and gastrointestinal tract [1]. It is considered that the initial translation product is the 30-Kd IL-33 precursor, and following activation of caspase-1, the IL-33 precursor is cleaved, released as an 18-Kd active cytokine [14]. Recent studies report that human IL-33 is processed at Asp178 but not Asp110 as previously claimed and is processed into mature bioactive forms independent of caspase-1 [15, 16]. Recent study also found that IL-33 was mainly localized in the nucleus of cells such as human high endothelial venules cells [3], and its nuclear function was chromatin associated [17, 18].

ST2L, specific receptor of IL-33, is mainly expressed on the surface of Th2 cells, mast cells, and NKT cells, but not on Th1 cells. IL-1R accessory protein (IL-1RAcP) is required for IL-33/ST2L signal transduction, and in IL-1RAcP−/− mouse-derived mast cells, IL-33 failed to induce IL-6 production [19, 20]. IL-33 signals through ERK1/2, p38MAPK, and JNKs

[1]. TRAF6 is a critical signal transducer in IL-33 signaling pathway to activate NF-κB and induce Th2 type cytokine production [21]. However, in recent years, ST2L has been reported to be principally expressed on the surface of several other types of cells, such as mast cells [22–24], eosinophils [25–27], basophils [28, 29], and NKT cells [11, 30]. Soluble ST2 (sST2) and transmembrane ST2L arise from a dual promoter system to drive differential mRNA expression [31, 32], and sST2 lacks the transmembrane and cytoplastic domains of ST2L [33]. Therefore, sST2 could serve as an antagonistic decoy receptor of IL-33 and was consistently used to antagonize the effect of IL-33 in experimental studies [34].

3. IL-33 and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammatory response, including synovial proliferation and excessive proinflammatory cytokine production, leading to eventual cartilage and bone destruction. Several proinflammatory cytokines are considered critical in forming the inflammatory process of RA [35, 36], including IL-1, IL-6, IL-8, IL-15, and TNF-alpha. Blockade of TNF-alpha activity has been widely used in ameliorating RA progression [37–40]. Until now, there has been much evidence confirming the involvement of IL-33 in rheumatoid arthritis. In earlier studies, it was reported that administration of sST2 fusion protein dramatically attenuated disease severity which contains reducing cellular infiltration in the joints, synovial hyperplasia, and joint erosion, by inhibiting the release of proinflammatory cytokines comprising IL-6, IL-12, TNF-alpha, and IFN-gamma [41]. After that, the high expression levels of IL-33 in human RA synovium and experimental arthritis were discovered. Moreover, treatment with an ST2 blocking antibody at the onset of disease attenuated the severity of CIA and reduced joint destruction, which totally suggested that locally produced IL-33 may contribute to the pathogenesis of joint inflammation and destruction [7]. Accordingly, the abnormally elevated level of IL-33 in RA patients was correlated with disease activity compared to the moderate or low activity group or healthy volunteers, and for synovial fluid, IL-33 levels were higher than those in sera. These observations revealed that IL-33 was mainly produced in inflamed joints [42]. Recently, Hong et al. also reported that in patients with RA, the serum level of IL-33 and sST2 was significantly higher than that of healthy controls. Accordingly, in the synovial fluid, the level of IL-33 was significantly higher than that of osteoarthritis patients [43]. All these results confirmed the fact that IL-33/ST2 signaling played a vital role in joint inflammation of human RA and experimental CIA model.

For the ways by which IL-33/ST2 was involved in the RA pathogenesis, most studies was focused on the relationship between IL-33 and TNF-alpha in RA pathogenesis. For RA patients, by administrating etanercept (a TNF-alpha inhibitor), the serum level of IL-33 significantly decreased at 3 and 6 months, and serum IL-33 levels showed a significant correlation with the number of tender joints, C-reactive protein, Disease Activity Core of 28 joints including CRP and the WBC count, and an inverse correlation with the RBC count and hemoglobin level [44]. This was in accordance with previous studies which reported that TNF-alpha could stimulate the production of IL-33 in vitro [45]. Otherwise, a newly reported study confirmed IL-33 as a target of anti-TNF therapy. They also pointed out that in mouse antigen-induced arthritis (AIA) which resembles human RA, IL-33 could induce and mediate neutrophil migration by activating synoviocytes and macrophages, and this induction was dependent on CXCL1, CCL3, TNF-alpha, and IL-1beta [46]. Furthermore, for patients who do not respond well to TNF-alpha inhibitors treatment, levels of IL-33 showed a significant positive correlation with IL-1beta. Therefore, it is concluded that IL-1beta might be inducing RA inflammation through producing proinflammatory IL-33 [47].

4. IL-33 and Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a multisystematic autoimmune disease characterized by chronic immune activation and multiple immunologic phenotypes, especially hypergammaglobulinemia and a plethora of autoantibodies [48]. Generally, there was evidence supporting the vital role of IL-33/ST2 signaling in the pathogenesis of SLE. For active SLE patients, the serum sST2 levels were significantly higher than those of inactive patients or healthy controls, whereas IL-33 was not comparable between SLE patients and controls [49]. However, another study discovered that, compared with healthy controls, the level of serum IL-33 was significantly increased in patients with SLE. Furthermore, serum sST2 level showed close correlation with SLEDAI, anti-dsDNA antibody, and prednisolone dosage but negatively with C3, and it was sensitive to change in disease activity longitudinally [50]. The discrepancy of these results may be due to different ways and devices of IL-33 detection. Furthermore, IL-33 level of patients with SLE was closely correlated with ESR, CRP, and IgA but showed significantly independent association of IL-33 with thrombocytopenia, erythrocytopenia, and anti-SSB antibody. Those results suggest that IL-33/ST2 signaling plays a role in SLE in the acute phase.

5. IL-33 and Ankylosing Spondylitis (AS)

Ankylosing spondylitis (AS), characterized by inflammation, bone erosion, and syndesmophyte formation, is a typical and the most common form of seronegative spondyloarthritis. Up to now, numerous studies have investigated the mechanism of AS development. However, there were only a few studies reporting the role of IL-33 in AS so far. It was discovered that in AS patients, serum IL-33 levels were elevated [51]; compared with inactive AS patients, the level of serum IL-33 was significantly higher in patients with active AS [52]. Moreover, serum IL-33 levels were positively correlated with IL-13, IL-4, IL-17, and TNF-alpha levels. Furthermore, these studies showed that IL-33 could enhance TNF-α and IL-6 production by peripheral blood mononuclear cells (PBMCs). Besides, neutrophil migration induced by IL-33 in AS patients were observed, which may also be an important mechanism

explaining the association between the elevated IL-33 concentrations and AS [52]. Consistently, in RA patients, suppression of ST2 expression in neutrophils reduces Synovial inflammation through preventing IL-33-induced neutrophils migration [46].

6. Other Rheumatic Diseases

Idiopathic infalmmatory myopathies (IIM), which includes dermatomyositis (DM) and polymyositis (PM), is a chronic systemic disease associated with high morbidity and functional disability. From the immunopathological viewpoint, in both, elevated concentrations of proinflammatory interleukins (TNF, IL-1, IL-6) and increased expression of molecules related to costimulation of T lymphocytes have been described [53]. It is reported that serum sST2 levels were significantly higher in DM and PM patients and correlated with markers of disease activity including CRP, CK, and LDH, and the level of serum sST2 decreased after therapy [54]. This indicates that sST2 may play a role in DM and PM. The role of IL-33 in DM and PM has not been reported yet, but considering the abnormal sST2 expression, it can be inferred that IL-33 may be involved in the pathogenesis of DM and PM.

Behçet's disease is a systemic inflammatory disorder with recurrent episodes of oral ulceration, skin lesions, genital ulceration, and intraocular inflammation (uveitis). The serum level of IL-33 in active BD patients was significantly higher than that of inactive BD patients or healthy controls. Moreover, IL-33 mRNA expression in the skin lesions of patients with active BD was significantly increased compared to that in healthy skin biopsies. Furthermore, a significant relationship was found between the levels of IL-33 and IL-17 and IL-33 and IL-6 in active BD patients [55]. These indicate that elevated IL-33 level in active BD patients was correlated with disease activity.

GCA is an inflammatory disease of blood vessels most commonly involving large and medium arteries of the head. Studies have demonstrated that both the innate and adaptive immune system contribute to GCA pathogenesis, such as Th1/Th17 [56, 57]. It was found that IL-33 and ST2 expression was significantly elevated in the inflamed arteries of GCA patients, but it was not accompanied by a concomitant increase of Th2 cytokines whereas elevated expression of IFN-γ, p-STAT6 and M2 macrophages polarisation were observed. Although IL-33 primarily induces Th2 immune responses, the role of IL-33 in the inflammation of GCA patients may not relied on inducing Th2 cytokines production, maybe inducing Th1 immune response [58].

Systemic sclerosis (SSc) is a disabling and incurable connective tissue disease with an unknown pathogenesis. In SSc, the combination of vascular abnormalities, collagen deposition, and autoimmunity leads to widespread tissue and organ fibrosis [59]. It has been found that, compared to healthy controls, IL-33 expression was significantly increased in SSc patients. Meanwhile, the serum level of IL-33 was correlated with early disease stage and microvascular involvement [60]. Moreover, some other investigators reported the same observations recently [61]. These data prompted us that IL-33 should be involved in the SSc pathogenesis, and the mechanism may be correlated with the role of IL-33 in promoting fibrosis [62].

7. Conclusion

Taken together, as a novel member of IL-1 family, IL-33 plays an important role in the development and progression of rheumatic diseases. For the autoimmune diseases above, either IL-33 or ST2 expression was altered in the serum of active patients, and this may be correlated with inflammatory cytokines, such as TNF-alpha and IL-1beta. So far, investigations on IL-33 and rheumatic diseases mostly focus on the expression level of IL-33 and disease activity, but the underlying mechanism and related clinical therapy still remain to be studied. Based on the aforementioned studies, we can infer that the clinical application of IL-33/ST2-related therapy in the treatment patients is full of prospects, although further studies are required to improve the details.

Authors' Contribution

Lihua Duan and Jie Chen contributed equally to this work.

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Research Article

Increased IL-33 in Synovial Fluid and Paired Serum Is Associated with Disease Activity and Autoantibodies in Rheumatoid Arthritis

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Objectives. IL-33, a newly found cytokine which is involved in joint inflammation, could be blocked by a decoy receptor—sST2. The expression and correlation of IL-33 and sST2 in rheumatoid arthritis (RA) are of great interest. *Methods*. Synovial fluid (SF) was obtained from 120 RA and 30 osteoarthritis (OA) patients, and paired sera were collected from 54 of these RA patients. The levels of IL-33 and sST2 were measured by ELISA. *Results*. SF IL-33 was significantly higher in RA than in OA, which was correlated with disease activity score 28, erythrocyte sedimentation rate, rheumatoid factor (RF)-IgM, RF-IgG, glucose phosphate isomerase (GPI), and immunoglobulin. Serum IL-33 was correlated positively with SF IL-33 in RA. Furthermore, it was correlated with RF-IgM and GPI. sST2 was partly detectable in RA (13 out of 54, 24.1%), while not in OA. Serum sST2 in RA had no significant correlation with serum IL-33 or SF IL-33. However, SFs from both RA and OA patients did not express sST2. *Conclusions*. This study supported that IL-33 played an important role in the local pathogenesis of RA. Considering the tight correlation between IL-33 and clinical features, it may become a new target of local treatment.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease characterized by the joints erosion and damage. Current research suggests that cytokines play important roles in the immunopathogenesis of RA.

IL-33, a member of the IL-1 cytokine family, is involved in the inflammation of RA via the IL-1 receptor-related protein ST2, and the recruitment of IRAK, IRAK4, MyD88, and TRAF6 to ST2, ultimately leading to the activation of NF-κB and MAP kinases [1]. The soluble form of ST2-sST2, is considered as a decoy receptor to block the effect of IL-33. IL-33 exacerbates the disease severity of collagen-induced arthritis (CIA) which is an animal model of RA disease [2]. Inhibition of IL-33 signaling pathway through blocking anti-ST2 antibody attenuated the severity of CIA [3]. It was reported that sST2 administration could also attenuate the severity of CIA [4]. Some recent studies have further shown that IL-33 is expressed by the synovial fibroblasts from RA patients [2, 3, 5] and the serum level of IL-33 is abnormally elevated in these patients [6–9]. However, the levels of IL-33 and sST2 in the synovial fluid (SF) and whether they are associated with disease activity are less known.

In this study, we compared the expression of IL-33 and sST2 in SF and paired serum samples of patients with RA to that with osteoarthritis (OA) and analyzed their association with clinical characteristics of RA disease.

2. Materials and Methods

2.1. Patients and Samples. SF samples were obtained from 120 patients with RA and 30 patients with OA in an outpatient clinic of the Department of Rheumatology and Immunology, Peking University People's Hospital, from December 2009 to December 2010. Paired sera of 54 RA patients and 12 OA

Table 1: Characteristics of enrolled patients with RA and OA.

Samples	Diseases	n	Gender		Age		Disease duration	
			M/F	P	Range (year, mean ± SD)	P	Range (year, mean ± SD)	P
SF	RA	120	23/97	0.457	12–81 (53.8 ± 16.1)	0.111	0.3–30 (9.4 ± 8.4)	0.847
	OA	30	4/26		40–77 (57.8 ± 11.3)		0.1–30 (11.1 ± 8.4)	
Serum	RA	54	6/48	0.347	23–81 (55.6 ± 13.4)	0.423	0.3–30 (8.2 ± 7.4)	0.524
	OA	12	3/9		53–72 (61.4 ± 10.4)		0.1–30 (6.6 ± 8.2)	

RA: rheumatoid arthritis; OA: osteoarthritis; SF: synovial fluid.

patients were also collected. All patients were grouped according to the revised criteria of the American College of Rheumatology for RA [10] or for OA [11]. The study was approved by the ethics committee of People's Hospital, Peking University, according to the Declaration of Helsinki. All patients had been informed and signed the consent for participation in the study. The details of the patients including gender, age, and disease duration were summarized in Table 1.

2.2. Measurement of IL-33 and sST2. Serum and SF IL-33 concentrations were determined with a commercial ELISA kit (DY3625, R&D Systems, Minneapolis, MN, USA). Serum samples were diluted 1 : 2 in sample dilution buffer. Soluble ST2 was assessed by ELISA using a commercial detection system (DST200, R&D Systems, Minneapolis, MN, USA). The experiments were performed according to the manufacturer's instructions.

2.3. Clinical Data and Inflammation Marker Analysis. Clinical data were recorded at the time of sample collection as the following index: age, sex, disease duration, number of swollen joints, and number of tender joints. Erythrocyte sedimentation rate (ESR) was evaluated by the Westergren method. Serum levels of immunoglobulins (IgG, IgM, and IgA,), complements (C3, C4), C-reactive protein (CRP), and rheumatoid factor (RF)-IgM were examined by immunonephelometry method. Antikeratin antibodies (AKA) and antiperinuclear factor (APF) were tested by indirect immunofluorescence assay. Anti-citrullinated peptide (anti-CCP) antibodies, glucose phosphate isomerase (GPI), and RF-IgG were tested by ELISA. The 28-joint count Disease Activity Score (DAS28) was evaluated as described [12].

2.4. Statistical Analysis. Data analyses were performed using SPSS 13.0 for Windows. Results are presented as the mean ± SD and percentage. Quantitative data were compared by the Mann-Whitney U test. Qualitative data were compared by the Pearson's chi-square test. Paired samples were analyzed using the Wilcoxon matched pairs test. A difference between groups was considered significant if $P < 0.05$. Spearman's rank correlation test was used to assess relationships between two variables. Correlation was considered significant if $P < 0.05$.

3. Results

3.1. IL-33 in Matched Serum-SF Samples of RA. IL-33 level was measured in the matched serum-SF samples of 54 RA patients. The level of IL-33 in SF (median 15.24 pg/mL) was lower than that in sera (median 31.64 pg/mL, Figure 1(a)). Spearman's correlation test was used to analyze the correlation of IL-33 levels in SF and in serum of RA. There is a significant correlation between two groups ($r = 0.578$, $P < 0.001$, Figure 1(b)).

3.2. IL-33 Levels in SF of RA and OA. The minimal concentration of standard (23.35 pg/mL) was assumed as the detection limit. SF IL-33 was detectable in 43 of the 120 patients with RA (35.8%), while none of the 30 OA patients had detectable level of IL-33 in their SF. SF IL-33 level in RA patients was significantly higher than that of OA patients (35.56 ± 62.56 pg/mL versus 3.66 ± 5.63 pg/mL, $P < 0.001$, Figure 2).

A correlation was analyzed between the clinical features and IL-33 levels in SF and serum of patients with RA. Comparing to the serum IL-33, SF IL-33 level in RA had more correlations with clinical features including disease activity features (ESR, DAS28 score) and autoantibodies (RF-IgM, RF-IgG, GPI, IgA, IgG, and IgM). The data was shown in Table 2 and Figure 3.

3.3. SF IL-33 Was Correlated with Disease Activity in RA. These 120 patients with RA were classified into 3 groups according to the DAS28-ESR: the high activity group (47 patients) was defined as DAS28-ESR > 5.1; the moderate activity group (56 patients) was defined as 5.1 ≥ DAS28-ESR > 3.2; the low activity group (17 patients) was defined as DAS28-ESR ≤ 3.2 [12]. SF IL-33 levels were significantly higher in the high and moderate activity groups than in the low activity group ($P = 0.0319$ and 0.0006, resp.; Figure 4).

Correspondingly, RA patients were divided into SF-IL-33-positive and SF-IL-33-negative groups according to the minimal detection limit of IL-33 (23.35 pg/mL), and the characteristics of these groups are shown in Table 3. It showed that the SF-IL-33-positive group had higher DAS28, ESR, and CRP than the SF-IL-33-negative group.

3.4. SF-IL-33 Was Correlated with Autoantibody Production in RA. It was shown in Table 2 that the level of SF-IL-33 was correlated with the levels of several auto-antibodies in serum including RF-IgM, RF-IgG, GPI, IgG, IgA, and IgM in patients with RA.

We also found in Table 3 that the SF-IL-33-positive group had higher levels of RF-IgM, IgG, IgA, and IgM than the SF-IL-33-negative group. The positive frequency of GPI was also

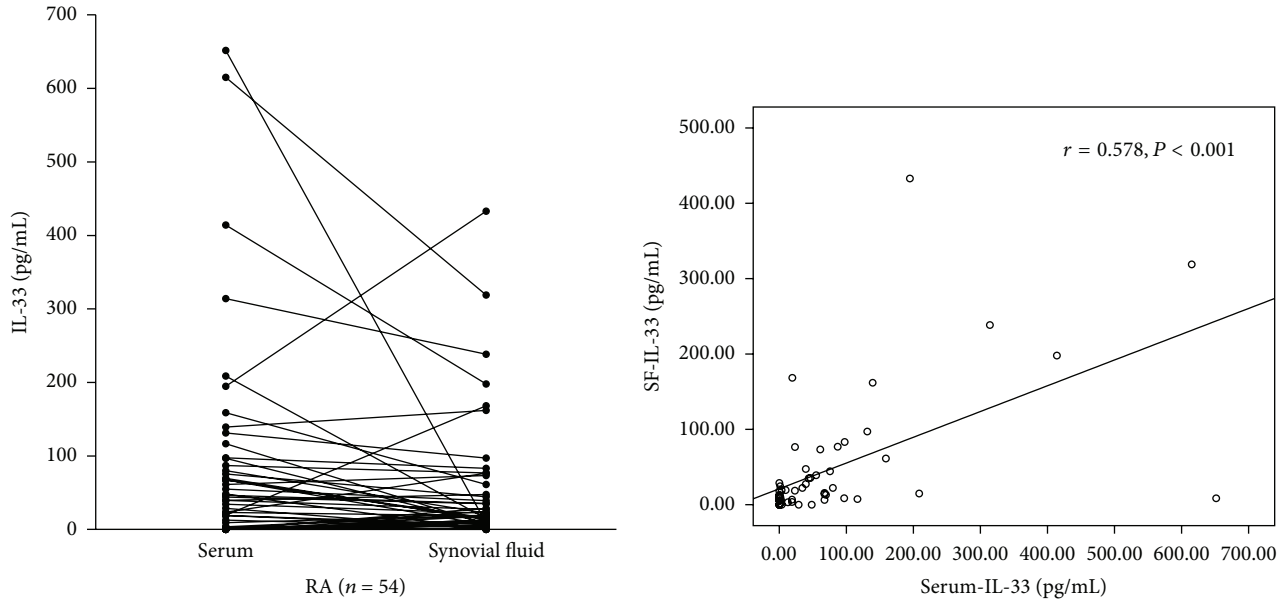

Figure 1: IL-33 levels in serum and SF samples in 54 RA patients were measured simultaneously. The levels in SF (median 15.24 pg/mL) were lower than those in sera (median 31.64 pg/mL (a)). A significant correlation between two groups was estimated by Spearman's correlation test ($r = 0.578$, $P < 0.001$ (b)).

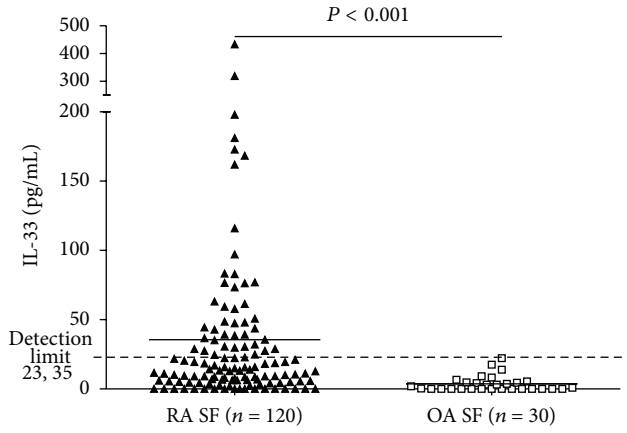

Figure 2: IL-33 levels in SF of RA and OA. SF IL-33 was detectable in 43 of the 120 patients with RA (35.8%), whereas it was nondetectable in patients with OA. The concentration in SF from RA was significantly higher than that from OA ($P < 0.001$).

higher in the SF-IL-33-positive group than in the SF-IL-33-negative group.

Similarly, SF IL-33 concentration in GPI-positive patients (53.96 ± 71.43 pg/mL) was higher than that in GPI-negative patients (24.85 ± 66.81 pg/mL, $P = 0.002$). A similar trend was identified in RF-IgM-positive patients (42.58 ± 59.85 pg/mL versus 22.98 ± 65.96 pg/mL, $P = 0.001$).

3.5. sST2 in Matched Samples of RA and OA. Soluble ST2 concentration in matched serum-SF samples of 54 RA patients and 12 OA patients was also measured by ELISA. The reference serum interval of kit (6.74–20.4 ng/mL) was used as the boundary. It showed that serum sST2 was elevated in 13 of the 54 patients with RA (24.1%) while none of the OA patients had a detectable level of sST2. No significant difference of SF sST2 concentrations was observed between RA (3.14 ± 1.94 ng/mL) and OA (3.20 ± 1.71 ng/mL) patients. Furthermore, there was no correlation between serum sST2

Table 2: Correlation analysis between SF-serum IL-33 (pg/mL) and clinical and laboratory variables.

Measurements	SF IL-33 ($n = 120$)		Serum IL-33 ($n = 54$)	
	r	P	r	P
Age (yrs)	−0.106	0.257	−0.014	0.921
Tender joint count, 0–46 joints	0.095	0.347	0.327	**0.020***
Swollen joint count, 0–48 joints	0.192	0.056	0.134	0.354
DAS28-ESR	0.209	**0.047***	0.253	0.102
ESR (mm/h)	0.256	**0.008***	0.213	0.150
CRP (mg/L)	0.166	0.073	0.219	0.116
C3 (g/L)	0.113	0.260	0.267	0.061
C4 (g/L)	0.039	0.698	0.180	0.211
IgA (g/L)	0.246	**0.012***	0.107	0.457
IgG (g/L)	0.309	**0.001***	0.136	0.343
IgM (g/L)	0.301	**0.002***	0.220	0.121
RF-IgM (U/mL)	0.327	**<0.001***	0.342	**0.011***
RF-IgG (IU/mL)	0.214	**0.037***	0.183	0.208
GPI (mg/L)	0.364	**0.001***	0.505	**0.000***
Anti-CCP (RU/mL)	0.104	0.284	0.052	0.716

*$P < 0.05$. Spearman's correlation test was used.

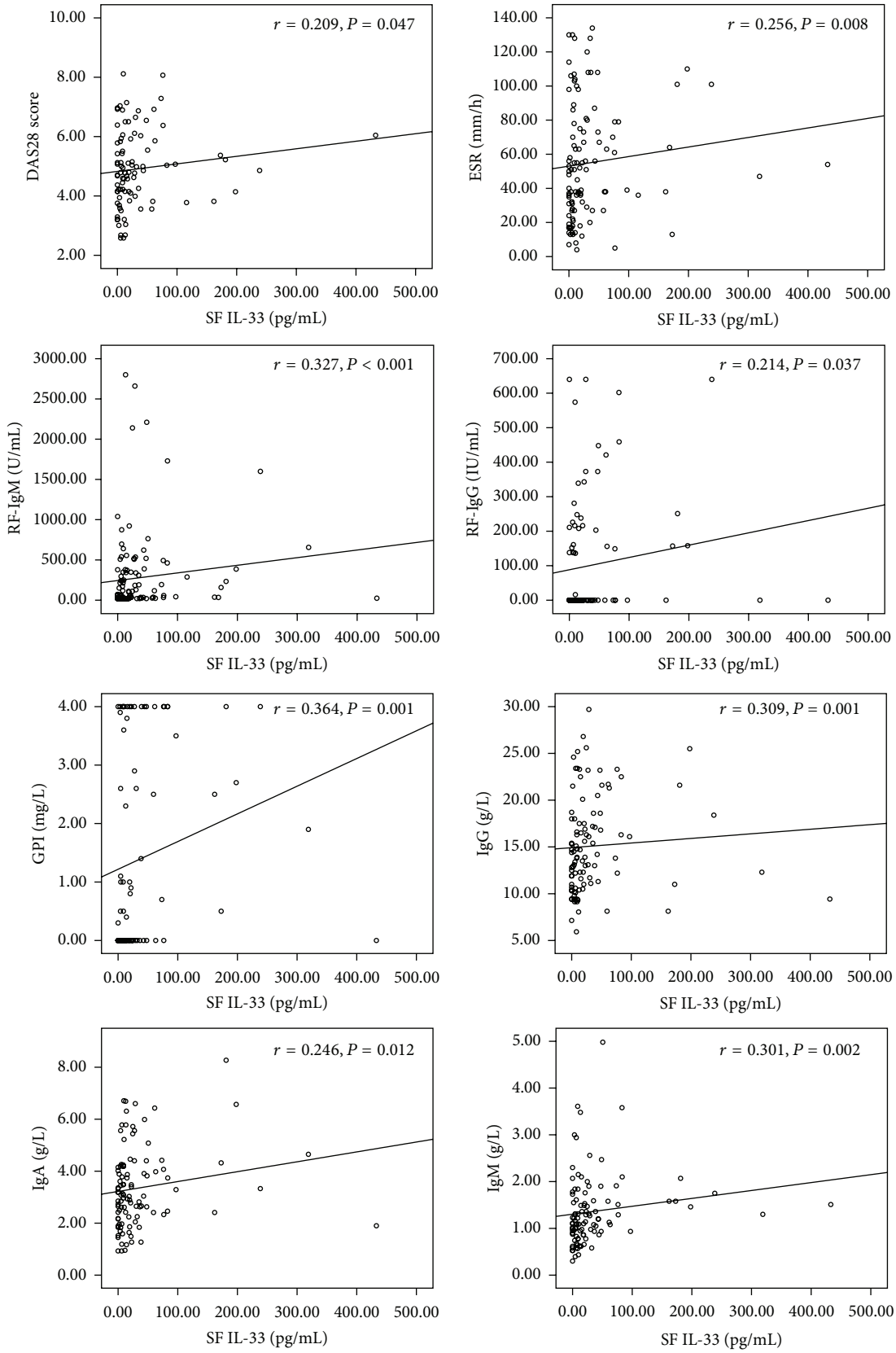

Figure 3: The significant correlations between SF IL-33 and clinical features including disease activity features (ESR, DAS28 Score) and autoantibodies (RF-IgM, RF-IgG, GPI, IgA, IgG, and IgM). ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; GPI: glucose phosphate isomerase.

Table 3: The comparison of clinical features between SF-IL-33-positive and SF-IL-33-negative groups.

Measurements	SF-IL-33-positive ($n = 43$)	SF-IL-33-negative ($n = 77$)	Z (x^2)	P
Age (yrs)	49.9 ± 18.5	56.0 ± 14.2	−1.490	0.136
Gender (M/F)	11/32	12/65	1.220	0.325
Disease duration (yrs)	10.2 ± 8.9	9.0 ± 8.0	−0.515	0.607
Tender joint count	8.1 ± 8.3	6.9 ± 7.8	−0.847	0.397
Swollen joint count	8.1 ± 8.0	5.4 ± 7.0	−1.789	0.074
DAS28	5.3 ± 1.2	4.7 ± 1.3	−2.103	**0.035***
ESR (mm/h)	66.7 ± 33.0	48.7 ± 33.2	−2.976	**0.003***
CRP (mg/L)	49.3 ± 39.4	34.9 ± 32.3	−2.055	**0.040***
RF-IgM (U/mL)	440.7 ± 646.0	186.8 ± 378.6	−3.564	**<0.001***
RF-IgG-positive	46.5% (20)	27.3% (21)	3.136	0.110
GPI-positive	67.4% (29)	41.6% (32)	5.332	**0.026***
IgA (g/L)	3.9 ± 1.6	3.1 ± 1.4	−2.258	**0.024***
IgG (g/L)	17.1 ± 5.4	14.0 ± 4.7	−2.888	**0.004***
IgM (g/L)	1.6 ± 0.8	1.2 ± 0.7	−3.064	**0.002***
C3 (g/L)	1.2 ± 0.4	1.1 ± 0.3	−1.002	0.317
C4 (g/L)	0.2 ± 0.1	0.2 ± 0.1	−0.077	0.939
Anti-CCP (RU/mL)	107.9 ± 80.3	97.0 ± 81.6	−0.937	0.349
AKA-positive	21 (48.8%)	42 (54.5%)	0.269	0.383
APF-positive	25 (58.1%)	50 (64.9%)	0.367	0.651

*$P < 0.05$.
SF IL-33-positive: SF IL-33 ≥ 23.35 pg/mL; SF IL-33-negative: SF IL-33 < 23.35 pg/mL.

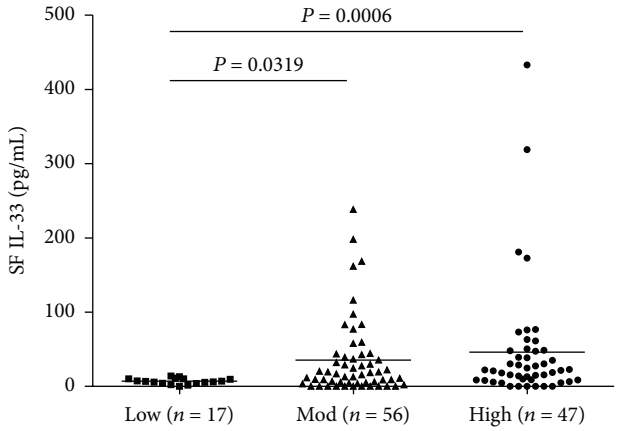

Figure 4: Interleukin 33 (IL-33) levels in synovial fluid (SF) from RA patients. RA patients were categorized into 3 groups according to the Disease Activity Score based on ESR. Low: low activity group; Mod: moderate activity group; High: high activity group. Symbols represent individual samples. Horizontal bars represent median IL-33 levels.

and serum IL-33 level or SF IL-33 level, showing that the relationship between sST2 and IL-33 in RA needs more effort to be understood.

4. Discussion

According to our knowledge, this is the largest study of IL-33 and sST2 in SF and matched serum of RA and OA patients. Comparing to the former studies [6–9], it could provide the more believable results about the expression of IL-33 in RA. Based on the present study, we found that there was an obvious difference of IL-33 levels between SF and matched serum. We also demonstrated that SF-IL-33 in RA patients had higher levels than OA patients, whether with positive rate or mean expression. Additionally, our study showed that there was a positive correlation between SF-IL-33 and disease activity or RA-associated auto-antibodies, which supported the hypothesis that IL-33 plays an important role in the pathogenesis of RA.

In our study, SF IL-33 levels were significantly higher in the high and moderate activity groups than in the low activity group. On the other hand, the SF-IL-33-positive group had higher DAS28, ESR, and CRP than the SF-IL-33-negative group. It suggested that SF IL-33 was closely associated with systemic inflammation.

Furthermore, the SF-IL-33-positive group had higher levels of RF-IgM, IgG, and IgA and positive frequency of GPI than the SF-IL-33-negative group. The result of our former study [7], that serum IL-33 is associated with antibody production, was also proved with SF-IL-33. All these antibodies are important features for diagnosis and prognosis [13–15], so we considered that IL-33 may be a risk factor for poor prognosis in RA. Further study is deserved about the mechanism of IL-33 in inducing antibody production.

As a specific ligand of IL-33, sST2 can serve as an anti-inflammatory mediator through binding with IL-33, sequentially decrease the interaction of IL-33 and ST2L [16]. Therefore, we need to know the expression of sST2 in RA SF and serum to further understand its function in the pathogenesis. In our study, the positive frequency of serum sST2 in RA patients was higher than that in OA patients, but there was no significant difference. The concentrations of SF sST2 also had

no difference between RA and OA. Since the expression of sST2 could be induced in various mesenchymal and hematopoietic cells by a lot of stimuli [17–20], therefore, it is possible that the inflammatory milieu is responsible for the level of sST2 in OA. We tried to find out the relationship between serum sST2 and serum IL-33 or the other clinical features in RA, but no correlation was observed. The similar result was also shown in a previous study [9], which was held by a relative low number of samples.

In conclusion, SF IL-33 levels that increased in RA patients could be treated as a sensitive marker of disease activity and were associated with the production of antibodies. The correlated expression of IL-33 between SF and serum suggests that IL-33 plays an important role in the local pathogenesis of RA. It may become a new target of local treatment. The role of sST2 and the therapeutic significance of IL-33/sST2 system in RA need further research.

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Clinical Study

A Clinical Analysis of Risk Factors for Interstitial Lung Disease in Patients with Idiopathic Inflammatory Myopathy

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Interstitial lung disease (ILD) is a common and severe complication of idiopathic inflammatory myopathies (IIM). The aim of our study was to identify risk factors for ILD by evaluating both clinical and biochemical features in IIM patients with or without ILD. From January 2008 to December 2011, medical records of 134 IIM patients in our rheumatology unit were reviewed. The patients were divided into ILD group (83 patients) and non-ILD group (51 patients). The clinical features and laboratory findings were compared. The univariable analyses indicated that arthritis/arthralgia (54.2% versus 17.6%, $P < 0.05$), Mechanic's hand (16.9% versus 2.0%, $P < 0.05$), Raynaud's phenomenon (36.1% versus 2.0%, $P < 0.05$), heliotrope rash (44.6% versus 19.6%, $P < 0.05$), fever (43.4% versus 21.6%, $P < 0.05$), elevated ESR (60.2% versus 35.3%, $P < 0.05$), elevated CRP (55.4% versus 31.4%, $P < 0.05$), or anti-Jo-1 antibody (20.5% versus 5.9%, $P < 0.05$) were risk factors for developing ILD in IIM. Multivariable unconditional logistic regression analysis that showed arthritis/arthralgia (OR 7.1, 95% CI 2.8–18.1), Raynaud's phenomenon (OR 29.1, 95% CI 3.6–233.7), and amyopathic dermatomyositis (ADM) (OR 20.2, 95% CI 2.4–171.2) were the independent risk factors for developing ILD in IIM.

1. Introduction

Idiopathic inflammatory myopathies (IIM) is a systemic autoimmune disease with unknown origin, characterized by proximal, symmetric muscle weakness, elevated serum creatine kinase (CK), characteristic electromyography findings, and lymphocytic infiltration in the muscle tissue [1, 2]. Polymyositis (PM) and dermatomyositis (DM) are the most common forms of IIM. In addition, amyopathic dermatomyositis (ADM) is a special type of DM [3]. In the case of DM, characteristic skin manifestations (heliotrope rash/Gottron papules) are also present [1]. PM and DM occur isolated or in connection with other connective tissue diseases (CTD) or malignancy [4].

Interstitial lung disease (ILD) is a common and severe complication of IIM. Concurrency of ILD in IIM has been reported to be 23.1–65% and considered the major cause of death [5]. Since ILD is associated with unfavorable clinical outcome, it requires more aggressive medications as corticosteroids and immunosuppressive drugs. The reported frequency of ILD is more than 70% in Jo-1 positive patients [6]. Anti-Jo-1 antibody can be found in 10–40% of patients with polymyositis (PM), 2–10% in dermatomyositis (DM), and 3–8% in overlap myositis [7]. The presence of this autoantibody helps to identify a subgroup of patients characterized by ILD, Raynaud's phenomenon, arthritis, and "mechanic's hand," referred to as "antisynthetase syndrome" [8, 9]. Anti-SS-A antibody can be found in 44–58% of patients with Jo-1 positive [10, 11]. Whether ILD in IIM is related to certain factors needs further research. The aim of this retrospective study was to investigate risk factors for ILD in patients with IIM.

2. Patients and Methods

134 patients with IIM from inpatient and outpatient department of our rheumatology unit between January 2008 and December 2011 were retrospectively reviewed. Diagnosis of PM (58 cases)/DM (58 cases)/ADM (18 cases) was established according to the criteria of ENMC workshop [3]. Patients who had other connective tissue diseases or malignancy concomitantly were excluded. Diagnosis of ILD was established based on the results of high-resolution computed tomography (HRCT).

Clinical data was obtained from patients' medical records. All patients underwent detailed laboratory examinations and clinical assessment to exclude malignancy and other connective tissue disease. Disease duration was determined from date of diagnosis to the latest follow-up visit. The clinical features include age, sex, proximal muscle weakness, myosalgia, arthritis/arthralgia, mechanic's hand, Raynaud's phenomenon, Gottron's sign, heliotrop rash, and fever. All patients also underwent routine laboratory examinations at diagnosis: CK and erythrocyte sedimentation rate (ESR) were detected by enzyme rate method and Westergren method, respectively. Laser nephelometry was used to detect the presence of C-reactive protein (CRP) (Dialab GmBH, Austria). Antinuclear antibodies (ANA) were detected by indirect immunofluorescence method using Hep-2 cell as substrate. Antibodies directed against extractable nuclear antigen (ENA) complex SS-A and Jo-1 were measured by immunoblotting (Euroline-WB, Euroimmun, Lübeck, Germany). All patients underwent electromyography (EMG) examination. The presence of polyphasic, short, small motor unit potentials, fibrillation, positive sharp waves, and repetitive high frequency discharges was considered typical of IIM changes. After informed consent, all patients underwent muscle biopsy.

Statistical analyses were performed with the SPSS version 19.0 software; P value was set at less than 0.05. The groups were analyzed with the following tests. In case of normal distribution the independent sample t test was used, and in nonnormal distribution Mann-Whitney test was adopted to compare the means. The chi-square test or Fisher's exact test was used to compare frequencies. However, caution is needed in interpreting statistical significance given the relative small number of patients. The unconditional multivariable logistic regression analysis was adopted to identify the risk factors.

3. Results

A total of 134 IIM patients were enrolled, including 83 (64.2%) with ILD (mean age 46.6 ± 12.4, range 16–82) and 51 (35.8%) without (mean age 40.4 ± 11.9, range 16–72). No significant differences were found between the two groups with regard to age, gender, and disease duration (Table 1).

The constituent ratio of three subtypes of IIM with ILD were summarized in Table 2. The constituent ratio was significantly different according to the result of chi-square test (chi-square value = 10.6, $P < 0.05$). It was no significant difference between PM with ILD and DM with ILD (chi-square value = 1.3, $P = 0.26$). ADM presented a statistically more frequent association with ILD than PM (chi-square value = 6.8, $P < 0.05$) and DM (chi-square value = 10.6, $P < 0.05$).

Clinical symptoms at presentation were summarized in Table 1. In both groups, no significant differences were found regarding myosalgia and Gottron's sign. The IIM with ILD patients had a statistically more frequent presence of arthritis/arthralgia, mechanic's hand, Raynaud's phenomenon, heliotrop rash, and fever ($P < 0.05$). However, this group had a statistically less frequent presence of proximal muscle weakness ($P < 0.05$) than IIM without ILD group.

Table 1: Univariable analysis of risk factors for ILD in patients with IIM.

Items	With ILD ($n = 83$)	Without ILD ($n = 51$)	P value
Mean age at diagnosis (years)	46.6 ± 12.4	40.4 ± 11.9	n.s.
Male : female	24 : 59	13 : 38	n.s.
Disease duration (months)	19.3 ± 7.6	22.5 ± 8.0	n.s.
Proximal muscle weakness	59 (71.1%)	45 (88.2%)	<0.05
Myosalgia	39 (47.0%)	25 (49.0%)	n.s.
Arthritis/arthralgia	45 (54.2%)	9 (17.6%)	<0.05
Mechanic's hand	14 (16.9%)	1 (2.0%)	<0.05
Raynaud's phenomenon	30 (36.1%)	1 (2.0%)	<0.05
Gottron's sign	33 (39.8%)	16 (31.4%)	n.s.
Heliotrope rash	37 (44.6%)	10 (19.6%)	<0.05
Fever	36 (43.4%)	11 (21.6%)	<0.05
Elevated ESR	50 (60.2%)	18 (35.3%)	<0.05
Elevated CRP	46 (55.4%)	16 (31.4%)	<0.05
Elevated CK	62 (74.7%)	47 (92.2%)	<0.05
ANA (+)	57 (68.7%)	34 (66.7%)	n.s.
Anti-SS-A antibody (+)	24 (28.9%)	11 (21.6%)	n.s.
Anti-Jo-1 antibody (+)	17 (20.5%)	3 (5.9%)	<0.05

IIM: idiopathic inflammatory myopathies; ILD: interstitial lung disease; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; CK: creatine kinase, ANA: antinuclear antibody; n.s.: not significant.

Table 2: The constituent ratio of three subtypes of IIM with ILD.

Subtypes	With ILD	Without ILD	Chi-square value	P value
PM	36 (62.1%)	22 (37.9%)	1.3*	0.26
DM	30 (51.7%)	28 (48.3%)	10.6**	<0.05
ADM	17 (94.4%)	1 (5.6%)	6.8***	<0.05

*PM versus DM, **DM versus ADM, and ***ADM versus PM
IIM: idiopathic inflammatory myopathies; ILD: interstitial lung disease; PM: polymyositis; DM: dermatomyositis; ADM: amyopathic dermatomyositis.

Elevated ESR was detected in 50 (60.2%) patients with ILD versus 18 (35.3%) patients without ILD, elevated CRP was detected in 46 (55.4%) patients with ILD versus 16 (31.4%) patients without ILD, and elevated CK was detected in 62 (74.7%) patients with ILD versus 47 (92.2%) patients without ILD. These data showed that IIM patients with ILD had statistically more frequent presence of elevated ESR and CRP, while in the other group elevated CK was more common.

The presence of ANA was not significantly different between patients with and without ILD (68.7% versus 66.7%). Anti-SS-A antibody was found in 24 (28.9%) patients with ILD versus 11 (21.6%) patients without ILD. The difference between the two groups did not reach a statistical significance. The presence of anti-Jo-1 antibody was statistically more frequent in ILD group ($P < 0.05$). Anti-SS-A antibody was found in 11 (55.0%) patients with anti-Jo-1 antibody

Table 3: Multivariable unconditional logistic regression analysis on risk factors associated with ILD in patients with IIM.

Variables	*B*	Wald	*P* value	OR	95.0% CI for OR
Arthritis/arthralgia	1.96	16.90	0.000	7.1	2.8–18.1
Raynaud's phenomenon	3.37	10.04	0.002	29.1	3.6–233.7
Amyopathic dermatomyositis	3.01	7.60	0.006	20.2	2.4–171.2

IIM: idiopathic inflammatory myopathies, ILD: interstitial lung disease, *B*: regression coefficient, OR: odds ratio, CI: credibility interval.

positive versus 24 (21.1%) patients with anti-Jo-1 antibody negative ($P < 0.05$).

Multivariable unconditional logistic regression analysis indicated that arthritis/arthralgia, Raynaud's phenomenon, and ADM were the independent risk factors for developing ILD in IIM (Table 3).

4. Discussion

IIM-related ILD was originally described by Mills and Mathews in 1956 [12]. In this study, we found that the concurrency of ILD in patients with IIM was 64.2% (83/134), which was consistent with previous reports [5]. The aim of our study was to identify risk factors for ILD in patients with IIM by evaluating clinical and biochemical features in IIM patients with and without ILD. The acknowledgement of predictors for ILD in IIM patients appears crucial to prompt management at an early stage of the disease. Our results showed that age, gender, and disease duration were incorrelation with ILD, which was similar to the published studies [13, 14]. As known before, arthritis/arthralgia and positive anti-Jo-1 were important predictive factors for ILD in IIM [14–19] and were suggested to constitute a distinct subgroup of myositis, which was named antisynthetase syndrome [8, 9]. In this study, we confirmed not only this viewpoint but also demonstrated that Mechanic's hand, Raynaud's phenomenon, heliotrop rash, fever, elevated ESR, and CRP were associated with ILD. However, anti-Jo-1, Mechanic's hand, heliotrop rash, fever, elevated ESR, and CRP were not the independent risk factors for developing ILD in IIM, which may attribute to the nature of disease, the multicollinearity of these factors, or the small sample in our study.

ADM is a special type of DM. In our study, prevalence of ILD was found 94.4% (17/18) in ADM patients. In PM and DM, however, the ratio was much lower to 62.1% and 51.7%, respectively. Cases with characteristic DM rash and little or no muscle involvement are regarded as ADM [15]. As the results shown in our study, ADM subtype closely correlated with ILD, which may lead to poor prognosis [15, 20–22]. Therefore, early diagnosis of ILD and aggressive approach in therapy is required in patients with ADM.

Anti-Jo-1 antibody is known as a marker for myositis [23, 24] and is found to be positive in 20%–30% of patients with IIM [25]. Previous literature showed that the prevalence of ILD approached 70%–90% among anti-Jo-1 antibody positive individuals [6, 26]. In our cohort, the prevalence of anti-Jo-1 antibody was 14.9%, and the prevalence of ILD was 85.0%, which was similar to previous investigations. In addition, the association of anti-Jo-1 with anti-SS-A antibodies has been reported in the literature, suggesting the coexistence of positive anti-Jo-1 and anti-SS-A could serve as a good predictor to identify candidate patients for severe progressive ILD [7, 10, 27]. The similar conclusion was made in our study that the presence of anti-SS-A antibody was statistically more frequent in patients with anti-Jo-1 antibody positive. But anti-SS-A antibody was incorrelation with ILD by comparing the frequency of anti-SS-A antibody in patients with or without ILD. Therefore, although anti-SS-A antibody was associated with anti-Jo-1 antibody, it could not be considered as an independent predictive factor for developing ILD in IIM.

In conclusion, our findings suggested that ILD was prevalent in patients with IIM, especially in patients with ADM. Patients who presented with arthritis/arthralgia and Raynaud's phenomenon tended to have a higher frequency of IIM-associated ILD. Furthermore, anti-SS-A antibody was associated with anti-Jo-1 antibody, but it was not an independent predictive factor for ILD in patients with IIM.

Conflict of Interests

There is no conflict of interests to be disposed.

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Review Article

Possible Implication of Fcγ Receptor-Mediated Trogocytosis in Susceptibility to Systemic Autoimmune Disease

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Leukocytes can "gnaw away" the plasma membrane of other cells. This phenomenon, called trogocytosis, occurs subsequent to cell-to-cell adhesion. Currently, two mechanisms of trogocytosis, adhesion molecule-mediated trogocytosis and Fcγ receptor-(FcγR-) mediated trogocytosis, have been identified. In our earlier study, we established an *in vitro* model of FcγR-mediated trogocytosis, namely, CD8 translocation model from T cells to neutrophils. By using this model, we demonstrated that the molecules transferred to neutrophils via FcγR-mediated trogocytosis were taken into the cytoplasm immediately. This result suggests that the chance of molecules transferred via FcγR-mediated trogocytosis to play a role on the cell surface could be time-limited. Thus, we consider the physiological role of FcγR-mediated trogocytosis as a means to remove antibodies (Abs) that bind with self-molecules rather than to extract molecules from other cells. This concept means that FcγR-mediated trogocytosis can be a defense mechanism to Ab-mediated autoimmune response. Moreover, the activity of FcγR-mediated trogocytosis was revealed to be parallel to the endocytotic activity of neutrophils, which was critically related to the susceptibility to systemic autoimmune diseases. The collective findings suggest that FcγR-mediated trogocytosis could physiologically play a role in removal of Abs bound to self-antigens and prevent autoimmune diseases.

1. Introduction

Trogocytosis is the exchange of plasma membrane fragments between immune cells which form a conjugate [1, 2]. The phenomenon was first described on $CD8^+$ T cells [3]. The $CD8^+$ T cells can take plasma membrane fragments of antigen-presenting cells (APCs) via T-cell receptor (TCR) and antigen (Ag)/class I major histocompatibility complex (MHC), when these cells form an immunological synapse. To date, it has been shown that not only $CD8^+$ T cells but also other immune cells, including $CD4^+$ T cells [4], $\gamma\delta$T cells [5], B cells [6], natural killer cells [7], dendritic cells [8], monocytes [9], and macrophages [10], have potential for trogocytosis. These cells are able to accept plasma membrane fragments of other cells after recognition of cell surface Ags by the specific receptors. This mechanism is called adhesion molecule-mediated trogocytosis.

Recently, another mechanism of trogocytosis mediated by Ag/antibody (Ab) immune complex and Fcγ receptor (FcγR), namely, FcγR-mediated trogocytosis, is advocated [11–13]. The major difference of FcγR-mediated trogocytosis from adhesion molecule-mediated trogocytosis is the intervention of Ab. This phenomenon is well characterized, wherein CD20 molecules on malignant B cells are lost after infusion of the humanized anti-CD20 monoclonal Ab, rituximab [14–16]. In this situation, $Fc\gamma R^+$ immune cells capture the plasma membrane fragments of B cells via the CD20/anti-CD20 immune complexes and FcγRs. Similar phenomenon is seen when CD22 [17] and CD25 [18] are targeted by therapeutic Abs.

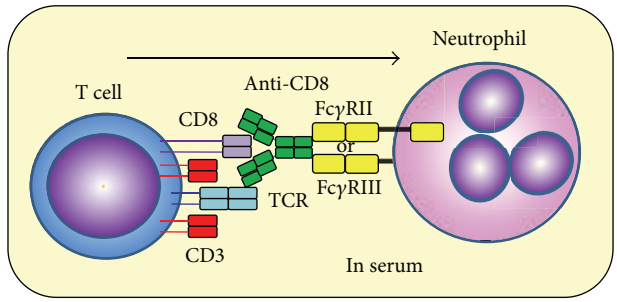

Figure 1: Schemas of CD8 translocation model from T cells to neutrophils CD8 molecules on T cells were transferred to neutrophils when these cells were incubated with anti-CD8 Ab in the serum. TCR and CD3 molecules on T cells were also transferred to neutrophils. FcγRs (FcγRII and FcγRIII) were involved in the mechanism.

Although the best known cells that can perform FcγR-mediated trogocytosis are monocytes and macrophages [12, 19–21], the most abundant FcγR$^+$ cells in peripheral blood, neutrophils, also have potential for FcγR-mediated trogocytosis [22, 23].

The physiological roles of trogocytosis have been discussed concerning T cells that acquired the Ag/MHC from the APCs in particular [24, 25]. The acceptor CD8$^+$ T cells for the Ag/MHC are prone to be killed by the fratricide mechanism. In another way, the acquisition of HLA-G1 from APCs via trogocytosis could change the T-cell phenotype from effector to regulatory phenotype [26, 27]. It has been also demonstrated that CD4 CD8 double negative regulatory T cells, which accept the Ag/MHC from APCs via trogocytosis, can function as cytotoxic cells toward the Ag-specific CD8$^+$ T cells [28]. These could be related to the convergence or suppression of the immune response. On the contrary, it is indicated that the T cells with the Ag/MHC can possibly function as new APCs resulting in the progression of the immune response. These controversial properties of T cells through adhesion molecule-mediated trogocytosis could be displayed in time and environment-dependent manners. In addition, recent studies have demonstrated that adhesion molecule-mediated trogocytosis could be related to the exercise of naturally occurring FOXP3$^+$ regulatory T cells [29]. However, the physiological role of FcγR-mediated trogocytosis remains unrevealed. In this perspective, the role of FcγR-mediated trogocytosis in the physiological immune system is discussed according to the current data.

2. Establishment of *In Vitro* Model of FcγR-Mediated Trogocytosis

Daubeuf et al. had established a simple method to detect adhesion molecule-mediated trogocytosis by flow cytometry (FCM) [30]. On the other hand, we earlier established an *in vitro* model of FcγR-mediated trogocytosis [23]. The protocol was simple and easy as follows: (1) heparinized peripheral blood samples were incubated with anti-CD8 and anti-CD15 Abs: (2) after removal of erythrocytes by treatment with ammonium chloride, cells were subjected to FCM. Results demonstrated the presence of CD8$^+$ cells in CD15$^+$ neutrophils. Since neutrophils do not express CD8 innately, the CD8 molecules detected on neutrophils seem to be derived from other cells. Our studies revealed that CD8 molecules on T cells were transferred to neutrophils via the anti-CD8 Ab and FcγRs (FcγRII and FcγRIII) on neutrophils. The usage of FcγRIII appears to be a specific characteristic of neutrophils because FcγRII, especially FcγRIIB, among the FcγRs mediates trogocytosis in monocytes and macrophages [19, 31]. Moreover, bystander molecules, such as TCR and CD3, were also transferred from T cells to neutrophils accompanied by CD8 molecules. Thus, this phenomenon was considered as FcγR-mediated trogocytosis (Figure 1). The polymerization of actin was involved in the process of neutrophil FcγR-mediated trogocytosis similar to T cells [32]. By using this model, we demonstrated that human anti-mouse IgG Abs in serum accelerated FcγR-mediated trogocytosis. This is consistent with the finding that FcγR-mediated trogocytosis is prone to occur in arthritic patients positive for rheumatoid factor, which is an anti-IgG Ab [33].

3. Immediate Intake of Molecules Deprived via FcγR-Mediated Trogocytosis

By using the *in vitro* model of FcγR-mediated trogocytosis, namely, CD8 translocation model from T cells to neutrophils, the dynamism of the molecules deprived by FcγR$^+$ cells via FcγR-mediated trogocytosis was determined. For this purpose, two anti-CD8 monoclonal Abs that could recognize diverse epitopes were applied. First, CD8 molecules on T cells were made to transfer to neutrophils via FcγR-mediated trogocytosis using the PE-labeled anti-CD8 Ab. After incubation for 0–8 hours, CD8 molecules that remained on the cell surface of neutrophils were detected by the other PECy5-labeled anti-CD8 Ab. Although the number of neutrophils labeled by PE (neutrophils that performed FcγR-mediated trogocytosis) was substantially stable during the incubation period, the number of PECy5-labeled CD8 molecules on the cell surface of neutrophils diminished and reached low level by 2 hours (Figure 2). These findings indicated that the molecules transferred to neutrophils via FcγR-mediated trogocytosis were taken into the cytoplasm immediately.

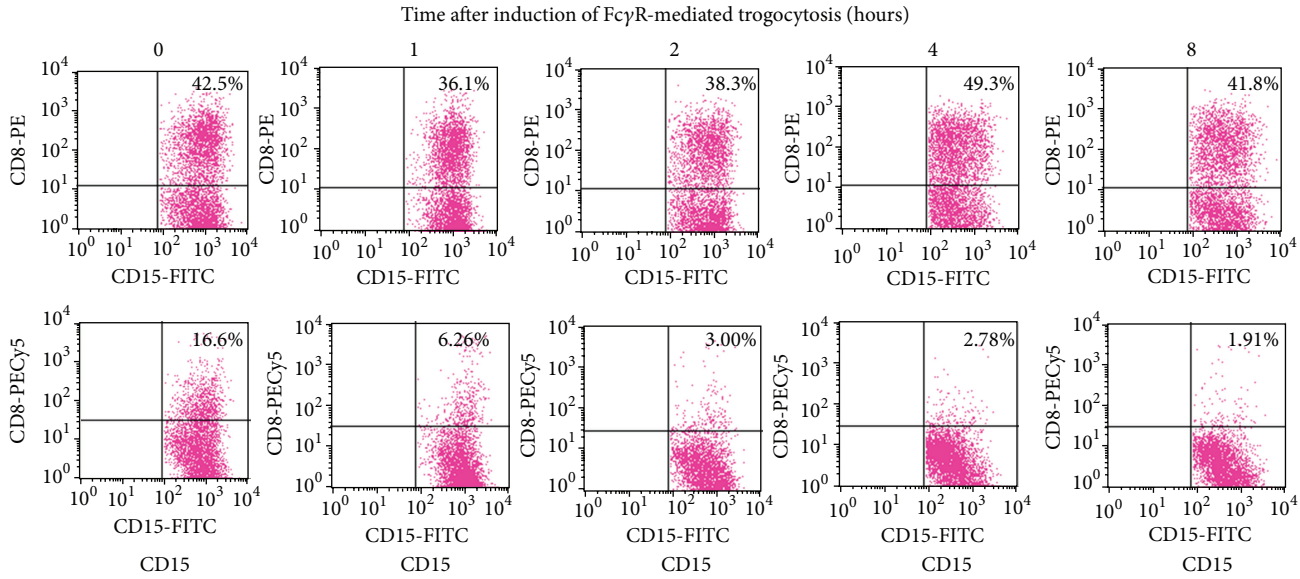

Figure 2: Dynamism of molecules transferred to neutrophils via FcγR-mediated trogocytosis. Heparinized peripheral blood samples (100 μL) were made to react with 0.1 μg/mL of PE-labeled anti-CD8 Ab for 30 min at room temperature. PE-labeled mouse IgG1 was used as an isotype-matched control. After depletion of erythrocytes, the cells were incubated in RPMI-1640 medium with 10% FBS for 0–8 hours at 37°C. Subsequently, the cells were resuspended in 100 μL of PBS and then made to react with 0.1 μg of PECy5-labeled anti-CD8 Ab for 20 min at room temperature. After removal of unbounded Ab, the cells were resuspended in 100 μL of PBS followed by reaction with 0.1 μg of FITC-labeled anti-CD15 Ab for 20 min at room temperature and then served for FCM. The PECy5-labeled anti-CD8 Ab used in this experiment could recognize a different epitope from that recognized by the PE-labeled anti-CD8 Ab. Experiments were repeated at least 3 times. Similar results were reproduced.

4. Association between FcγR-Mediated Trogocytosis and Endocytosis

Next, the association between FcγR-mediated trogocytosis and endocytosis was examined using the CD8 translocation model from T cells to neutrophils. Endocytosis is a fundamental function of neutrophils. Two diverse mechanisms of endocytosis include phagocytosis and pinocytosis, wherein the difference between the two is the size of target molecules. Pinocytosis is used for the absorption of extracellular fluids, and in contrast to phagocytosis, the target molecules are very small. To determine the association between FcγR-mediated trogocytosis and endocytosis, experiments were performed using yellow-green carboxylate-modified latex beads and FITC-labeled ovalbumin (OVA). The process of intake of latex bead and OVA represents phagocytosis and pinocytosis, respectively. After enhancing FcγR-mediated trogocytosis by the CD8 translocation model from T cells to neutrophils, fluorescence-labeled latex beads and OVA were added to the cells. Subsequently, the engulfment of these molecules by $CD8^+$ neutrophils that underwent FcγR-mediated trogocytosis was examined, and the data were compared with those by $CD8^-$ neutrophils that did not perform FcγR-mediated trogocytosis. The activities of both phagocytosis and pinocytosis were higher in $CD8^+$ neutrophils than those in $CD8^-$ neutrophils (Figure 3). These findings suggest that the activity of FcγR-mediated trogocytosis is parallel to the phagocytic and pinocytic activities of neutrophils.

5. Physiological Role of FcγR-Mediated Trogocytosis

It was revealed that the CD8 molecules transferred to neutrophils via FcγR-mediated trogocytosis were taken into the cytoplasm immediately. This evidence suggests that the chance of molecules transferred via FcγR-mediated trogocytosis to play a role on the cell surface could be time-limited. Thus, we consider the physiological role of FcγR-mediated trogocytosis as a means to remove Abs that bind with self-molecules rather than to extract molecules from other cells. Essentially, a living body is always exposed to invading pathogens. The immune system produces Abs to compete with the pathogens; however, these Abs sometimes cross-react with self-Ags. In such situation, the presence of mechanism that can prevent Ab-mediated autoimmune response is beneficial. FcγR-mediated trogocytosis can be a defense mechanism to remove Abs, which unexpectedly bind to self-Ags (Figure 4).

In this study, the relationship between FcγR-mediated trogocytosis and endocytosis of neutrophils was examined using the established CD8 translocation model from T cells to neutrophils. Results clearly indicated that the activity of FcγR-mediated trogocytosis was closely linked to the endocytosis activity of neutrophils. The low activity of endocytosis of neutrophils is critically related to the high susceptibility to systemic autoimmune diseases including systemic lupus erythematosus [34]. Therefore, it is reasonable to consider that the low ability of FcγR-mediated trogocytosis could be also

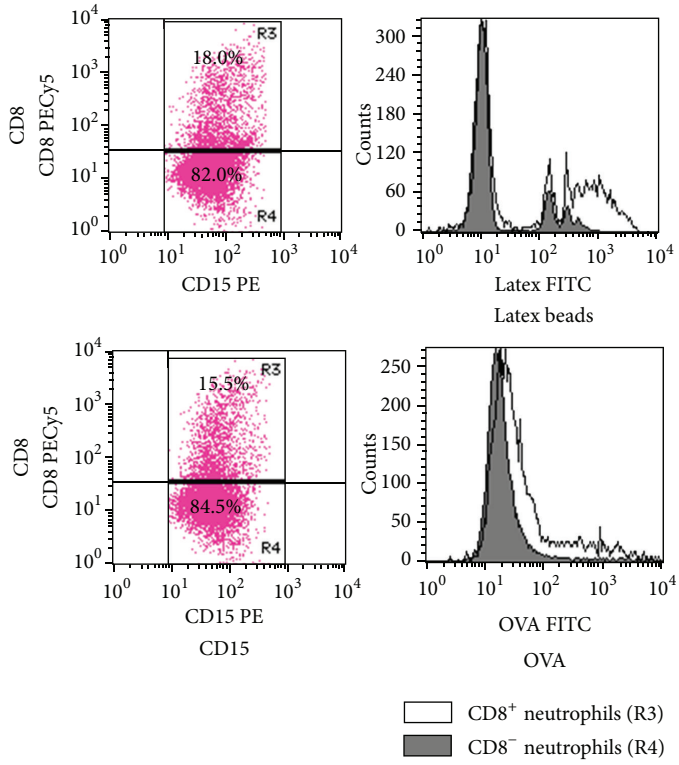

Figure 3: Comparison of endocytotic activities between neutrophils with and without display of FcγR-medicated trogocytosis. Peripheral blood polymorphonuclear cells (0.5×10^6) and mononuclear cells (0.5×10^6) were mixed and preincubated in 100 μL of the autologous serum for 20 min at 37°C. The cells were made to react with 0.1 μg of PECy5-labeled anti-CD8 Ab for 1 hour at room temperature. After washing with PBS, the cells were resuspended in 1 mL of RPMI-1640 medium with 10% FBS. Subsequently, the cells were made to react with 2 μL of yellow-green carboxylate-modified latex beads for 90 min at 37°C or with 100 μg of FITC-labeled OVA for 30 min at 37°C. After washing 3 times with PBS, the cells were re-suspended in 100 μL of PBS and then made to react with 0.1 μg of PE-labeled anti-CD15 Ab, followed by serving for FCM. Experiments were repeated at least 3 times. Similar results were reproduced.

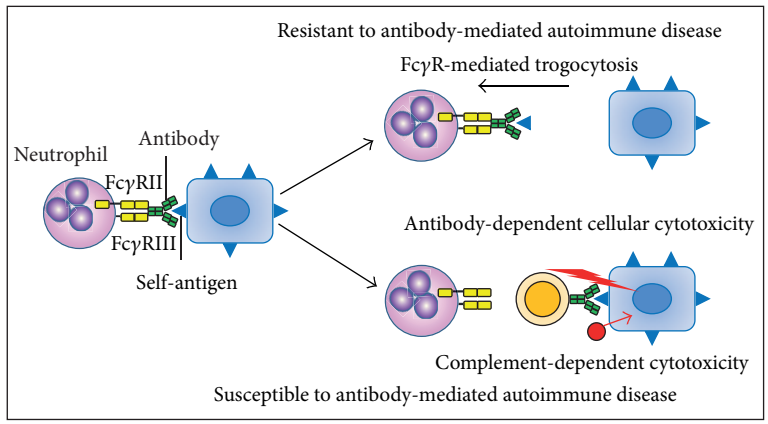

Figure 4: Relation between FcγR-mediated trogocytosis and susceptibility to Ab-mediated autoimmune disease. FcγR-mediated trogocytosis can play a role in the removal of Abs that bind to self-Ags on the cell surface and prevent Ab-mediated autoimmune diseases.

linked to the high susceptibility to systemic autoimmune diseases. This is compatible with our concept that FcγR-mediated trogocytosis can play a role in the removal of autoantibodies and prevent autoimmune diseases. However, in autoimmune hemolytic anemia, trogocytosis mediated by anti-red blood cell (RBC) polymeric IgA Abs and Fcα receptors is involved in the destruction of RBCs [35]. Thus, further prospective studies are needed to clarify if the ability of FcγR-mediated trogocytosis would be actually related to the susceptibility to systemic autoimmune diseases.

Conflict of Interests

The authors have no financial conflict of interests.

Acknowledgments

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Research Article

A Possible Role of HMGB1 in DNA Demethylation in CD4$^+$ T Cells from Patients with Systemic Lupus Erythematosus

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The aberrant activity of CD4$^+$ T cells in patients with systemic lupus erythematosus (SLE) is associated with DNA hypomethylation of the regulatory regions in CD11a and CD70 genes. Our previous studies demonstrated that Gadd45a contributes to the development of SLE by promoting DNA demethylation in CD4$^+$ T cells. In this study, we identified proteins that bind to Gadd45a in CD4$^+$ T cells during SLE flare by using the method of co-immunoprecipitation and mass spectrometry, High mobility group box protein 1 (HMGB1) is one of identified proteins. Furthermore, gene and protein expression of HMGB1 was significantly increased in SLE CD4$^+$ T cells compared to controls, and HMGB1 mRNA was correlated with CD11a and CD70 mRNA. A significant, positive correlation was found between HMGB1 mRNA and SLEDAI for SLE patients. Our data demonstrate that HMGB1 binds to Gadd45a and may be involved in DNA demethylation in CD4$^+$ T cells during lupus flare.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease that affects multiple organ systems. The etiology and pathogenesis of SLE remain to be fully defined. Recent studies have shown that T-cell DNA demethylation plays an important role in the pathogenesis of SLE [1]. We previously demonstrated that inhibition of DNA methylation in T cells resulted in the demethylation of regulatory sequences and increased gene expression of CD11a (ITGAL) and CD70 (TNFSF7) [2–4]. Increased expression of these genes results in T-cell autoreactivity and autoantibody production by the B lymphocyte lineage. The molecular mechanisms that mediate gene demethylation in SLE CD4$^+$ T cell are not fully understood, but it is possible that growth arrest and DNA damage-inducible gene (Gadd45a/Gadd45) may contribute since increased expression of Gadd45a gene has been shown to repress DNA methylation, and factors that induce lupus flare such as ultraviolet have been shown to promote DNA demethylation in SLE CD4$^+$ T cells [5–7].

In this study, we identified protein(s) that bind to Gadd45a, assessed their gene and protein expression and their association with CD11a, CD70 mRNA, and lupus disease activity. HMGB1 was identified as a major protein that bind to Gadd45a, which may contribute to DNA demethylation.

2. Materials and Methods

2.1. Patients and Control Subjects. Ten SLE patients (28.50 ± 10.23 years of age) were recruited from the outpatient clinics and inpatient Department of Dermatology at the Second Xiangya Hospital, Central South University. All patients fulfilled at least 4 of the SLE classification criteria of the American College of Rheumatology [8]. Disease activity was quantified by the SLE Disease Activity Index (SLEDAI) [9], and the mean SLEDAI score of patients recruited into this study was 7.20 ± 1.93 (Table 1). Age- and sex-matched healthy controls (25.80 ± 6.89 years of age) were recruited from the medical staff at the Second Xiangya Hospital. This study

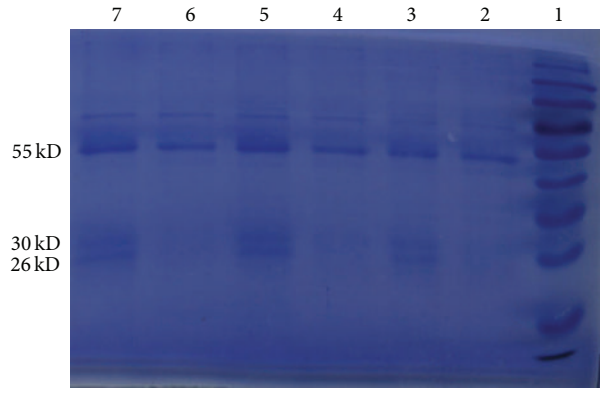

Figure 1: Representative gel showing protein bands immunoprecipitated with anti-human Gadd45a antibody. Lane 1: prestained markers, lanes 3, 5, and 7: immunoprecipitated with anti-Gadd45a antibody, and lanes 2, 4, and 6: immunoprecipitated with isotype-matched control IgG.

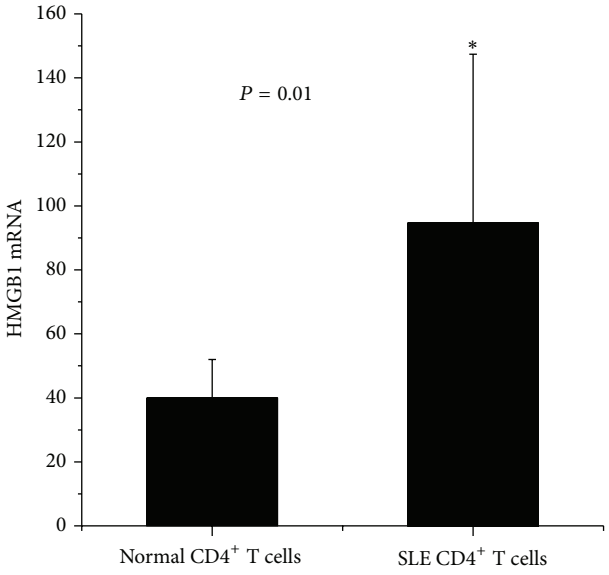

Figure 2: Real-time PCR quantification of HMGB1 mRNA in $CD4^+$ T cells. HMGB1 mRNA in $CD4^+$ T cells isolated from patients with SLE and healthy controls was determined by quantitative real-time PCR. Results are normalized to β-actin and presented as mean + SD.

was approved by the human ethics committee of the Second Xiangya Hospital.

2.2. Isolation of $CD4^+$ T Cells. Peripheral blood mononuclear cells were isolated from heparin-anticoagulated whole blood using Ficoll-Hypaque density gradient centrifugation (Shanghai Hengxin Chemical Reagent Co., Ltd., Shanghai, China), and $CD4^+$ T cells were isolated by positive selection using Miltenyi beads, according to the manufacturer's instructions (Miltenyi, Germany). The purity of the enriched subsets was validated by flow cytometry and was >95%.

Table 1: Patient demographics and treatment.

Patient	Age/gender	SLEDAI	Treatment
1	49/F	12	None
2	38/F	6	Pred 20 mg/d
3	23/F	6	None
4	35/F	6	Pred 5 mg/d
5	15/F	8	None
6	34/F	6	None
7	24/F	8	None
8	25/F	6	None
9	22/F	8	Pred 30 mg + CTX
10	20/F	6	Pred 10 mg

F: female; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; Pred: Prednisone; CTX: Cyclophosphamide.

2.3. Coimmunoprecipitation and LS-MS/MS. $CD4^+$ T cells were solubilized with cell lysis buffer (KeyGEN, China). Aliquots of $CD4^+$ T-cell lysates were incubated overnight at 4°C with rabbit anti-human Gadd45a antibody (Santa Cruz Biotechnology, USA) or isotype-matched rabbit IgG (Beyotime, China) prior to the absorption with protein A/G PLUS-agarose beads (Millipore, USA) at 4°C for 1 hour. Proteins that bound to Gadd45a were centrifuged and eluted. Immunoprecipitated samples were incubated with 2 × SDS electrophoresis sample buffer at 95°C for 5 min and subjected to SDS-PAGE followed by staining with Coomassie blue. Bands of interest were excised, and sent to BGI (Shenzhen, China) for identification using LS-MS.

2.4. Western Blot. Aliquots of $CD4^+$ T-cell proteins were denatured, separated by SDS-PAGE, and transferred to PVDF membranes (Amersham Bioscience, GE Healthcare Company, USA). The membranes were incubated overnight at 4°C with rabbit anti-human HMGB1 polyclonal antibody (diluted 1 : 500, Abcam, USA) followed by incubation with horseradish peroxide-conjugated mouse anti-rabbit antibody (diluted 1 : 3000, Santa Cruz, USA). Bands were detected with Pierce chemiluminescence detection system (Thermo Scientic, Rockford, IL, USA). All bands were normalized to β-actin.

2.5. Real-Time Polymerase Chain Reaction. Total RNA was isolated from $CD4^+$ T cells obtained from healthy controls and patients with SLE using TRIzol (Invitrogen, USA) and 1 μg total RNA reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Canada). Quantitative real-time PCR was performed in duplicate using SYBR Premix Ex Tag kit (Takara, Japan) in a Rotor-Gene 3000 thermocycler. Primers used included

HMGB1 Forward: 5′-TCACAG CCATTG CAG TACATT GAG-3′

Reverse: 5′-GGATCT CCT TTG CCC ATG TTT AGT T-3′

CD70 Forward: 5′-CACACTCTGCACCTCACT-3′

Reverse: 5′-CACCCACTGCACTCCAAAGA-3′

CD11a	Forward:	5′-TGAGAGCAGGCTATTTGGGT TAC-3′
	Reverse:	5′-CGGCCCATGTGCTGGTAT-3′
β-actin	Forward:	5′-CGCGAGAAGATGACCCAGAT-3′
	Reverse:	5′-GCACTGTGTTGGCGTACAGG-3′

2.6. Statistical Analysis. Results were expressed as mean + SD. Intergroup analysis was performed using Student's t-test. Correlation of HMGB1 mRNA with CD11a, CD70 mRNA and SLEDAI was assessed by Pearson's correlation coefficient. P values < 0.05 were considered significant. All analyses were performed with SPSS version 15.0 software.

3. Results

3.1. Identification of Proteins That Bound to Gadd45a. To identify proteins that bind to Gadd45a, total proteins extracted from $CD4^+$ T cell were immunoprecipitated with anti-human Gadd45a antibodies, separated by SDS-PAGE, and stained with Coomassie blue. Anti-Gadd45a antibodies but not isotype-matched control immunoprecipitated two bands with MW of ~30 and ~26 kDa (Figure 1). A protein band with MW ~55 kDa was detected by both anti-Gadd45a antibodies and control IgG. These bands were excised, subjected to in-gel trypsin digestion, and subjected to Tandem MS. Data obtained were analyzed against the Gene Ontology annotations to proteins in the UniProt Knowledgebase using Mascot 2.3.02 software. (Proteins listed in Table 2 are MW of ~30 KDa and ~26 KDa proteins precipitated by anti-Gadd45a antibodies and MW ~55 KDa proteins which are different proteins between the anti-Gadd45a antibodies precipitation group and control antibody precipitation group.)

3.2. Gene and Protein Expression of HMGB1 in $CD4^+$ T Cells from SLE Patients with Active Disease. HMGB1 was identified as the protein that bound to Gadd45a by immunoprecipitation. Elevated expression was confirmed in PBMC and serum in SLE patients in previous studies [10–12]. In addition, it may interact with various proteins, including methy-CpG binding protein2 (MeCP2) [13]. So we compared HMGB1 mRNA levels in $CD4^+$ T cells from SLE patients and healthy controls. Gene and protein expression of HMGB1 was significantly increased in SLE $CD4^+$ T cells compared to $CD4^+$ T cells isolated from healthy subjects ($P = 0.01$ and $P = 0.018$, resp.) (Figures 2, 3(a), and 3(b)).

Gene expression of HMGB1 was significantly correlated with CD11a and CD70 mRNA in $CD4^+$ T cell ($R = 0.737$, $P = 0.000$; $R = 0.650$, $P = 0.002$, resp.) (Figures 4(a) and 4(b)). Furthermore, we analyzed the correlation between HMGB1 mRNA and lupus disease activity as measured by the SLEDAI. A positive correlation was also found between HMGB1mRNA and SLEDAI ($R = 0.797$, $P = 0.006$) (Figure 5).

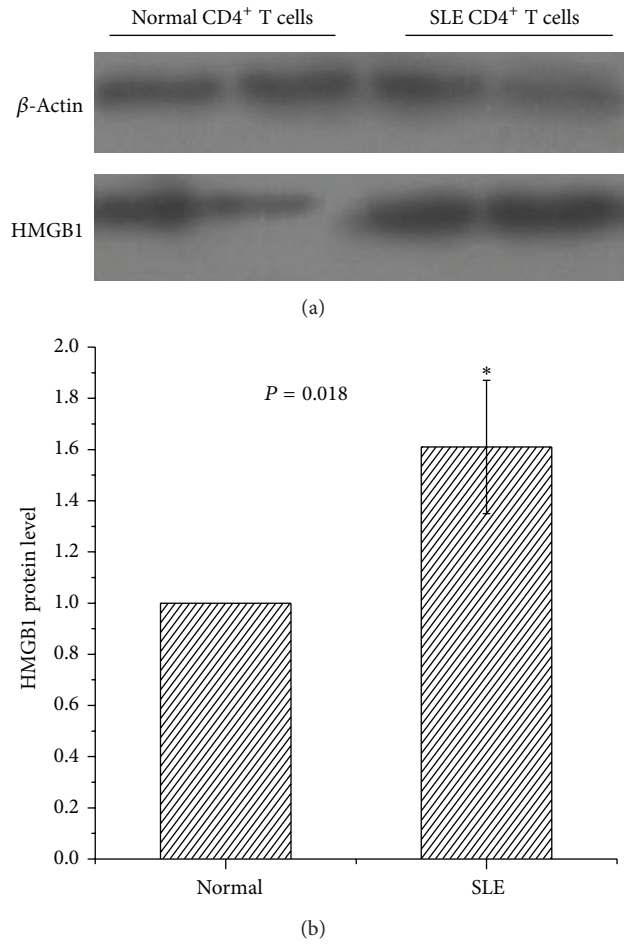

Figure 3: Protein expression of HMGB1 in $CD4^+$ T cells using western blot. (a) Representative western blot showing protein expression of HMGB1 in $CD4^+$ T cells isolated from patients with SLE and healthy controls. (b) Bar chart showing the mean intensity of the bands as determined by densitometric scan, normalized to the house-keeping gene, β-actin.

4. Discussion

Over the past two decades, global DNA hypomethylation and gene-specific DNA demethylation have been observed in T cells in patients with idiopathic lupus and drug-induced lupus. Gadd45a (Gadd45a/Gadd45) is a nuclear protein that plays an important role in the maintenance of genomic stability, DNA repair, and suppression of cell growth. In our previous studies, we have demonstrated that Gadd45a contributes to autoimmune diseases by promoting DNA demethylation [5]. In this study, we identified proteins that bind to Gadd45a in SLE $CD4^+$ T cells that may assist in DNA demethylation. Using LS-MS/MS, we identified HMGB1 as one of the proteins that bind to Gadd45a. Gene and protein expression of HMGB1 was increased in SLE $CD4^+$ T cells compared to control $CD4^+$ T cells. HMGB1 is a nuclear DNA binding protein secreted by monocytes and macrophages and plays a critical role in innate immunity, inflammation, and sterile injury [14]. Serum HMGB1 levels are increased in SLE

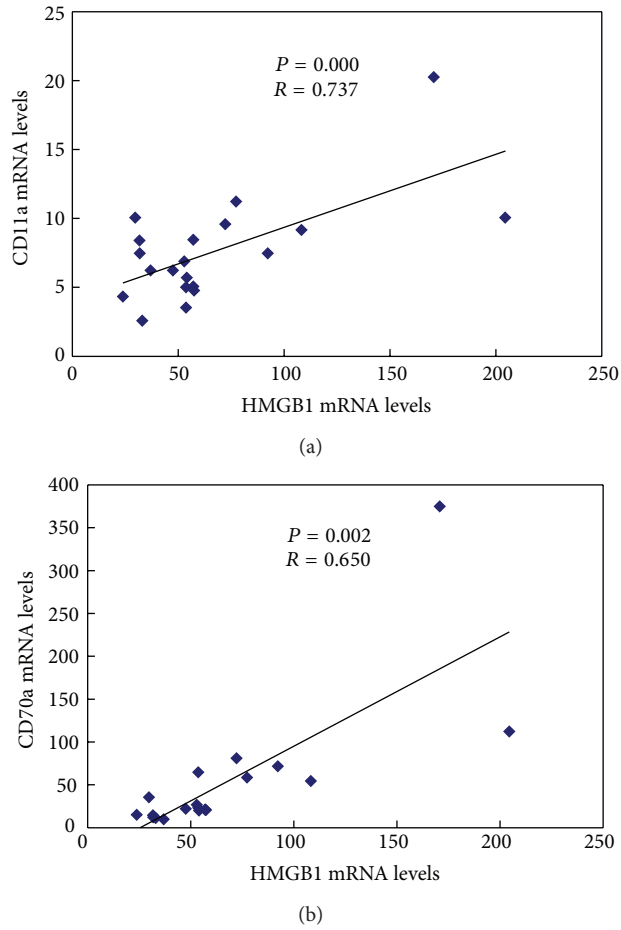

Figure 4: Correlation between HMGB1 mRNA and CD11a and CD70 mRNA. Gene expression of HMGB1, CD11a, and CD70 was assessed by real-time PCR. Correlation between HMGB1 mRNA and (a) CD11a mRNA and (b) CD70 mRNA is shown for $CD4^+$ T cells in SLE patients and controls.

patients and its expression is increased in skin lesions in lupus patients [10–12, 15].

HMGB1 interacts with numerous proteins such as methyl-CpG binding protein-2 (MeCP2), a protein that participates in the recognition of methylated DNA in CpG islands [13]. In this study, we demonstrated that gene and protein expression of HMGB1 in $CD4^+$ T cells is significantly increased in SLE patients compared to healthy controls thereby corroborating previous studies. Furthermore, HMGB1 mRNA in $CD4^+$ T cells is correlated with SLEDAI for SLE patients. Given that $CD4^+$ T cells promote inflammation and autoimmunity, it is plausible to suggest that HMGB1 contributes to the pathogenesis of SLE [16].

CD11a plays a key role in cellular adhesion and costimulatory signaling. Its expression is increased in T cells consequent to hypomethylation and correlates with the development of T-cell autoreactivity in SLE patients [3, 4]. CD70 is a methylation-sensitive gene present in T cells. Costimulatory signals mediated through CD70-CD27 interactions regulate

Table 2: Proteins identified by LS-MS/MS.

Protein mass	Description
	~26 KDa, ~30 KDa
25049.23	**HMGB1 High mobility group protein B1**
29807.14	RPS4X 40S (ribosomal protein S4, X isoform)
27898.79	YWHAZ 14-3-3 protein zeta/delta
14126.95	HIST1H2AB; HIST1H2AE; HIST1H2AD; HIST1H2AG; HIST1H2AK; HIST1H2AI; HIST1H2AL; HIST1H2AM; HIST1H2AJ Histone H2A type 1-B/E
38837.09	HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1
28876.08	PSME1 Proteasome activator complex subunit 1
32179.71	HNRNPCL1 Heterogeneous nuclear ribonucleoprotein C-like 1
36201.46	GAPDH Glyceraldehyde-3-phosphate dehydrogenase
18885.87	RPS10 40S ribosomal protein S10
33059.24	SLC25A5 ADP/ATP translocase 2
37463.75	HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1
15215.62	PFN1 Profilin-1
25119.3	IGLC2; IGLV2-14 IGL@ protein
24820.32	RAB27B Ras-related protein Rab-27B
22449.98	DRAP1 Isoform 1 of Dr1-associated corepressor
32867.15	TNFAIP8L3 Tumor necrosis factor alpha-induced protein 8-like protein 3
16982.46	LYZ Lysozyme C
31881.8	STOM Erythrocyte band 7 integral membrane protein
24764.75	SNRPB Isoform SM-B′ of Small nuclear ribonucleoprotein-associated proteins B and B′
19545.99	SRSF3 Serine/arginine-rich splicing factor 3
17707.86	RPS18 40S ribosomal protein S18
38805.89	ANXA2P2 Putative annexin A2-like protein
26688.86	AK2 Isoform 1 of Adenylate kinase 2, mitochondrial
21950.62	IMP3 U3 small nucleolar ribonucleoprotein protein IMP3
22113.26	RPS7 40S ribosomal protein S7
30729.42	ASB13 Isoform 1 of Ankyrin repeat and SOCS box protein 13
	~55 KDa
52076.78	PPP2R2B Isoform 1 of Serine/threonine-protein phosphatase 2A 55
52324.86	CAP1 Isoform 1 of Adenylyl cyclase-associated protein 1
56456.49	EEF1G cDNA FLJ56389, highly similar to Elongation factor 1-gamma
52586.54	EIF3E Eukaryotic translation initiation factor 3 subunit E

B-cell activation and autoantibody production, thereby highlighting the role of CD70 in the pathogenesis of SLE. In this

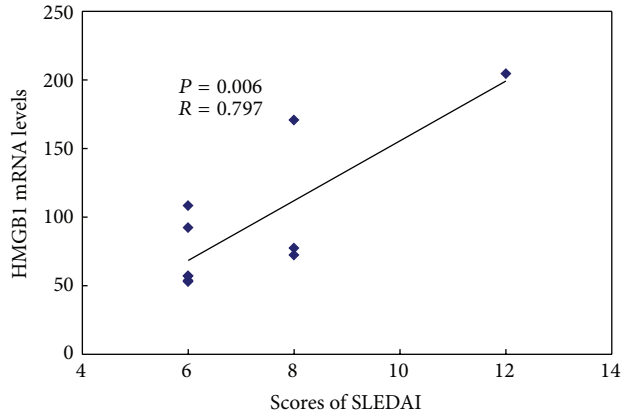

Figure 5: Correlation between HMGB1 mRNA and SLEDAI. Lupus disease activity was quantified by the SLE Disease Activity Index (SLEDAI). Correlation between HMGB1 mRNA in lupus $CD4^+$ T cells and SLEDAI for lupus patients is shown.

study, we demonstrated that HMGB1 mRNA is significantly correlated with CD11a and CD70 mRNA.

In conclusion, we have demonstrated that HMGB1 binds to Gadd45a and its increased gene expression which is associated with a concomitant increase in CD11a and CD70 mRNA may be involved in DNA demethylation in $CD4^+$ T cells, thus contributing to the pathogenesis of SLE. Further studies are currently ongoing to delineate the function of HMGB1 and its underlying mechanism contributing to DNA demethylation in $CD4^+$ T cells during pathogenesis of SLE.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Replication of British Rheumatoid Arthritis Susceptibility Loci in Two Unrelated Chinese Population Groups

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Previous genome-wide association study by WTCCC identified many susceptibility loci of common autoimmune diseases in British, including rheumatoid arthritis (RA). Because of the genetic heterogeneity of RA, it is necessary to replicate these susceptibility loci in other populations. Here, three SNPs with strong RA association signal in the British were analyzed in Han Chinese, and two SNPs (rs6457617 and rs11761231) were genotyped in the test cohort firstly. The rs6457617 was significantly associated with RA in the test cohort. The individuals bearing the homozygous genotype CC had 0.39-fold risk than these bearing the wild-type genotype TT ($P = 0.004$, OR 0.39, [95% CI 0.21–0.74]). And the protective effect of allele C was confirmed in another validation cohort with 1514 samples ($P_{genotye\,CC/TT} = 5.9 \times 10^{-10}$, OR 0.34, [95% CI 0.24–0.48]). The rs6457617 can be used as a tagSNP of HLA-DQA1*03 which encoded MHC-II α chain. Since MHC restriction is important for primary T-cells in positive selection and negative selection stages, MHC protein polymorphisms may be implicated in shaping the T-cell repertoire, including the emergence of a T-cell clone involved in the inflammatory arthritis.

1. Introduction

Rheumatoid arthritis (RA (MIM 180300)) is a common autoimmune disease characterized by chronic inflammatory, destructive, and debilitating arthritis. The etiology of RA, like that of other autoimmune disorders, is complex and remains elusive. The occurrence of RA is relatively constant with a prevalence of between 0.5 and 1.0% in several European populations [1, 2], North-American populations [3], Japan [4], and China [5]. But some native American-Indian populations have high prevalence of RA such as the Pima Indians (5.3%) [6] and Chippewa Indians (6.8%) [7]. Although it is difficult to elucidate whether the environmental or genetic effect influence the differences between populations in different countries, it is thought to have both a genetic and an environment basis [8–11]. The heritability of RA has been estimated to be about 60% [12]. The highly polymorphic HLA region is estimated to account for about one-third of the total genetic component of susceptibility [13]. Many genes outside the HLA region also account for the RA risk genetic factor.

There are two strategies used commonly to detect the RA risk genetic loci. One is the candidate gene association study, and another is the genome-wide association study (GWAS). Candidate gene association studies rely on prior knowledge of the biology of the disease and the highly subjective selection of potential genes. It is hard to find new RA susceptibility loci outside of HLA. Genome-wide association studies (GWAS) have an advantage that it entails a systematically search of the entire genome for susceptibility variants without any clue about pathogenesis. With the advent of GWAS, relatively large number of new potential susceptibility loci for RA in some populations has been identified [14, 15]. One of the most impressive GWASs was the one by the WTCCC, which scanned 500,568 SNPs (Affymetrix Chip) in 14,000 cases of seven major autoimmune diseases, including 2000 United Kingdom RA cases and 3000 controls [16]. Among the loci

Table 1: PCR primers and tagged extension probes used for SNP detection.

SNPs	Locus	Gene	Primer	Sequence 5′-3′
rs6457617	6q	*MHC*	Forward	AAATGCAGTCAGTGGACTCAA
			Reverse	AAAACAAAAAAAACCCTTCAATC
			Probe	GACCTGGGTGTCGATACCTACTGTTTGTTGAGTCCATGAGCAGAT
rs11761231	7q32	*Unknown*	Forward	TGTCTTATCATGAGAACGTGCA
			Reverse	TTTTGTGTTCAAAGAATTCTGTCTT
			Probe	AGAGCGAGTGACGCATACTATGAAATCAAGAAGGTCTGAAAA

Table 2: Clinical characteristics of the test and validation cohorts.

Subjects	Test cohort	Validation cohort
Number of RA cases	190	757
Number of healthy controls	190	757
Study design	Unrelated cases with individually matched controls	Unrelated cases with individually matched controls
Ethnicity	Han Chinese	Han Chinese
City of residence	Chengdu	Chongqing
Female, number (%)	300 (78.9)	1253 (82.7)
Age, mean ± SD years	46.4 ± 8.0	46.8 ± 10.2
Age at RA onset, mean ± SD years*	39.4 ± 9.6	41.0 ± 10.1
Disease duration, mean ± SD years*	6.97 ± 6.2	5.7 ± 5.4

*Refers to rheumatoid arthritis (RA) patients only.

showing the strongest association signals, there were 3 RA susceptibility SNPs (rs6679677, rs6457617, and rs11761231) with a P value of less than 4×10^{-7}.

Meanwhile, much evidence shows that there is the genetic heterogeneity of RA across the major racial groups. For example, the haplotype in STAT4 gene associated with RA in Caucasians is not associated in the Han Chinese population, but with the presence of rheumatoid factor [17]. The rs247661 in PTPN22 gene, which was associated with RA in Europeans [18], does not exist in Han Chinese. The PADI4 gene, which was originally identified as RA susceptibility gene in large Japanese and Korean cohorts [19], was not associated with RA and unlikely to be responsible for the presence of anti-CCP autoantibodies in Caucasian populations [20]. Therefore, it is necessary to test RA susceptibility loci of GWAS in different populations. Here, the three SNPs identified by the WTCCC with a P value of less than 4×10^{-7} were taken into our consideration to investigate whether they are associated with RA in Han Chinese population.

2. Materials and Methods

2.1. Subjects Evaluated in the Association Study. Total 1894 RA patients and healthy individuals were recruited for this study. The test cohort was 380 Chengdu residents recruited from the Chengdu Medical College, and the validation cohort was 1514 Chongqing residents recruited from the Southwest Hospital, as reported in our previous study [21]. All the subjects were unrelated ethnic Han Chinese, according to self-reported ancestry. Patients have an established diagnosis of RA according to the 1987 revised criteria of the ACR for the classification of the disease. In each cohort, healthy controls were individually matched to RA cases on the basis of sex, age, ethnicity, and local residential region. There was no significant difference between the 2 cohorts in terms of the distribution of age, age at RA onset, or percentage of female subjects (Table 2). Written informed consent was obtained from all subjects, and the study was performed with the approval of the ethical committee of the Chengdu Military General Hospital.

2.2. SNP Selection and Genotyping Methods. Three SNPs with strong RA association signal in British population were rs6457617, rs11761231, and rs6679677. The rs6679677 has no diversity in Han Chinese population based on the HCB data of HapMap and did not genotyped in our study. The rs6457617 is in the MHC region, and the minor allele frequency (MAF) in HCB is 0.465. The rs11761231 locates on chromosome 7q32, and the MAF is 0.233. The rs6457617 and rs11761231 were genotyped using the SNPStream Ultra High Throughput Genotyping system (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. The primers and probes of each SNP were listed in Table 1. The genotype concordance rate was 100% as assessed by random retyping across different plates.

2.3. Statistical Analysis. Hardy-Weinberg equilibrium was assessed using a chi-square goodness-of-fit test. Case-control association analyses were performed by chi-square or Fisher's exact test, as appropriate. P values, odds ratios (ORs), and 95% confidence intervals (95% CIs) were calculated.

3. Results and Discussion

All SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$) in both patients and healthy controls, indicating that our subjects were random-mating population with no selection, mutation, or migration. As shown in Table 3, rs6457617 was significantly associated with RA in the test cohort. The frequency of the minor allele C was significantly lower in RA patients compared to healthy control (28.9% of the patients versus 40.8% of the controls; $P = 0.001$, OR 0.59, [95% CI 0.44–0.80]), implying that the minor allele C was protective against RA. When we compared the distribution of the homozygous genotype CC or the heterozygous genotype

Table 3: Allele and genotype frequencies of SNPs in test cohort.

SNP	Chromosome	Gene	Allele	Allele analysis				Genotype	Genotype analysis			
				Controls number (%)	Cases number (%)	OR (95% CI)	*P*		Controls number (%)	Cases number (%)	OR (95% CI)	*P*
rs6457617	6q	MHC	T	218 (59.2)	263 (71.1)	1 (reference)	—	TT	68 (37.0)	97 (52.4)	1 (reference)	—
			C	150 (40.8)	107 (28.9)	**0.59 (0.44–0.80)**	**0.001**	CT	82 (44.6)	69 (37.3)	**0.59 (0.38–0.92)**	**0.02**
								CC	34 (18.5)	19 (10.3)	**0.39 (0.21–0.74)**	**0.004**
rs11761231	7q32	Unknown	T	287 (75.9)	290 (76.7)	1 (reference)	—	TT	108 (57.1)	112 (59.3)	1 (reference)	—
			C	91 (24.1)	88 (23.3)	0.96 (0.68–1.34)	0.80	CT	71 (37.6)	66 (34.9)	0.90 (0.59–1.37)	0.62
								CC	10 (5.3)	11 (5.8)	1.06 (0.43–2.60)	0.90
rs11761231 in female group	7q32	Unknown	T	218 (73.2)	220 (73.8)	1 (reference)	—	TT	79 (53.0)	82 (55.0)	1 (reference)	—
			C	80 (26.8)	78 (26.2)	0.97 (0.67–1.39)	0.85	CT	60 (40.3)	56 (37.6)	0.90 (0.56–1.45)	0.66
								CC	10 (6.7)	11 (7.4)	1.06 (0.43–2.63)	0.90
rs11761231 in male group	7q32	Unknown	T	69 (86.3)	70 (87.5)	1 (reference)	—	TT	29 (72.5)	30 (75.0)	1 (reference)	—
			C	11 (13.7)	10 (12.5)	0.90 (0.36–2.25)	0.82	CT	11 (27.5)	10 (25.0)	1.14 (0.42–3.08)	1.0
								CC	0	0		

Data shown in *n* (%). OR = odds ratio; 95% CI = 95% confidence interval; MHC: major histocompatibility complex; allele analyses for association were used by Pearson chi-square test, and genotype analyses for association were used by logistic regression.

Table 4: Validation of SNPs in test cohort, validation cohort, and combined analyses (combined validation cohort with test cohort).

	Genotype	Subjects number (%)		Codominant genetic model		Dominant genetic model		Recessive genetic model	
		Controls	Cases	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Test cohort rs6457617	TT	68 (37.0)	97 (52.4)	1 (ref)	—	**0.53 (0.35–0.81)**	**0.003**	**0.51 (0.28–0.92)**	**0.025**
	CT	82 (44.6)	69 (37.3)	**0.59 (0.38–0.92)**	**0.02**				
	CC	34 (18.5)	19 (10.3)	**0.39 (0.21–0.74)**	**0.004**				
Validation cohort rs6457617	TT	263 (35.7)	353 (48.2)	1 (ref)	—	**0.60 (0.49–0.74)**	**1.5×10^{-6}**	**0.41 (0.30–0.56)**	**2.3×10^{-8}**
	CT	340 (46.1)	319 (43.5)	**0.70 (0.56–0.87)**	**1.0×10^{-3}**				
	CC	134 (18.2)	61 (8.3)	**0.34 (0.24–0.48)**	**5.9×10^{-10}**				
Combined samples rs6457617	TT	331 (35.9)	450 (49.0)	1 (ref)	—	**0.58 (0.48–0.70)**	**1.4×10^{-8}**	**0.43 (0.32–0.57)**	**2.2×10^{-9}**
	CT	422 (45.8)	388 (42.3)	**0.68 (0.56–0.82)**	**1.1×10^{-4}**				
	CC	168 (18.3)	80 (8.7)	**0.35 (0.26–0.47)**	**9.4×10^{-12}**				

Data shown in *n* (%). OR = odds ratio; 95% CI = 95% confidence interval; allele analysis and genotype analysis in dominant genetic model or in recessive genetic model for association were used by Pearson chi-square test, and genotype analyses in codominant genetic model for association were used by logistic regression.

CT among the RA patients and controls to the one of the wild type genotype TT, the protective effect of allele C was obvious and presented a dose effect relationship. The individuals bearing the homozygous genotype CC had 0.39-fold decreased risk than these bearing the wild-type genotype TT ($P = 0.004$, OR 0.39, [95% CI 0.21–0.74]). while individuals bearing the heterozygous CT had 0.59-fold decreased risk for RA than those bearing wild-type genotype ($P = 0.02$, OR 0.59, [95% CI 0.38–0.92], Table 3).

The strong association signal of rs6457617 was replicated in the validation cohort with 1514 subjects. Among 1470 samples successfully genotyped, each genotype was assessed with the use of codominant, dominant, and recessive genetic models (Table 4). The significant association was found in all genetic models. The individuals with the homozygous genotype CC had the lowest risk than those with wild-type genotype ($P = 5.9 \times 10^{-10}$, OR 0.34, [95% CI 0.24–0.48]). Because the allele frequencies were similar across the controls tested in both cohorts, we combined all of the data and obtained stronger statistical evidence of an association between this SNP and RA susceptibility. Compared to individuals with genotype TT, the Han Chinese bearing genotype CC had a lower decreased risk ($P = 9.4 \times 10^{-12}$, OR = 0.35, 95% CI: 0.26–0.47) than heterozygous carriers had ($P = 1.1 \times 10^{-4}$, OR = 0.68, 95% CI: 0.56–0.82). The allele T was RA risk allele both in the British and the Han Chinese. But the Han Chinese bearing genotype TT had a 2.86-fold (1/0.35) increased risk compared to those with genotype CC, much lower than it in the British (OR = 5.21).

This rs6457617 was identified in another GWAS in 800 Spanish subjects and replicated in an independent cohort of 794 Spanish subjects [22]. For the Asian population, this SNP was also replicated in the Korean [23]. The minor allele was C in Korean and Chinese but was T in the WTCCC British samples. With the data of each genotype frequency in RA cases and controls in Korean in their Table 3 of the previous report [23], we found that the genotype TT in Korean had a 4.77-fold increased risk compared to the Korean with genotype CC. The OR in Korean is between the one (OR 2.86) in Chinese and another (OR 5.21) in British. Recently, this SNP was replicated in another replication study in north Indians ($P = 1.6 \times 10^{-9}$) [24]. But this report has not shown more detail data about rs6457617. Although there are different frequencies of the alleles C and T in different populations, the association between rs6457617 and RA susceptibility exists in all these different populations, and the risk allele for RA is the same T allele, strongly suggesting that rs6457617 in HLA-DQ region is the real RA susceptibility locus.

The rs6457617 locates at 32,663,850 on 6p21.3 between the HLA-DQB1 (32,627,241-32,634,466) encoded β chain of MHC-II protein and HLA-DQA2 (32,709,163-32,714,664) encoded α chain of MHC-II protein. The MHC protein is an α β heterodimer protein receptor that is typically expressed on the surface of antigen-presenting cells. MHC loci are the most genetically variable loci in the human population. HLA DQ is highly variable, the β subunit more so than the α chain. rs6457617 can be used as a tagSNP for HLA-DQA1*03 [25, 26], which encodes MHC-II α chain. As we know, DRB1*0401, *0404, *0408, and *01, DQA*01, *0201, and *04 are the shared epitope (SE) associated with RA [27], while HLA-DQA1*03 has been reported to be associated with chronic HCV infection [28], type 1 diabetes [29, 30], and childhood-onset ocular myasthenia gravis [31]. Up to now, whether HLA-DQA1*03 is associated with RA has not been identified. Since rs6457617 is the RA susceptibility locus and can be a tagSNP for HLA-DQA1*03, HLA-DQA1*03 may

be a SE of RA. Highly variable MHC-II molecules recognize and present different antigens to T-cells. Since MHC restriction is particularly important for primary T-cells in positive selection and negative selection stages, MHC protein polymorphisms may play an important role in shaping the T cell repertoire, including the emergence of an unusual T cell clone characterized by the potential of inflammatory arthritis. Whether the rs6457617 is a functional SNP and influences the expression or the structure of β chain is needed to identify.

As for rs11761231, no association evidence was found in the allele and genotype analyses. We noticed that in the male Han Chinese, including RA patients and healthy controls, none of them bears genotype CC. The stratification of patients and controls for gender was performed. However, there is no association between rs11761231 and RA disease either in female group or in male group (Table 3). This SNP locates within the eukaryotic translation elongation factor 1 beta 2 pseudogene. Although it had very strong association signal of RA in British population, it did not show any evidence of association in Han Chinese populations. The minor allele C frequency (MAF) in Han Chinese is only 0.278, quite different from the MAF in CEU (0.407). Moreover, the prevalence of RA in China is lower than it in European. Therefore, the test cohort with 380 subjects might have no enough statistical power to detect the genetic effect of rs11761231. So, to investigate the association between rs11761231 and RA in Han Chinese, the case-control study with sufficiently large sample size is needed.

4. Conclusions

In this study, rs6457617 in the MHC region, which was identified as RA susceptibility locus in previous GWAS study in British, was associated with RA in the Han Chinese populations. The minor allele C has dependent protective effect of RA risk. The rs6457617 is a real RA susceptibility locus and could be a good predictor of RA risk.

Conflict of Interests

The authors declare that there is no conflict of interests in this study.

Acknowledgments

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Research Article

Serum Interleukin-6 Expression Level and Its Clinical Significance in Patients with Dermatomyositis

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Objective. To analyze serum interleukin-6 (IL-6) expression level and its clinical significance in patients with dermatomyositis. *Methods*. Blood samples from 23 adult patients with dermatomyositis (DM), 22 with systemic lupus erythematosus (SLE), 22 with rheumatoid arthritis (RA), 16 with Sjögren's syndrome (SS), and 20 healthy controls were collected. The IL-6 concentration was detected by chemiluminescence immunoassay. Correlations between IL-6 expression levels and clinical features or laboratory findings in patients with DM were investigated. *Results*. IL-6 expression level of DM patients was significantly higher than that of normal controls, significantly lower than that of RA patients, and slightly lower than that of SLE or SS patients with no significant differences. The incidence of fever was significantly higher in the IL-6 elevated group. Serum ferritin (SF) and C-reactive protein (CRP) were positively correlated with IL-6. *Conclusions*. IL-6 plays a less important role in DM than in RA. IL-6 monoclonal antibodies may have poor effect in patients with DM.

1. Introduction

Idiopathic inflammatory myopathy (IIM) is a series of disorders characterized by chronic muscle inflammation of unknown cause. Dermatomyositis (DM) is one of the most common forms of IIM with muscle, skin, and internal organs involved [1, 2]. Interleukin-6 (IL-6) is a potent, pleiotropic Th2 cytokine that regulates the immune defense response. IL-6 acts as both a proinflammatory and an anti-inflammatory cytokine and plays a central role in the transition from the acute to the chronic phase of the inflammatory process [3, 4]. Elevated levels of IL-6 have been documented in a variety of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, glomerular nephritis, and so forth [5–7]. In this study, we tried to determine whether IL-6 could contribute to the inflammation associated with DM. We compared serum IL-6 level in DM patients with other three connective tissue diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS). Clinical significance of IL-6 was evaluated.

2. Materials and Methods

2.1. Patients and Controls. All patients were enrolled in West China Hospital from August 2012 to March 2013. Patients with DM satisfied the criteria of Bohan/Peter [8, 9]. The disease controls were patients with SLE, RA, and SS, based on the Criteria of ACR for SLE [10], the Criteria of ACR for RA [11], and the revised American-European Criteria for SS [12], respectively. Patients with an overlapping syndrome were excluded. Healthy controls were matched to DM patients by age and gender. This study was approved by the Ethical Committee of West China Hospital of Sichuan University, and informed consent was obtained from all patients.

2.2. Methods. Serum IL-6 levels were measured by chemiluminescence immunoassay (ROCHE Modular Analytics E170) in DM patients and controls. Blood parameters including creatine phosphokinase (CPK), CRP, erythrocyte sedimentation rate (ESR), SF, antinuclear antibody (ANA)

and anti-Jo-1 antibody were measured by standard methods. Clinical data were obtained from medical records on admission.

2.3. Statistics. Statistical analysis was performed by using Fisher's exact test for comparison of frequencies and Mann-Whitney U test for comparison of median levels. Correlation coefficients were established by using Spearman's correlation coefficients. The data were analyzed by SPSS 17.0 software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Serum IL-6 Levels in DM Patients and Controls. Twenty-Three patients with DM, 22 with SLE, 22 with RA, 16 with SS, and 20 healthy controls were enrolled in this study. The demography and the IL-6 levels in DM patients and controls were shown in Table 1 and Figure 1. The age at onset and sex were not significantly different between DM patients and controls. The serum level of IL-6 was 21.5 ± 30.3, 43.3 ± 55.6, 102.6 ± 82.8, 26.7 ± 29.9, and 1.2 ± 2.6 pg/mL in DM, SLE, RA, SS patients, and healthy controls, respectively. The level of IL-6 was significantly higher in DM patients than that in healthy controls ($P < 0.01$), and was significantly lower than that in RA patients ($P < 0.001$). Compared with SLE and SS patients, IL-6 level was slightly lower in DM patients, but no significant difference was found.

3.2. Correlation between IL-6 and Clinical Features or Laboratory Markers. Correlation coefficients were established by using Spearman's correlation coefficients. Considering the clinical characteristics, significantly positive correlation was found between IL-6 and fever ($r_s = 0.569$; $P = 0.005$, Table 2). As shown in Figure 2, the receiver-operator characteristic curve (ROC curve) analysis was used to determine the level of IL-6 when fever occurred. The area under the curve was 0.831, and the cutpoint value was 22.35 pg/mL. All DM patients had fever when IL-6 level was above the cutpoint. The sensitivity and specificity for IL-6 cutoff point value predicting fever in DM patients were 60% and 100%, respectively. In addition, no correlation was found between IL-6 and sex, age, course of disease, muscle weakness, interstitial lung disease, Gottron's sign, heliotrope eruption, or arthritis. As for laboratory markers, IL-6 was found positively correlated with CRP ($r_s = 0.595$; $P = 0.004$) and SF ($r_s = 0.789$; $P = 0.004$, Table 3). No correlation was found between IL-6 and abnormal electromyogram, CPK, positive muscle biopsy findings, ESR, ANA, or anti-Jo-1.

4. Discussion

It is widely accepted that DM arises from CD_4^+ T cell- and B cell-mediated muscle inflammation, in which the complement system is activated, resulting in membrane deposition of attack complex within muscle capillaries [13]. It is unclear which pathway mediates the inflammation and perivasculitis [14]. As far as we know, IL-6 is an important proinflammatory cytokine and contributes to the inflammatory process.

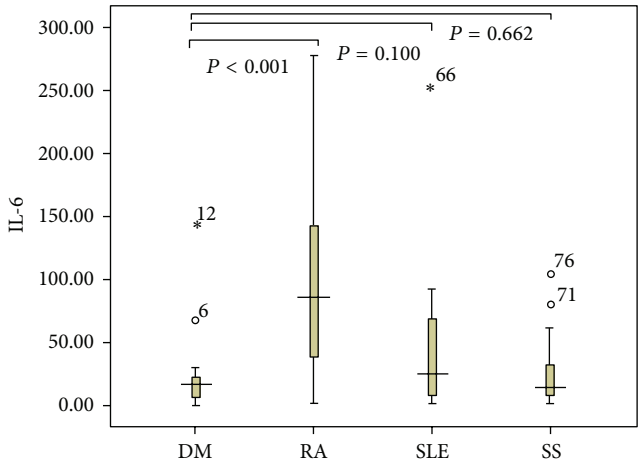

Figure 1: Serum IL-6 levels in patients with DM, RA, SLE, and SS.

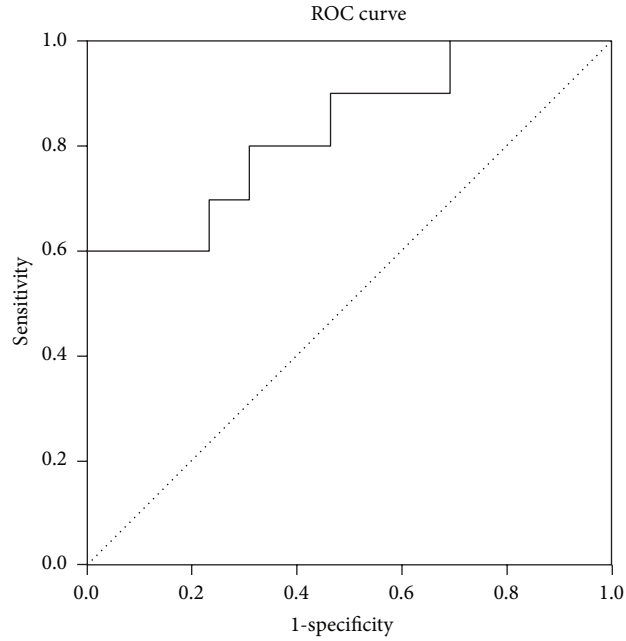

Figure 2: The ROC curve of DM patients with fever.

Elevated levels of IL-6 have been documented in a variety of autoimmune diseases, including rheumatoid arthritis, colitis, Crohn's disease, glomerular nephritis, and so forth [15]. Besides, IL-6 plays central roles in the regulation of both innate and adaptive inflammatory immune responses, as well as both humoral and cell-mediated autoimmune reactions, such as B cell differentiation activity and the T cell growth and differentiation [16]. In literature, evidence concerning the pathogenic role of IL-6 in DM is sparse. Our study demonstrated that the level of serum IL-6 was significantly higher in DM patients than in healthy controls, and IL-6 had significant correlation with the inflammatory marker CRP or serum ferritin (SF). In pathophysiology, tissue injury of skin, muscle, or other internal organs activates monocytes and macrophages to release cytokines, inducing in turn hepatic synthesis of acute phase reactants (APRs) [17]. CRP and SF

Table 1: The demography and the IL-6 levels in DM patients and controls.

Variables	DM	SLE	RA	SS	Healthy controls
	$n = 23$	$n = 22$	$n = 22$	$n = 16$	$n = 20$
Age, years	49 ± 11	41 ± 16	53 ± 14	44 ± 14	45 ± 12
Male/female	9/14	1/21	8/14	1/15	10/10
IL-6, pg/mL	21.5 ± 30.3	43.3 ± 55.6	102.6 ± 82.8	26.7 ± 29.9	1.2 ± 2.6
P value		0.100	<0.001	0.662	<0.01

P value was obtained from the statistical comparisons of serum IL-6 between the DM group and the SLE, RA, SS, and healthy groups. DM: dermatomyositis, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, SS: Sjögren's syndrome, and IL-6: interleukin-6.

Table 2: Correlation between IL-6 and clinical features in patients with DM.

Variables	r_s	P value
Sex	−0.013	0.952
Age (years)	0.009	0.968
Course of DM (months)	−0.322	0.134
Muscle weakness	−0.011	0.961
ILD	0.100	0.651
Gottron's sign	0.215	0.325
Heliotrope eruption	0.121	0.582
Arthritis	0.079	0.721
Fever	0.569	0.005

ILD: interstitial lung disease.

Table 3: Correlation between IL-6 and laboratory markers in patients with DM.

Variables	r_s	P value
EMG	0.081	0.715
CPK	0.405	0.055
Biopsy	0.228	0.295
ESR	−0.019	0.930
CRP	0.595	0.004
SF	0.789	0.004
ANA	−0.079	0.721
Anti-Jo-1	−0.111	0.613

EMG: electromyogram, CPK: creatine phosphokinase, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, SF: serum ferritin, and ANA: antinuclear antibody.

are classic APRs, and IL-6 is a mainly stimulator of APRs [18]. These findings showed that IL-6 played a role in the inflammatory process of DM. Interestingly, this conclusion was verified in an experimental mouse model of myosin-induced myositis, indicating that the deficiency of IL-6 led to marked amelioration of the clinical signs and pathologic findings of muscle injury [19].

Furthermore, we found that the level of IL-6 in DM patients was significantly lower than that in RA patients, which suggesting a less important role of IL-6 in DM than in RA [20]. The level of IL-6 in DM was even slightly lower than those in SLE and SS. In DM patients, the degree of inflammatory reaction is not always consistent with clinical severity, and inflammation decrescence may be accompanied with aggravated systemic injuries. The effect of anti-inflammatory drugs as glucocorticoid on DM is limited to some degree. These phenomena provided clinical evidence to our deduction that inflammation is only a small chapter in the pathogenesis of DM, and IL-6 plays a minor role in it. Therefore, we can conclude that although blockade of IL-6 and IL-6 signaling have been shown to be effective in treating several inflammatory diseases (e.g., tocilizumab for rheumatoid arthritis and inflammatory bowel disease [21, 22]), its effect in patients with DM should be limited.

RA, SLE, and SS are known to have immune activation, producing a lot of antibodies. However, few DM patients have myositis-specific antibody (MSA) or myositis-associated antibody (MAA) [23]. Autoantibody production relies on B cell differentiation and activation. As mentioned above, IL-6 plays central roles in B cell differentiation activity. Thus, lower serum IL-6 level in DM than in RA, SLE, and SS may be an explanation to the differences in autoantibody production in these rheumatic diseases. In this study, we found that the level of IL-6 was closely related to fever. DM patients whose IL-6 level was above the cutoff point almost had fever. In view of pathology and physiology, IL-6 plays an important role in the mechanism of fever by changing the thermoregulation set-point [24]. It is interesting that IL-6 is significantly higher in RA patients, but fever is seldom seen. The mechanism for this needs further research.

In conclusion, we believe that IL-6 plays a minor role in DM. Large samples and longitudinal studies are required to elucidate the exact relationship between IL-6 and DM.

5. Conclusions

IL-6 plays a less important role in DM than in RA. The effect of the therapy targeting IL-6 on DM may be limited.

Conflict of Interests

There is no conflict of interests to disclose.

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Research Article

Rapamycin Ameliorates Proteinuria and Restores Nephrin and Podocin Expression in Experimental Membranous Nephropathy

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Objective. Recent studies have shown a beneficial effect of rapamycin in passive and active Heymann Nephritis (HN). However, the mechanisms underlying this beneficial effect have not been elucidated. *Methods*. Passive Heymann Nephritis (PHN) was induced by a single intravenous infusion of anti-Fx1 in 12 Sprague-Dawley male rats. One week later, six of these rats were commenced on daily treatment with subcutaneous rapamycin 0.5 mgr/kg (PHN-Rapa). The remaining six rats were used as the proteinuric control group (PHN) while six more rats without PHN were given the rapamycin solvent and served as the healthy control group (HC). All rats were sacrificed at the end of the 7th week. *Results*. Rapamycin significantly reduced proteinuria during the autologous phase of PHN. Histological lesions were markedly improved by rapamycin. Immunofluorescence revealed attenuated deposits of autologous alloantibodies in treated rats. Untreated rats showed decreased glomerular content of both nephrin and podocin whereas rapamycin restored their expression. *Conclusions*. Rapamycin monotherapy significantly improves proteinuria and histological lesions in experimental membranous nephropathy. This beneficial effect may be mediated by inhibition of the alloimmune response during the autologous phase of PHN and by restoration of the normal expression of the podocyte proteins nephrin and podocin.

1. Introduction

Membranous nephropathy (MN) is a common cause of nephrotic syndrome (NS), accounting for approximately 20% of cases in Caucasians [1]. MN is characterized by thickening of the glomerular basement membrane (GBM) and deposition of immune complexes and complement on its subepithelial aspect.

Spontaneous complete or partial remission of proteinuria occurs in 5–32% and 25–40%, respectively, at five years [2–5]. The probability of end-stage renal disease (ESRD) in untreated patients is approximately 15% at five years, 35% at 10 years, and 40% at 15 years [2–4, 6]. Due to the relatively benign clinical course, immunosuppressive agents are considered only in patients at risk of progressive disease or with severe symptomatic NS [2, 7, 8].

Recent evidence suggests that the majority of patients with idiopathic MN have circulating antibodies against phospholipase A2 receptor (PLA2R), which is present on podocytes, as is megalin in rat models of MN [9]. Similarly, neutral endopeptidase has been found as the target antigen in newborns' podocytes with alloimmune neonatal membranous nephropathy [10] and cationic bovine serum albumin as a planted antigen in early childhood MN [11]. Additional circulating autoantibodies against human podocytic antigens have recently been described [12]. It is speculated that as a result of podocyte injury by complement, various intracellular proteins and cryptic epitopes may be exposed, thus inducing "a second wave of immunisation" [13, 14].

Heymann Nephritis is a faithful experimental model of the disease that has been extensively studied since first described by Heymann et al. in 1959 [15]. The active model

of HN is induced by immunization of Lewis rats with preparations of brush-border proteins. The passive model of HN (PHN) is induced by a single i.v. injection of heterologous anti-brush border antiserum (anti-Fx1A) that produces heterologous IgG subepithelial deposits within hours to days. Proteinuria occurs in almost all animals within five days. This "heterologous phase" is followed, two weeks later, by an "autologous phase" during which rat IgG antibodies are produced against the heterologous IgG. The autologous IgG alloantibodies are also deposited at the subepithelial space, inducing a further increase in proteinuria [16]. The second (autologous) phase of PHN mimics idiopathic MN because during the autologous phase there is production of autoantibodies (similar to human disease), against a planted exogenous antigen (similar to cationic bovine serum albumin in humans) but also against neoantigens that are exposed in the subepithelial space during the initial injury (again similar to the second wave of immunization that is believed to happen in human idiopathic MN).

Therefore it is the autologous phase of passive HN that shares the same pathophysiological mechanisms to those recently identified in idiopathic MN in humans.

To date the therapeutic approach has not changed substantially. The monthly alteration of cyclophosphamide or cyclosporine and corticosteroids remains the standard therapy for severe and persisting proteinuria. Given the significance of IgG antibodies in MN, strategies to target B lymphocytes and antibody formation may be effective in inducing remission of the NS [14]. Indeed there is recent evidence that anti-CD20 antibody administration can effectively treat patients with idiopathic MN [17, 18].

The immunosuppressive effect of rapamycin was first attributed to the inhibition of cytokine-induced proliferation and clonal expansion of T cells. More recently, it has become evident that rapamycin (in contrast to tacrolimus and cyclosporine) inhibits the proliferation of B cells [19] and restricts B cells capable of producing immunoglobulins [20].

Bonegio et al. demonstrated that low dose rapamycin ameliorated proteinuria in experimental PHN and limited tubulointerstitial inflammation and interstitial fibrosis in association with reduced expression of proinflammatory and profibrotic genes [21]. The beneficial effects of rapamycin have also been observed in active HN [22]. Here we tried to investigate more specific effects of rapamycin, beyond the known antifibrotic ones. In particular we examined the effect of rapamycin on podocytes architecture and slit diaphragm proteins, as well as on the deposition of pathogenic autoantibodies that coincides with the autologous phase of PHN.

2. Materials and Methods

2.1. Experimental Design. Eighteen male Sprague-Dawley rats (Pasteur Institute, Athens, Greece) were used in this study. The experiment was carried out in accordance with current legislation on animal experiments in the European Union and approved by our institution's Safety and Ethics Committee for Animal Research. All animals were housed in a room with 12 h light/12 h dark cycle, constant temperature of 22°C, and had free access to standard diet and water. PHN was induced in 12 rats by a single i.v. infusion of 0.5 mL sheep anti-Fx1 per 100 gr of body weight. Anti-Fx1 antiserum was kindly provided by Dr. Kerjaschki. Rats were anesthetized by intraperitoneal infusion of Ketamine 67 mg/kg and Xylazine 10 mg/kg.

One week after anti-Fx1 infusion all rats became proteinuric. Six of them were randomly selected to commence daily subcutaneous injections of rapamycin (Sigma, St Louis, MO, USA) at a dose of 0.5 mgr/kg (PHN-Rapa group). Another six rats with PHN were given subcutaneously only the rapamycin solvent (DMSO) and served as the passive HN proteinuric group (PHN group). The remaining six, age and weight matched healthy rats without PHN, received only DMSO and served as the healthy control group (HC group). Urine collections were performed weekly in metabolic cages (Tecniplast, Italy). Body weight was also determined weekly and rapamycin dose was adjusted accordingly. All animals were sacrificed 7 weeks after anti-Fx1 administration.

2.2. Isolation of Glomeruli. Glomeruli were isolated by differential sieving by utilizing sieves (Retsch, Haan, Germany) of different pore sizes: 150 μm, 106 μm, and 75 μm. Isolated glomeruli were retained on the bottom screen of 75 μm pore size. Purity of the glomerular isolate was estimated to be >95%. After several washings with PBS, glomeruli were collected and centrifuged for 4 min at 1200 r.p.m. The pellet was homogenized in RIPA-buffer containing protease inhibitors and was stored in −80°C till analysis.

2.3. Western Blot (WB) Analysis. Kidney cortex tissue was homogenized in RIPA-buffer containing protease inhibitors (Roche Diagnostics, Hellas, SA). Forty μg of glomerular lysate was electrophorized per lane on 7.5% SDS-gels. The proteins were transferred electrophoretically on nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Germany). Membranes were blocked with 5% BSA (Sigma-Aldrich) in TBS-1X Tween-20 0.1% and incubated overnight at 4°C with guinea pig nephrin pAb (1 : 500) (Progen Biotechnik GmbH, Germany), rabbit podocin pAb (1 : 500) (Abcam, Cambridge, UK), and with mouse anti-actin mAb (1 : 3000) (C4; Chemicon International, Temecula, CA). Appropriate HRP-linked antibodies (Cell Signaling Technology) were applied for 60 minutes at room temperature. Signal was detected using appropriate chemiluminescence reagent (Amersham Biosciences, GE Healthcare, UK). Bands were normalized to actin expression. Image-J (NIH, MD, USA) densitometry analysis system was used for measurements.

2.4. Real Time RT-PCR (qRT-PCR). Renal tissue was homogenized in Trizol Reagent (Life Technologies; Gibco BRL, Paisley, UK). One μg of total RNA was reverse transcribed (Superscript-II; Gibco) and amplified by RT-PCR. Products were normalized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Measurements were performed using the ABI-Prism 7000 System (Applied Biosystems; California, USA). iTaq SYBR-Green Supermix with ROX (Bio-Rad) was used for the reactions. Results were normalized to GAPDH and analysis was performed using the $2^{-\Delta\Delta Ct}$ method. All samples were tested in duplicate.

Table 1: Clinical and biochemical characteristics of groups under study.

	Group			*P*		
	PHN	PHN-Rapa	HC	PHN versus PHN-Rapa	PHN versus HC	PHN-Rapa versus HC
Initial body weight (gr)	170 ± 2.8	169 ± 5.2	180 ± 2.1	0.9	0.12	0.1
Final body weight (gr)	335.7 ± 7.3	268.3 ± 12.1	432.5 ± 9.2	<0.001	<0.001	<0.001
Kidney mass (gr)	4.3 ± 0.3	2.8 ± 0.2	4.04 ± 0.02	0.001	0.47	0.009
Kidney mass over body weight	0.013 ± 0.0002	0.010 ± 0.0004	0.009 ± 0.0003	0.013	0.002	0.27
Serum creatinine (mg/dL)	0.30 ± 0.001	0.31 ± 0.03	0.40 ± 0.001	0.55	0.006	0.016
Serum total protein (mg/dL)	5.91 ± 0.14	6.33 ± 0.12	6.77 ± 0.075	0.032	0.001	0.04
Serum albumin (mg/dL)	2.8 ± 0.09	3.1 ± 0.1	3.6 ± 0.04	0.03	0.002	0.04
Serum cholesterol (mg/dL)	187.5 ± 21	313.3 ± 53	66 ± 5	0.03	0.05	0.001

2.5. Microscopy Studies. Left kidney sections were fixed in neutral formalin and examined by a renal pathologist (L. Nakopoulou) who was blinded to the group assignment.

For immunofluorescence (IF) studies left kidney sections were embedded in OCT compound (Sakura Finetek USA, Inc), snap frozen in liquid nitrogen, and stored in −80°C until examination. Five-micrometer thick cryosections were incubated overnight with the same primary antibodies used in western blot. Dilution for nephrin was 1 : 250 and for podocin was 1 : 100. Secondary antibodies used included Alexa Fluor-488 conjugated goat anti-rabbit or anti-guinea pig or anti-rat IgG at 1/1000 (Molecular Probes, Inc). RNAse (Sigma) diluted in BSA1% PBS Tween1X (1 : 500) was applied for 30 min and then samples were incubated with propidium iodide 1 : 1000 (Sigma) for 5 minutes. At least 30 glomeruli were examined per animal. The intensity of the fluorescence was scored on a scale of 0 to 3+, where 0 = absent, 1+ = mild, 2+ = moderate, and 3+ = strong staining.

For EM studies left kidney sections were processed as usual and examined under a transmission electron microscope (JEM100CX-II; JEOL Inc., Tokyo, Japan). Twenty random glomeruli were examined for each mouse. Microphotographs were analyzed using the Digital Micrograph software (Gatan GmbH, Munchen, Germany). The entire curved length of the GBM of all open capillary loops (loop length, LL) and the number of foot processes (FPN) overlying capillary loops were measured. The foot process width (FPW) in each loop was calculated using the formula: FPW = $(\pi/4 \times$ LL)/FPN [23]. The foot process density (FPD) in each loop was measured using the formula: FPD = FPN/LL.

2.6. Measurement of Proteinuria, Serum Creatinine, and Rapamycin Levels. Urinary protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Serum creatinine levels were measured at sacrifice by an autoanalyzer (Olympus 600, Tokyo, Japan). Rapamycin blood levels were determined by EIA in whole blood (Imx Analyzer, Abbott Lab, USA).

2.7. Statistical Analysis. Analysis of variance (ANOVA) was performed to compare serum creatinine and IF scores between groups. Continuous variables are expressed as

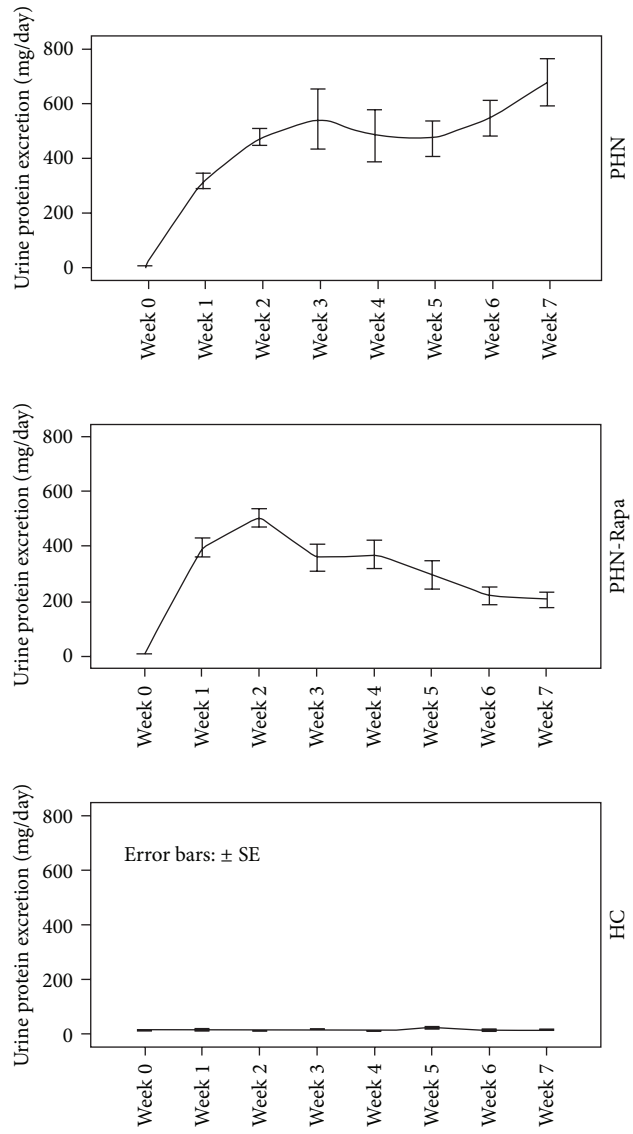
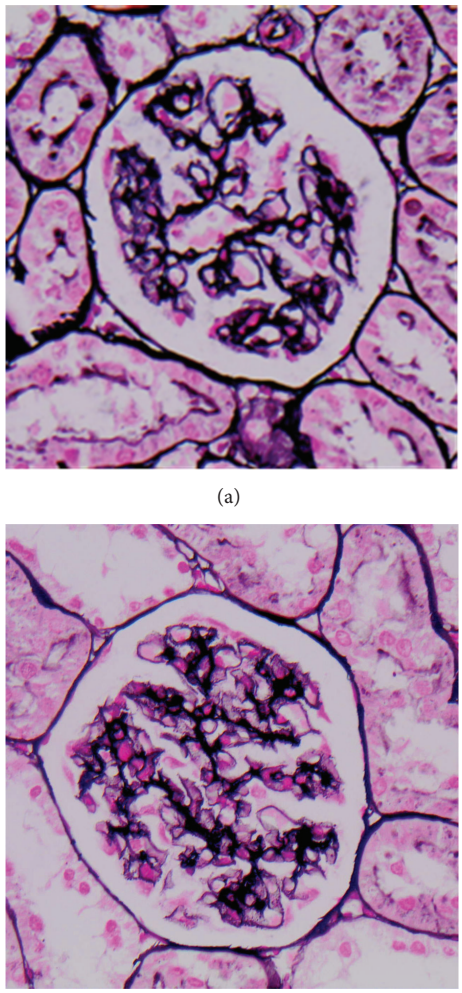


Figure 1: 24-hour urine protein excretion in study groups. Rapamycin was administered at week one in the PHN-Rapa group resulting in gradual amelioration of proteinuria in contrast to the PHN group in which proteinuria continued deteriorating.



(a)

(b)

Figure 2: Photon microscopy. (a) Passive Heymann Nephritis. Moderate to severe irregular thickening of glomerular capillary basement membranes (Silver Methenamine, ×400). (b) Passive Heymann Nephritis after rapamycin administration. Mild to moderate thickening of glomerular capillary basement membranes (Silver Methenamine, ×400).

mean ± SE. Repeated measures analysis of variance was used to compare the weekly measurements of proteinuria and body weight throughout the study. Independent samples Kruskal-Wallis and median tests were used for nonparametric comparisons. Differences were considered significant for a P less than 0.05 (two tailed). SPSS19-IBM software was used for statistical analysis.

3. Results

3.1. Clinical and Biochemical Characteristics. The 24-hour urinary protein of both PHN and PHN-Rapa groups increased at nephrotic levels at day 7 and remained so until the 2nd week after nephritis induction. Thereafter proteinuria began to decline in the PHN-Rapa group while it continued deteriorating in the PHN group. At the end of the study (week 7) urine protein levels in the PHN-Rapa group were 1/3 of those in the PHN group ($P = 0.007$, by repeated measures ANOVA). Although proteinuria in the PHN-Rapa group declined continuously, it did not reach the urine protein levels of HCs at the time of sacrifice (Figure 1).

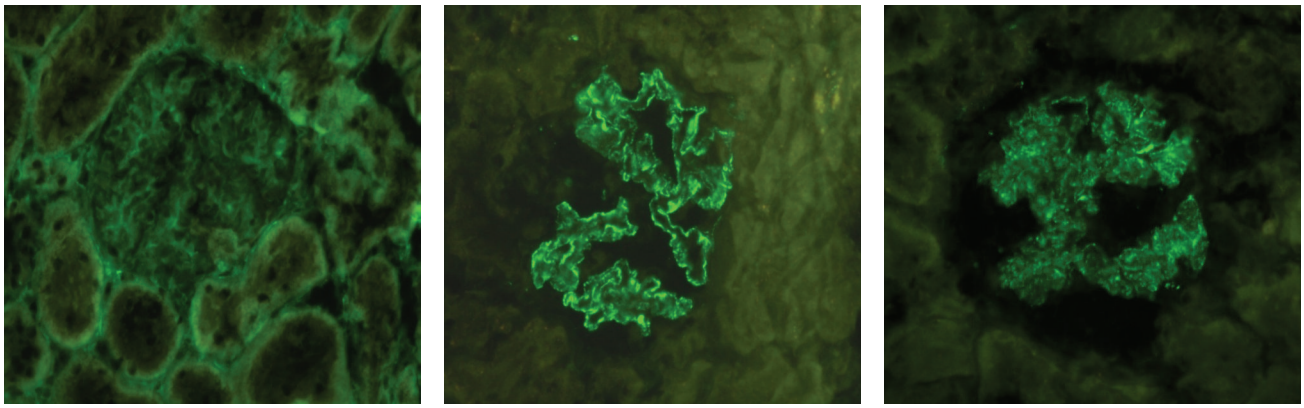
Rats in the PHN-Rapa group did not increase BW at the same pace as the other groups. At the end of the study their BW was 66% and 45% of that in the PHN and HC groups (Table 1). The ratio of renal to body mass at the end of the study was higher ($P < 0.01$) in the PHN group (0.013) compared to PHN-Rapa group (0.010) and HC group (0.009). Serum creatinine, total protein, and total cholesterol are presented in Table 1. Serum creatinine levels were higher in the HC group due to the higher body weight in this group at the end of the study. Serum total protein and albumin levels in the PHN-Rapa group were significantly higher as compared to the PHN group but did not reach those of HCs (Table 1). The trough rapamycin levels averaged at 12.5 ± 0.76 ng/mL in treated rats.

3.2. Photon Microscopy, Immunofluorescence, and Electron Microscopy. After staining with silver methenamine the glomeruli in the PHN group revealed moderate to severe thickening of the GBM, while rapamycin treated rats displayed less severe histological lesions with only mild or moderate thickening (Figure 2). Cryosections stained for anti-Rat IgG showed intense (3+) granular and irregular fluorescence along the glomerular capillary walls of the PHN group, whereas staining was attenuated (1+ to 2+) in the PHN-Rapa group and absent in the control group (Figure 3). Electron microscopy showed massive subepithelial deposits in almost all capillary loops in the PHN group with severely affected podocytes (Figure 4). In particular the harmonic mean and the median value of FPW were 830.78 nm and 789.26 nm, respectively, while the mean foot process density per μm of GBM length (FPD) was 0.94 ± 0.42. In the PHN-Rapa group the deposits and the podocytic injury were markedly attenuated compared to the PHN group (harmonic mean of FPW 613.3 nm, median 654.1 nm, and FPD 1.28 ± 0.9; all $P < 0.001$). Despite this improvement, the respective values in the HC were much lower (harmonic mean FPW 349 nm, median FPW 355 nm, and mean FPD 2.24 ± 0.43; all $P < 0.001$ as compared to other groups).

3.3. Nephrin and Podocin Expression. By WB the levels of nephrin and podocin protein levels in glomerular lysates were significantly lower (all $P < 0.05$) in the PHN group as compared to the HC and PHN-Rapa groups. In the PHN-Rapa group, nephrin and podocin levels were similar to HCs (Figure 5).

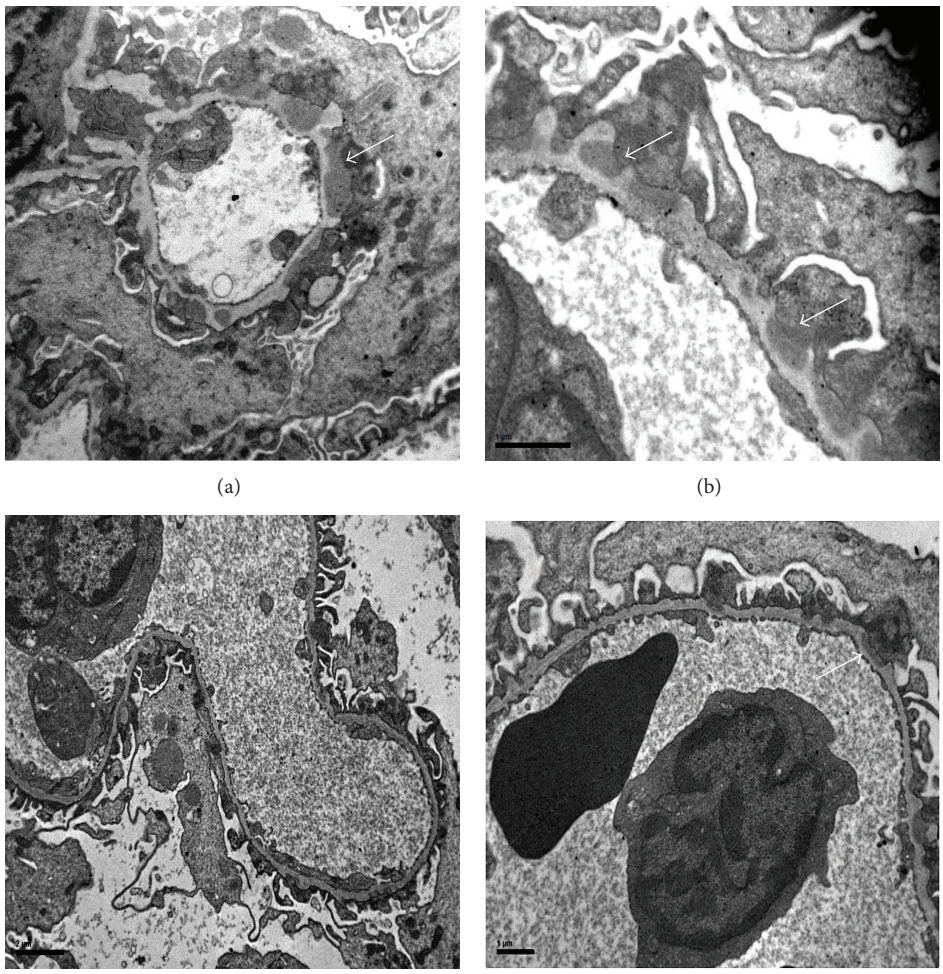
By RT-PCR the expression of nephrin mRNA was lower in the PHN group compared to HC and PHN-Rapa groups ($P = 0.011$ and $P = 0.039$, resp.; Figure 6). In contrast podocin mRNA was increased in the PHN and PHN-RAPA groups as compared to HCs (Figure 6).

Immunofluorescence for nephrin and podocin showed intense (3+) and regular linear staining in all examined glomeruli in the HC group. Normal staining for both proteins was also evident in the majority of glomeruli (72.8%) in the



(a) (b) (c)

Figure 3: Immunofluorescence staining with anti-Rat IgG. (a) Healthy controls showed absence of staining. Original magnification ×400. (b) Passive Heymann Nephritis; glomeruli showed intense (3+) granular and irregular fluorescence along the capillary walls (×400). (c) Passive Heymann Nephritis after rapamycin administration; staining was significantly attenuated (1+ to 2+) in almost all glomeruli (×400).



(a) (b)

(c) (d)

Figure 4: Electron microscopy. (a) Passive Heymann Nephritis. Massive subepithelial deposits (white arrow) with severe fusion of podocyte foot processes; original magnification ×12 k. (b) Passive Heymann Nephritis. Subepithelial deposits in higher magnification (white arrows); original magnification ×26 k. (c) Passive Heymann Nephritis after rapamycin administration. The deposits and the podocytic injury were attenuated compared to the PHN group; original magnification ×8 k. (d) Passive Heymann Nephritis after rapamycin administration (higher magnification ×20 k). Small subepithelial deposit (white arrow).

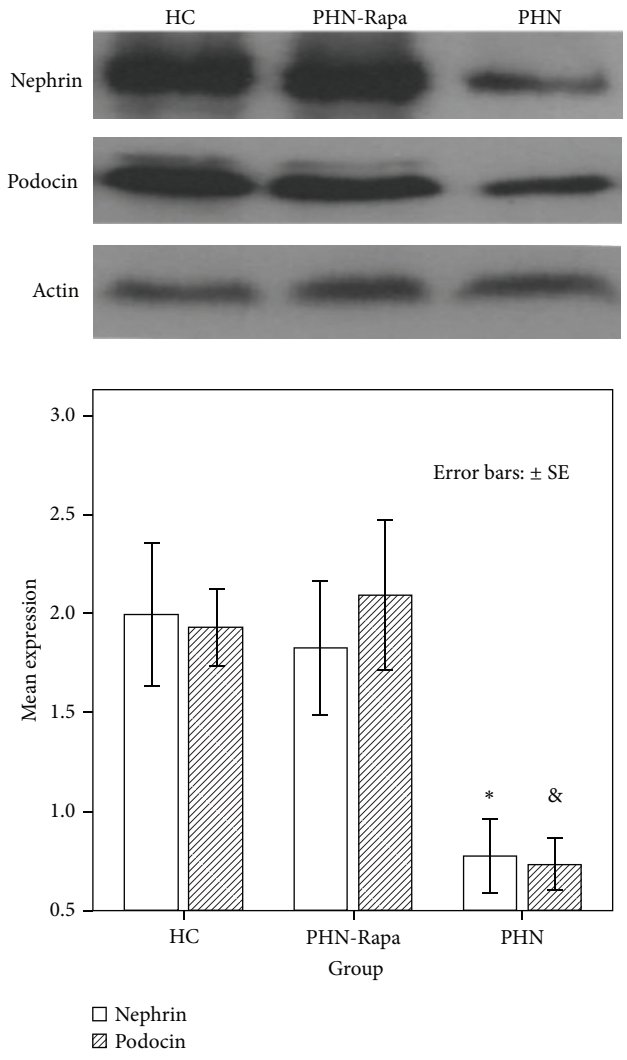


Figure 5: Western blot analysis for nephrin, podocin, and actin in glomerular lysates. HC: healthy controls; PHN: Passive Heymann Nephritis; PHN-Rapa: Passive Heymann Nephritis after rapamycin administration. Bars represent nephrin (white columns) and podocin (shaded columns) protein content corrected to actin concentration. $^{*}P = 0.01$, $^{\&}P = 0.007$. Error bars: ±SE.

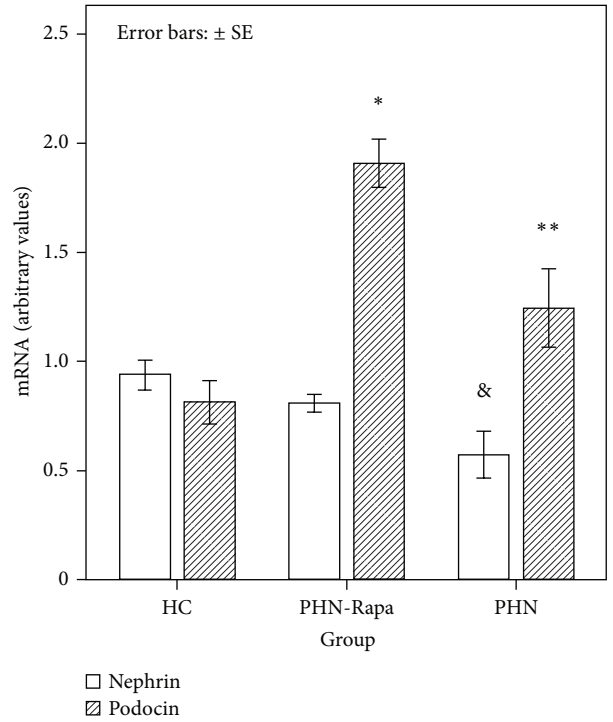


Figure 6: Real time PCR for nephrin and podocin mRNA in glomerular lysates. HC: healthy controls; PHN: Passive Heymann Nephritis; PHN-Rapa: Passive Heymann Nephritis after rapamycin administration. Bars represent nephrin mRNA (white columns) and podocin mRNA (shaded columns) corrected to GAPDH mRNA concentration. $^{*}P < 0.001$, $^{\&}P = 0.011$, $^{**}P = 0.038$. Error bars: ±SE.

PHN-Rapa group. On the contrary, staining for nephrin and podocin was irregular and attenuated (<3+) in almost all glomeruli in the PHN group (Figure 7).

4. Discussion

Rapamycin treatment has shown either protective [21, 22, 24–31] or untoward [32–36] results in various forms of experimental or human kidney disease. In summary, rapamycin displays dual opposing effects, with proteinuria and podocyte damage aggravation in the toxicoimmunological glomerular models and a nephroprotective effect in the chronic inflammatory glomerulotubulointerstitial models [37]. Rapamycin inhibits the proliferation of both T and B cells [19] and reduces the number of B cells capable of producing immunoglobulins in contrast to cyclosporine and tacrolimus [20]. Rapamycin can also promote the generation of regulatory T cells which suppress the immune system and control autoimmunity [38]. These combined properties make sirolimus an attractive agent for the treatment of autoimmune diseases such as MN.

In the present study rapamycin was given after induction of HN when severe proteinuria, and by inference histological lesions, had already been established. Nevertheless, rapamycin was able to abrogate the second rise of proteinuria during the autologous phase. This clinical result was escorted by significant alleviation of the histological lesions. More precise podocyte indices such as FPW and FPD were markedly improved by rapamycin whereas the expression of slit diaphragm proteins nephrin and podocin was almost completely restored. It is interesting that in the PHN group, podocin mRNA levels were increased, nephrin mRNA levels were decreased, and the respective protein levels were both decreased. This discrepancy implies that nephrin decreased owing to suppressed translation, whereas the decreased podocin levels may be due to loss or destruction of the protein with a compensatory increase in podocin mRNA levels. Residual histological lesions seen in the PHN-Rapa group should be attributed to the initial insult during the heterologous phase when the drug had not been given yet. The attenuation of anti-Rat IgG staining in IF indicates that rapamycin blocked the production of pathogenic autologous

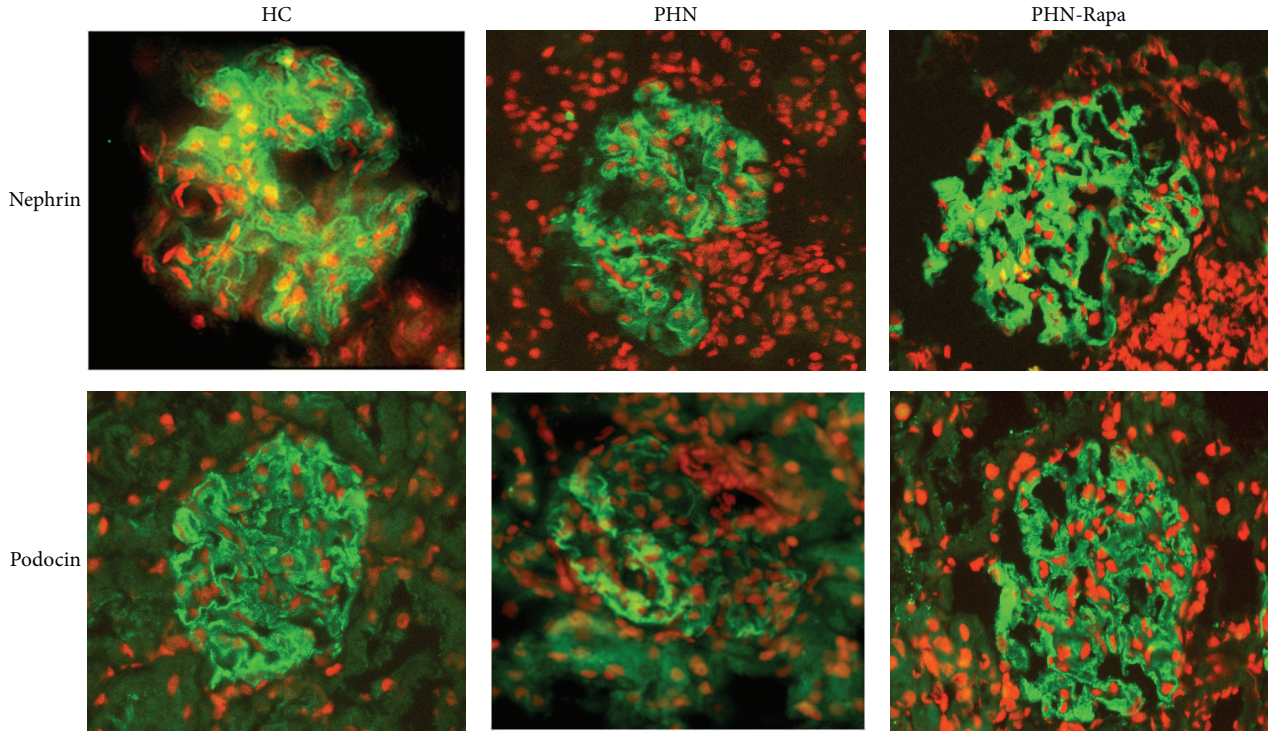


Figure 7: Immunofluorescence staining for nephrin and podocin. HC: healthy controls; PHN: Passive Heymann Nephritis; PHN-Rapa: Passive Heymann Nephritis after rapamycin administration. Nuclei have been stained with propidium iodide (red). Intense (3+) linear staining in the HC and PHN-Rapa groups in contrast to the PHN group which presented attenuated and irregular pattern of staining for both nephrin and podocin (middle panel).

alloantibodies (possibly via its B-cell inhibitory effects) that are responsible for the second boost of proteinuria, resulting thus in the gradual resolution of the NS. These results are in line with previous studies in animal models of MN [21, 22] and offer further insights into possible mechanisms for the therapeutic effect of rapamycin in experimental MN.

5. Conclusions

Rapamycin significantly improves proteinuria and histological lesions during the autologous phase of PHN, an effect that may be mediated by inhibition of the autoimmune response and by restoration of the normal expression of the podocyte proteins nephrin and podocin. If our results are confirmed by future studies, rapamycin may prove to be an effective treatment for MN.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Decreased PERP Expression on Peripheral Blood Mononuclear Cells from Patient with Rheumatoid Arthritis Negatively Correlates with Disease Activity

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Background. PERP, p53 apoptosis effector related to PMP-22, is a p53-dependent apoptosis in diverse cell types and has cell type-specific roles in p53-mediated apoptosis. However, its role in PBMCs of RA patients has remained largely unclear. *Objectives*. The aim of this study was to detect the expression levels of PERP on PBMCs of RA patients and healthy controls and analyze the role of PERP in the pathogenesis of RA. *Methods*. The mRNA expression levels of PERP and IL-17 were detected by real-time PCR in PBMCs from patients with RA ($n = 40$) and healthy controls ($n = 40$). The correlations of PERP expression levels to IL-17 transcripts and disease activity parameters were analyzed. *Results*. The PERP and IL-17 expression levels in the PBMCs were significantly decreased and increased in comparison of which in healthy controls. The mRNA expression levels of PERP in PBMCs from patients with RA were negatively correlated with IL-17 and disease activity parameters DAS28, RF, CRP, and ESR rather than Anti-CCP and ANA. *Conclusions*. These results demonstrated that PERP might be involved in the pathogenesis and a potential therapeutic target of RA by regulating the expression of IL-17.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammatory of synovial tissues in multiple joints and destruction of articular cartilage and bone [1]. The abnormalities of immune regulation and function are implicated in the pathogenesis of RA. Activation and recruitment of immune cells, especially lymphocytes and monocytes, into joints are characteristic features of this disease [2]. In normal inflammatory responses, proper apoptosis is critical for maintaining the lymphocyte homeostasis and tissue growth by initiation of apoptotic cascades [3]. Furthermore, the immune system heavily depends on apoptosis to ameliorate inflammation for preventing misdirected damage to normal tissues [4]. However, defection of the apoptotic cascades usually contributes to human autoimmune diseases, such as rheumatoid arthritis (RA) [5].

PERP, as a tetrapan protein, was first identified as a p53 transcriptional target proapoptotic gene which expresses high levels during apoptosis rather than cell cycle arrest and has cell-specific and tissue-specific roles in p53-mediated apoptosis [6, 7]. PERP is a member of peripheral myelin protein 22/growth arrest specific 3 (PMP-22/gas3) family, which includes PMP-22 and the epithelial membrane proteins 1, 2, and 3 [8]. The apoptotic mechanism of PERP may differentiate with the BH3-containing proteins such as Noxa and Puma, since it localizes to the plasma membrane and secretory pathway rather than the mitochondria [9]. Prior study demonstrates that PERP contributes to radiation-induced apoptosis in $CD4^+CD8^+$ thymocytes which undergo

a well-characterized p53-dependent apoptotic response using $PERP^{-/-}$ mice [6, 10]. In autoimmune diseases, researcher found that the tumor suppressor molecule p53, which is significant in apoptosis and cell-cycle control, is greatly reduced in peripheral blood mononuclear cells from patients with RA, systemic lupus, and multiple sclerosis compared with that in normal controls [11]. This observation demonstrates that the decreased expression of p53 might contribute to the pathogenesis of autoimmune diseases by failing to eliminate potentially pathogenic cells. However, the role of PERP in autoimmune diseases is still unknown.

Interleukin-17 (IL-17) is a proinflammatory cytokine predominantly produced by a specific subset of CD T helper cells called Th17 cells and is critical for inducing and perpetuating chronic inflammation, cartilage damage, and bone erosion [12, 13]. Several studies have suggested that IL-17 plays a key role in the pathogenesis of RA [14, 15], and the expression of IL-17 gene is associated with the inflammatory process and disease activity of RA disease [16]. Recently, two investigational monoclonal antibodies which neutralize IL-17 appeared to be safe and effective in the early stage clinical trials in RA patients [17, 18]. The expression of IL-17 can downregulate apoptosis in RA and p53 can regulate Th1 and Th17 functions in patients with RA participating in the pathogenesis of RA [19, 20]. However, as a p53 transcriptional target, the role of PERP in regulating IL-17 and involving in the pathogenesis of RA is still unclear.

The aim of this study was to investigate the role of PERP in disease activity and progression of human and to explore the possibility of PERP p as a target for the treatment of RA. We measured the mRNA expression levels of PERP and IL-17 in the peripheral blood mononuclear cells (PBMCs) from RA patients and analyzed the correlation between them. Furthermore, we investigated the correlation of PERP expression levels with different activity parameters. The results showed that the expression of PERP mRNA in the peripheral blood mononuclear cells (PBMCs) from patients with RA was significantly decreased compared with healthy controls (HCs). The expression levels of PERP were negatively correlated with IL-17 mRNA levels in PBMCs and the disease activity parameters including disease activity scores (DAS28), rheumatoid factor (RF), C-reaction antibodies (anti-CCP), and erythrocyte sedimentation rate (ESR). These data suggested that the reduced expression of PERP might participate in regulating IL-17 and involving in the pathogenesis of RA.

2. Materials and Methods

2.1. Patients and Controls. A total of 40 patients with rheumatoid arthritis (RA) (5 men and 35 women, age 23–76, mean 45.3 years), all from the outpatient clinic of the Department of Rheumatology, West China Hospital, Sichuan University, were included in the present study. The RA patients were diagnosed based on the 1987 criteria of the American College of Rheumatology [21]. The disease duration of RA patients was between 2 months and 12 years. Some patients were taking nonsteroidal anti-inflammatory drugs. None of the patients had ever taken disease-modifying antirheumatic drugs. Patients taking corticosteroids or vitamin D and those who had renal insufficiency were excluded. The study was approved by the Ethics Committee of West China Hospital. All procedures involving specimens obtained from human were performed with informed consent from each patient. The disease activity score calculated for 28 joints (DAS28) [22], has been widely used in clinical trials and for the assessment of patients in the clinic to monitor disease activity of patients with RA. Healthy controls peripheral blood mononuclear cells (PBMCs) were obtained from 40 healthy volunteers (5 men and 35 women, age 28–63, mean 43.8 years). The demographic and clinical characteristics of healthy controls and patients with RA were summarized in Table 1.

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

	RA patients ($n = 40$)	HCs ($n = 40$)
Age, mean (range) years	45.3 (23–76)	43.8 (28–63)
Male : female	5 : 35	8 : 32
Rheumatoid factor (RF) (IU/mL)	362.1 (22.6–1560)	21.34 (19.3–43.8)
DAS28 mean (range) score	4.05 (1.05–6.59)	—
C-reactive protein (CRP) (mg/L)	27.1 (0.3–90.6)	—
Erythrocyte sedimentation rate (ESR)	26.9 (1.25–90.9)	—
Disease duration, mean (range) months	28.4 (2–136)	—

DAS28: disease activity score 28.

2.2. PBMCs Isolation. Peripheral blood mononuclear cells (PBMCs) were purified from peripheral blood of healthy controls and patients by standard density-gradient centrifugation, using Ficoll-Paque Plus (Axis-Shied PoC AS, Oslo, Norway). Briefly, blood was centrifuged at 500 g for 20 min at room temperature. After centrifugation, the top plasma layer was collected and stored at −80°C. Then the precipitation was diluted 1 : 1 with sterile phosphate buffered saline (PBS) before to layering over the Ficoll. PBMCs were carefully aspirated with spinning at 1600 rpm for 30 min. The cells were then washed three times with PBS before adding trizol (Invitrogen, USA).

2.3. RNA Extraction and Real-Time PCR. Total RNA extracted from PBMCs was extracted with Trizol (Invitrogen, USA) following the manufacturer's instructions. Briefly, 1 μg total RNA was first reverse-transcribed to cDNA with reverse transcription reagent kits according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). A 20 μL reverse-transcribed reaction mixture was acted including 4 μL 5×iScript reaction mix, 1 μL iScript reverse transcriptase and 15 μL with nuclease-free water, and 1 μg total RNA. The reaction conditions were: 25°C for 5 min, then 42°C 30 mins, and last 85°C 5 min. The expression levels of *PERP, IL-17A,* and *GAPDH* were determined by real-time quantitative

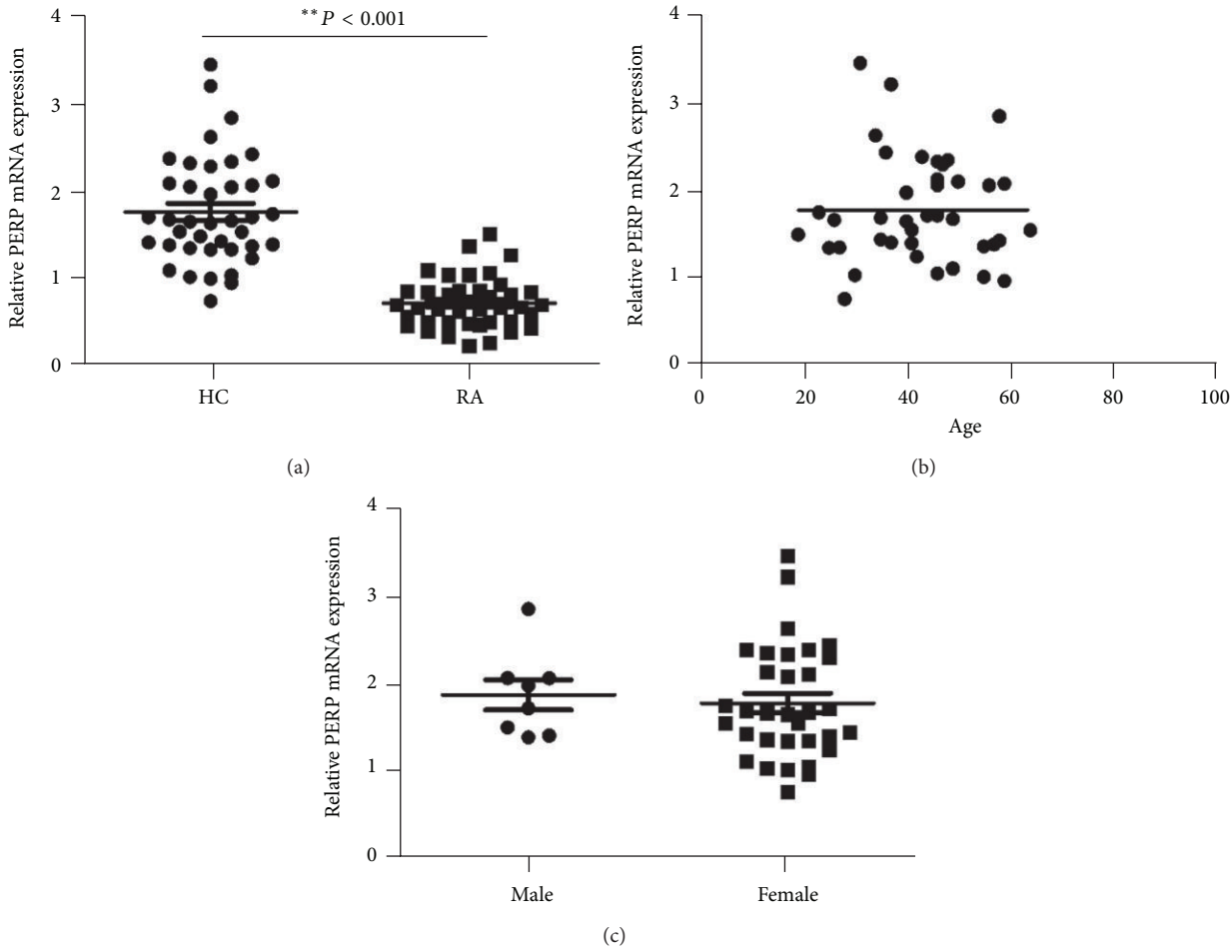

Figure 1: Decreased expression of PERP in peripheral blood lymphocytes (PBMCs) from patients with rheumatoid arthritis (RA) and PERP expression levels in age and sex in healthy controls (HCs) have no differences. (a) Expression of PERP mRNA in PBMCs from RA patients ($n = 40$) and healthy controls (HCs; $n = 40$) ($P < 0.05$). (b) The correlation between age and PERP expression levels in healthy control (HCs; $n = 40$) ($P > 0.05$). (c) The expression levels of Perp between male and female in healthy control (HCs; $n = 40$) ($P > 0.05$). P value was determined by Mann-Whitney test. $P < 0.05$ deems significantly different.

PCR, using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA). A 10 μL SsoFast EvaGreen PCR reaction mixture was used containing 2 μL of cDNA, 0.2 μL of sense primer, 0.2 μL of antisense primer, 2.6 μL ddH_2O, and 5 μL SsoFast EvaGreen Supermix. Quantitative PCR was performed on the iQ5 and MyiQ Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA, USA). The PCR reaction conditions were: 95°C for 1 min for denature, followed by 40 cycles of three-step PCR including melting for 10 s at 95°C, annealing for 10 s at 60°C, and extending for 10 s at 72°C. The mRNA expression was normalized to the expression levels of *GAPDH*, and relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method. The following sense and antisense primers were used: for PERP, sense 5′-AGAGCCTCATGGAGTACGC-3′ and 5′-CCTCACTTGCCGAAACAGC-3′; for IL-17A, sense 5′-CTACAACCGATCCACCTCAC-3′ and 5′-TGTGGTAGTCCACGTTCCCAT-3′; for GAPDH, sense 5′-AGAAGGCTGGGGCTCATTTG-3′ and antisense 5′-AGGGGCCATCCACAGTCTTC-3′.

2.4. Statistical Analysis. The statistical significance of the data was analyzed by PRISM software (GraphPad Software, San Diego, CA, USA) using Mann-Whitney test. Spearmen's rank test was utilized to test the correlations between the levels of PERP expression and clinical parameters of RA patients. P values <0.05 were considered significant.

3. Results

3.1. Decreased Expression of PERP in PBMCs from Patients with RA. Peripheral blood mononuclear cells (PBMCs) were separated from 40 RA patients and 40 age and sex matched healthy controls. Quantitative-Real-time PCR was used to analyze the mRNA expression level of PERP. Results showed that Perp transcripts were significantly lower in RA patients than healthy controls (Figure 1(a)) ($P < 0.001$), while the expression of Perp mRNA levels in healthy controls (HCs) have no correlations with age and sex (Figures 1(b) and 1(c)) ($P > 0.05$).

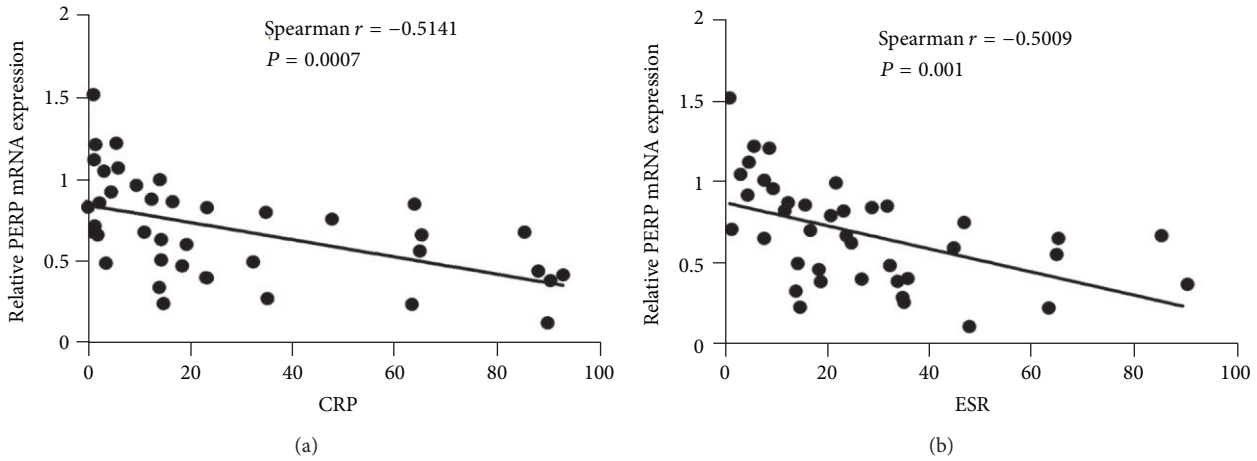

Figure 2: Expression of PERP on PBMCs from RA patients correlates with CRP and ESR. The expression levels of PERP in PBMCs of patients with RA negatively correlate with C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) ($P < 0.01$; $P < 0.01$). $P < 0.05$ deems significantly different.

3.2. Expression Levels of PERP in PBMCs Correlated with Parameters of Disease Activity in Patients with RA. In order to detect whether the low expression levels of PERP may involve in the disease pathogenesis and progression of RA, we analyzed the correlation between PERP transcripts and different disease activity parameters. A significant negative correlation was seen between PERP mRNA levels in RA patients and serologic parameters of disease activity, including CRP (Figure 2(a)) ($P = 0.0066$), ESR (Figure 2(b)) ($P = 0.0014$), DAS28 (Figure 3(a)) ($P = 0.0014$), and RF (Figure 3(c)) ($P = 0.0008$). However, the PERP mRNA expression levels have no correlations with anti-CCP (Figure 4(a)) ($P > 0.05$) and ANA (Figure 4(b)) ($P > 0.05$). There were no correlations between the expression levels of PERP in RA patients and sex, age, or duration of RA. (data not shown) ($P > 0.05$). We define the disease activity states criteria of RA by disease activity score in 28 joints (DAS28), with remission (DSA28 < 2.4), low disease activity (DAS28 between 2.4 and 3.6), active RA (DAS28 between 3.6 and 5.5), and high disease activity (DAS28 > 5.5) [23]. Then we compared the PERP expression in PBMCs from RA patients and healthy controls. It is showed that the high disease activity group had the lowest expression of PERP compared with remission group, low disease activity group, and healthy controls group. However, there was no statistical significance between active RA group and high disease activity group (Figure 3(b)).

3.3. The Correlation between the Expression of PERP and IL-17 in RA Patients. The prior study demonstrates that Th17 cells and their specific transcription factor or related cytokines are being recognized as important mediators in inflammatory and autoimmune diseases including RA [24]. To assess the relationships between the mRNA levels of PERP and IL-17 in RA patients, we first detected the IL-17 mRNA expression levels on PBMCs in healthy control, (HCs) and patients with RA, and then we examined the correlation between the mRNA levels of PERP and IL-17 in PBMCs of RA patients. The IL-17 mRNA expression levels were significantly increased (Figure 5(a)) ($P < 0.01$), and there was a significantly negative correlation between the expression of PERP and IL-17 (Figure 5(b)) ($P < 0.01$).

4. Discussion

Apoptosis is an evolutionarily conserved, multi-step processes cell death pathway which occurs in a variety of physiological conditions. The balance of apoptosis is main mechanism of physiological cell death, which is significant for homeostasis in multi-cellular organisms including the immune system [4]. Abnormal elevates in apoptosis can result to immunodeficiency and a failure to undergo apoptosis can contribute to the development of autoimmunity [25, 26]. In RA, apoptosis plays divergent roles in the pathogenesis of this disease. In joint of acute patients, there are fewer apoptotic cells than healthy controls, and experimental data demonstrate that elevated apoptosis in the joint contributes to beneficial roles [27]. The impaired of PBMCs apoptosis also detected in RA patients [28].

Previous studies have shown that the expression of p53 was decreased in PBMCs from patients with RA [11] and there are p53 mutations in rheumatoid arthritis synovium [29]. The defects in downstream p53 target gene play a vital role in promoting systemic autoimmunity diseases. For example, p21, a downstream cyclin dependent kinase inhibitor and transcriptional target of p53, is also downexpressed in patients with RA [30]. PERP, another direct p53 target gene, may also play an important role in human RA disease. In this study, we first examined the PERP expression levels on PBMCs in patients with RA compared with healthy controls and further explored their correlation with disease activity parameters. Results showed that PERP mRNA expression levels in PBMCs from patients with RA were significantly decreased compared with healthy controls. Furthermore, we found that the PERP mRNA expression levels were inversely correlated with disease activity referred to as disease activity

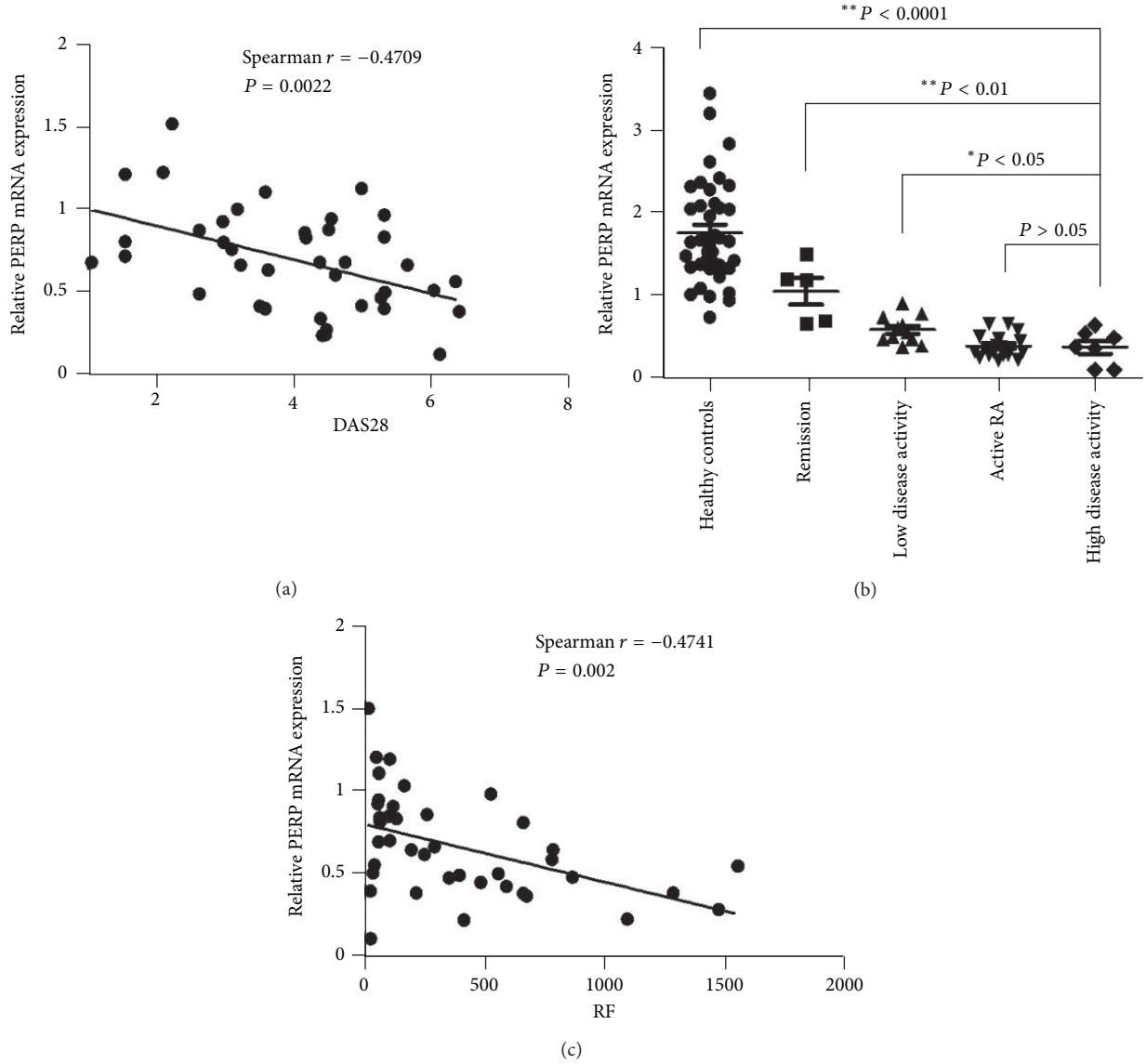

FIGURE 3: Correlations of PERP mRNA expression on PBMCs in RA patients with DAS28 and RF. PERP mRNA transcripts negatively correlate with disease activity score (DAS28) (a) ($P < 0.01$) and rheumatoid factor (RF) (c) ($P < 0.01$). (b) The expression levels of PERP of PBMCs from healthy controls (HCs; $n = 40$) and RA patients in different stages of disease activity determined by DAS28. $P < 0.05$ deems significantly different.

score 28 (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF). Although there are no correlation relationships with anti-CCP and ANA, the prior studies demonstrated that anti-CCP and ANA have no significant relationship with both disease activity and severity [31, 32].

Proper apoptosis of lymphocytes was vital for tolerance and autoimmunity, and insufficient apoptosis of lymphocytes contributes to the persistence of RA disease [5]. It seems plausible that PERP mRNA expression levels reflect the disease severity since the negative correlation with disease activity parameters of RA. To further explore whether PERP expression can serve as a biomarker for disease activity in RA, we choose DAS28 as criteria to define the disease activity states and analyze the relations with PERP mRNA expression levels and each disease activity state. The results showed that the high disease activity group has statistical significance with remission group and low disease activity group. However, there is no difference between active RA group and high disease activity group. The data above suggest that PERP might be a potential diagnostic marker for RA severity and as a therapeutic target for treatment.

Interleukin-17 (IL-17) was first discovered in 1993 and found exerting various biological functions in vivo that might be involved in the pathogenesis of a wide range of inflammatory, infectious and autoimmune diseases [13, 33]. Previous study showed that IL-17 expression has associated with disease activity and make IL-17 as a key player in RA pathogenesis [34, 35]. Latest study demonstrated IL-17 gene expression in PBMCs of patients with RA is significantly

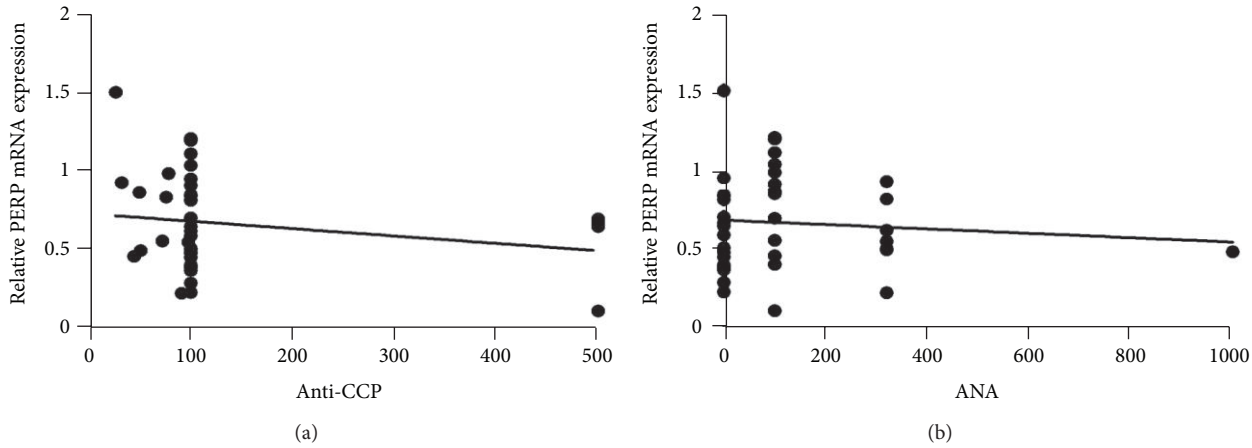

Figure 4: Correlations of PERP mRNA expression on PBMCs in RA patients with DAS28 and RF. The PERP mRNA expression levels do not have correlations with anti-cyclic citrullinated protein antibodies (anti-CCP) (a) ($P > 0.05$) and antinuclear antibodies (ANA) (b) ($P > 0.05$). $P < 0.05$ deems significantly different.

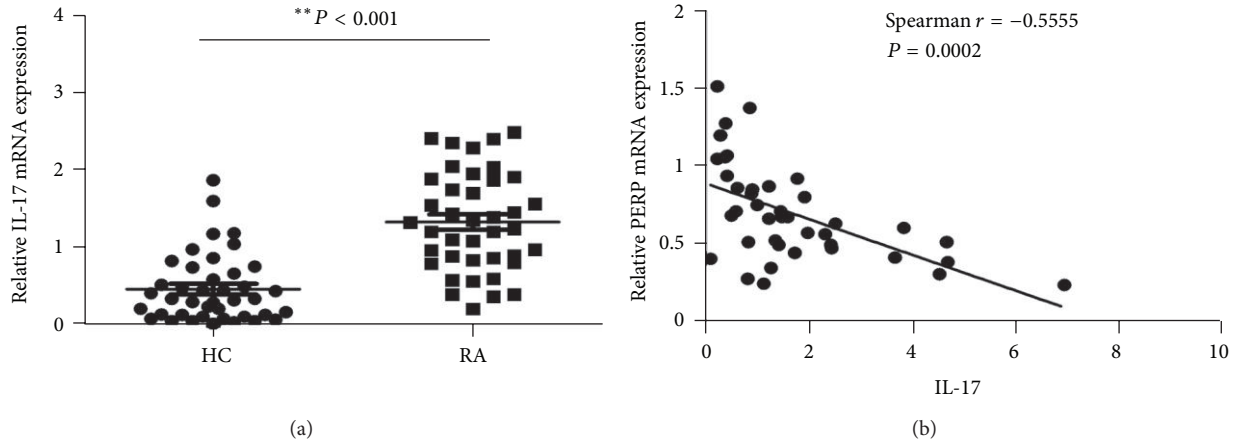

Figure 5: Correlations of PERP and IL-17 mRNA expression levels in RA patients. The mRNA expression levels of patients with RA ($n = 40$) were determined by real-time PCR. (a) The increased expression levels of PERP in patients with RA ($P < 0.01$). (b) There is a significant negative correlation between PERP and IL-17 mRNA expression levels ($P < 0.01$). $P < 0.05$ deems significantly different.

higher than healthy controls and suggested that IL-17 had an important role in the pathogenesis of RA [16]. Also the tumor suppressor gene p53 can regulate Th17 functions by inhibiting production of IL-17 in patients with RA and participate in the pathogenesis of RA [20], and the downstream of p53 transcriptional target gene *Bax* involved in the effect of sulforaphane (SFN) inhibit the production of IL-17 by rheumatoid T cells in vitro [36]. In order to explore the potential mechanism of PERP, which is another transcriptional target beside *Bax*, participate in the pathogenesis of RA, and then we detect the mRNA expression levels of IL-17 on PBMCs of patients with RA and analyzed the expression correlation between them. The result showed that the IL-17 transcripts increased significantly in patients with RA compared with healthy controls, and have significantly negative correlation with the Perp mRNA expression levels. This data demonstrate that Perp may be one of the regulators of IL-17 expression participating in the pathogenesis of RA.

Collectively, the expression of PERP is downregulated on peripheral blood mononuclear cells (PBMCs) from patients with RA, which significantly inverse-correlated with IL-17 gene expression levels in PBMCs and disease activity. Our data suggest that PERP might be a regulator of IL-17 participating in the pathogenesis of RA and a potential diagnostic marker for RA severity and a therapeutic target for treatment. However, the exact role of PERP in RA disease still needs to be further elucidated in the future.

Conflict of Interests

The authors of this paper have no conflict of interests.

Authors' Contribution

Yanchun Du and Lin Deng contributed equally to this work.

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Research Article

Elevated Apoptosis and Impaired Proliferation Contribute to Downregulated Peripheral $\gamma\delta$ T Cells in Patients with Systemic Lupus Erythematosus

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Objective. To investigate the frequency of peripheral $\gamma\delta$ T cells in patients with systemic lupus erythematosus (SLE) and its correlation with disease activity and to analyze the apoptotic status, proliferation ability, and intracellular cytokine profile of these cells. *Methods*. Flow cytometry was performed to detect the percentage and intracellular cytokine expression of peripheral $\gamma\delta$ T cells from SLE patients. Annexin-V/PI double staining was applied to determine the proportion of apoptotic $\gamma\delta$ and $CD3^+$ T cells. $\gamma\delta$ T cell proliferation was analyzed by CFSE labeling technique. *Results*. The percentage and absolute number of $\gamma\delta$ T cells were remarkably decreased in active SLE patients compared to those in inactive patients and healthy controls, with $\gamma\delta$ T cell count negatively correlated with disease activity. Compared with healthy controls, peripheral $\gamma\delta$ T cells from active SLE patients exhibited higher apoptotic rate and lower proliferation ability, as well as elevated expression of intracellular IFN-γ, IL-4, IL-10, and TGF-β, but not IL-17 or Foxp3. *Conclusion*. Decreased $\gamma\delta$ T cells in the peripheral blood of SLE patients might be caused by upregulated apoptosis and downregulated cell proliferation. These $\gamma\delta$ T cells may secret both pro- and anti-inflammatory cytokines to perform their functions in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease involving multiple organs and tissues. Although the pathogenesis of SLE has not yet been fully elucidated, it has generally been accepted that activated T/B lymphocytes and enhanced production of proinflammatory cytokines and autoantibodies can cause damage to specific organs and tissues. Various pro- and anti-inflammatory cytokines, including IFN-γ, IL-4, IL-17, IL-10, and TGF-β, play crucial roles in the pathogenesis of SLE [1].

$\gamma\delta$ T cells expressing TCR $\gamma\delta$ chains are a minor population of T cells. Based on different TCR $\gamma\delta$ chain expressions, human $\gamma\delta$ T cells can be divided into two subsets: $V\delta1^+$ T cells that are mainly distributed in epithelial and mucosal surfaces, and $V\delta2^+$ T cells that generally coexpress Vγ9 and exist primarily in the peripheral blood and lymphatic system. In normal human peripheral blood, $\gamma\delta$ T cells, 70–90% of which are $V\gamma9^+V\delta2^+$ T cells, account for about 1–5% of total T cells, and they can be activated by small nonpeptide phosphoantigens (e.g., isopentenyl pyrophosphate, IPP) in a TCR-dependent and non-MHC-limited manner [2, 3]. In the early stage of immune responses, $\gamma\delta$ T cells may bridge innate and adaptive immunity through induction of DC maturation, thus playing important roles in anti-infection, antitumor, and autoimmunity [4–6].

It has ever been demonstrated that $\gamma\delta$ T cells play important roles in the development of autoimmune diseases through their capacity of antigen presenting, release of proinflammatory cytokines, interaction with $CD4^+CD25^+$Tregs, and promotion of antibody production by providing B cell help [7]. Increased percentage of $\gamma\delta$ T cells has been found in the synovial fluids and synovium of patients with active rheumatoid arthritis (RA) [8], and lesions of chronic

cutaneous lupus erythematosus displayed the expansion of the Vγ2/Vδ2 subset [9]. TCR$\beta^{-/-}$ MRL/lpr mice developed a moderate disease while TCR$\delta^{-/-}$ MRL/lpr mice showed exacerbated renal disease and increased mortality, indicating that $\gamma\delta$ T cells may participate in the development of lupus [10]. The number of $\gamma\delta$ T cells was abnormal in the peripheral blood, skin, and panniculus of SLE patients [11, 12], but the precise role of $\gamma\delta$ T cells in the pathogenesis of SLE remains elusive.

In this study, we aimed to investigate the distribution of $\gamma\delta$ T cells in the peripheral blood of SLE patients and its relation to disease activity and to analyze the apoptotic status, proliferation ability, and intracellular cytokine profile, including IFN-γ, IL-4, IL-10, IL-17, and TGF-β, in these $\gamma\delta$ T cells.

2. Materials and Methods

2.1. Patients and Controls. Forty-two SLE patients fulfilling the 1997 SLE classification criteria revised by American College of Rheumatology (ACR) [13] and 20 age- and sex-matched healthy controls (HC) were enrolled in this study. All patients were not complicated with infection, tumor, or other autoimmune diseases. The median age of these patients was 33 years (range 14–57 years, female/male: 36/6) and that of normal subjects was 30 years (range 18–48 years, female/male: 17/3). The mean score of SLE disease activity index (SLEDAI) was 8.9 ± 3.1 (range 2–16). The mean disease duration was 60.1 ± 57.0 months (range 10 days–252 months). All subjects signed informed consent before the study. Z. M. Lu was responsible for the collection of clinical data and measurement of SLEDAI scores.

2.2. Preparation of Peripheral Blood Mononuclear Cells (PBMCs). Peripheral blood of SLE patients and healthy controls were collected. 4 mL of heparinized blood was diluted with the same volume of phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, washed in RPMI 1640 culture medium (Gibco) twice, and then resuspended at a concentration of 2×10^6 cells/mL.

2.3. Cell Stimulation and Culture. PBMCs were resuspended in RPMI 1640 medium (Gibco) supplemented with 1% penicillin-streptomycin solution (Gibco) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco). 50 ng/mL of Phorbol myristate acetate (PMA) and 1 ug/mL of ionomycin (Io) (both from Sigma) were added for polyclonal stimuli. 10 ug/mL of Brefeldin A (Sigma) was used for inhibition of cytokine secretion. Samples not stimulated with PMA and Io but exposed to Brefeldin A were served as negative controls. Cells were cultured for 5 h at 37°C in a 5% CO_2 atmosphere.

PBMCs from SLE patients and healthy controls were incubated with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) solved in PBS for 10 minutes at 37°C. An excess of ice-cold RPMI 1640 with 10% FBS was added to the cells to quench the reaction. After being washed with RPMI 1640 culture medium twice, these CFSE-labeled cells (1×10^6/well) were cultured in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, and 10% FBS for 7 days with addition of rhIL-2 (200 U/mL) in the presence or absence of precoated immobilized anti-human $\gamma\delta$ TCR (1 μg/mL, Biolegend). The proliferation of $\gamma\delta$ T cells was measured by flow cytometry to determine the CFSE fluorescence intensity.

2.4. Flow Cytometry Analysis. Freshly isolated and cultured PBMCs were suspended in PBS. For the staining of surface antigens, cells were incubated with FITC-conjugated anti-CD3 and APC-conjugated anti-TCR$\gamma\delta$. For the detection of intracellular cytokines, cells were stained with appropriate amount of PE-conjugated anti-IL-4, anti-IL-10, anti-IL-17, anti-Foxp3 (all from BD), anti-TGF-β monoclonal antibodies (R&D), or PE-Cy7-conjugated anti-IFN-γ (BD) for 20 min at 4°C after the fixation/permeabilization process. Foxp3 staining was performed according to the manufacturer's manual. Mouse anti-human FITC-, PE-, and PE-Cy7-conjugated IgG1 were used as isotype controls. All cell samples were assayed by a FACSCalibur flow cytometer (BD Bioscience) and the acquired data were further analyzed using FCS express V3 analysis software. Flow cytometric results were represented as positive percentages or mean fluorescence intensity (MFI). Annexin-V/PI double staining flow cytometry was employed to detect the proportion of the apoptotic $\gamma\delta$ T and $CD3^+$ T cells in 6 active SLE patients and 6 healthy controls.

2.5. Statistical Analysis. Data are presented as mean ± standard deviation if not otherwise stated. All data were analyzed using SPSS version 17.0 software. Mann-Whitney U test was used to compare means between SLE patients and healthy controls. Pearson correlation analysis was performed to evaluate the correlation between variables of peripheral $\gamma\delta$ T cells and SLEDAI scores. The P values < 0.05 were considered statistically significant.

3. Results

3.1. Peripheral $\gamma\delta$ T Cells Were Reduced in SLE Patients. We compared the frequency and numbers of peripheral $\gamma\delta$ T cells between healthy controls and SLE patients. Flow cytometric analysis showed that the percentage of $\gamma\delta$ T cells was remarkably decreased in active SLE patients ($2.96 \pm 1.84\%$) compared to that in inactive patients ($5.31 \pm 3.05\%$) and healthy controls ($6.83 \pm 2.85\%$, both $P < 0.01$, Figure 1(c)), while there was no difference between inactive SLE patients and healthy controls. The absolute number of peripheral $\gamma\delta$ T cells was also decreased in active SLE patients ($(1.72 \pm 1.58) \times 10^7$/L) compared to that in inactive patients ($(5.27 \pm 3.60) \times 10^7$/L) ($P < 0.01$), and both of which were lower than that in healthy controls ($(10.07 \pm 4.99) \times 10^7$/L) (both $P < 0.01$, Figure 1(d)).

3.2. Peripheral $\gamma\delta$ T Cells Were Associated with SLE Disease Activity and Other Clinical Indices. Correlation analysis showed that in those 42 SLE patients, the absolute number of

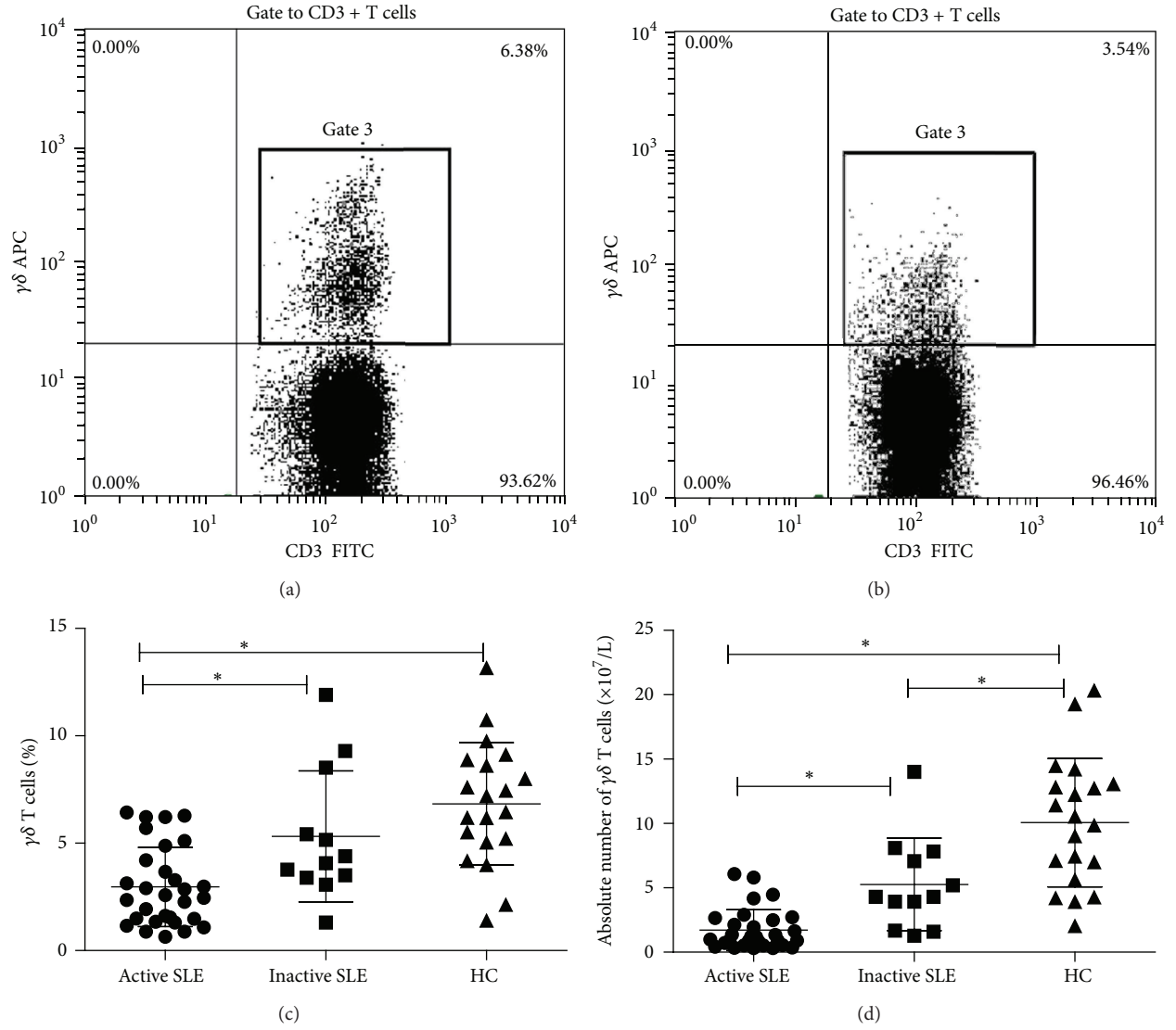

Figure 1: Reduction of $\gamma\delta$ T cells in peripheral blood of SLE patients. (a) and (b) Representative flow cytometric plot of $CD3^+\gamma\delta^+$ T cells from one healthy control (HC) and one SLE patient, respectively. (c) Comparison of the percentage of peripheral $\gamma\delta$ T cells among active SLE patients, inactive SLE patients, and healthy controls. (d) Comparison of the absolute number of peripheral $\gamma\delta$ T cells among active SLE patients, inactive SLE patients, and healthy controls. ($^*P < 0.01$).

peripheral $\gamma\delta$ T cells was negatively correlated with SLEDAI score ($r = -0.456$, $P = 0.002$, Figure 2(a)). The mean blood hemoglobin (Hb) level of all 42 patients was (109.33 ± 21.76) g/L and erythrocyte sedimentation rate (ESR) was 49.60 ± 33.34 mm/h. Our data showed that the numbers of peripheral $\gamma\delta$ T cells were negatively correlated with ESR level ($r = -0.410$, $P = 0.007$, Figure 2(b)) and positively correlated with Hb level ($r = 0.409$, $P = 0.007$, Figure 2(c)) in SLE patients. As the frequency of $\gamma\delta$ T cells was reportedly elevated in the normal cutaneous tissue of SLE patients [14], we compared the number of peripheral $\gamma\delta$ T cells between patients with or without erythema, but no significant difference was observed ($(2.11 \pm 1.93) \times 10^7$/L ($n = 17$) versus $(3.16 \pm 3.24) \times 10^7$/L ($n = 25$)). No significant correlation was found between the number of peripheral $\gamma\delta$ T cells and the level of serum complement, 24-hour urinary protein, antinuclear antibody (ANA), and anti-dsDNA antibody of these SLE patients.

We also analyzed the difference of peripheral $\gamma\delta$ T cell distribution in patients treated with various doses of prednisone. Before recruitment into the study, 17 patients were treated with low-dose prednisone (≤15 mg/d, mean dose (9.4 ± 4.4) mg/d) and 19 patients with median- to high-dose prednisone (>15 mg/d, mean dose (39.5 ± 20.4) mg/d). The number of peripheral $\gamma\delta$ T cells in these two groups was $(3.37 \pm 2.32) \times 10^7$/L versus $(2.58 \pm 3.45) \times 10^7$/L, respectively, (Figure 2(d)), with no significant difference observed. We recruited additional 6 SLE patients without prednisone treatment, and our data showed no significant difference between the numbers of peripheral $\gamma\delta$ T cells in

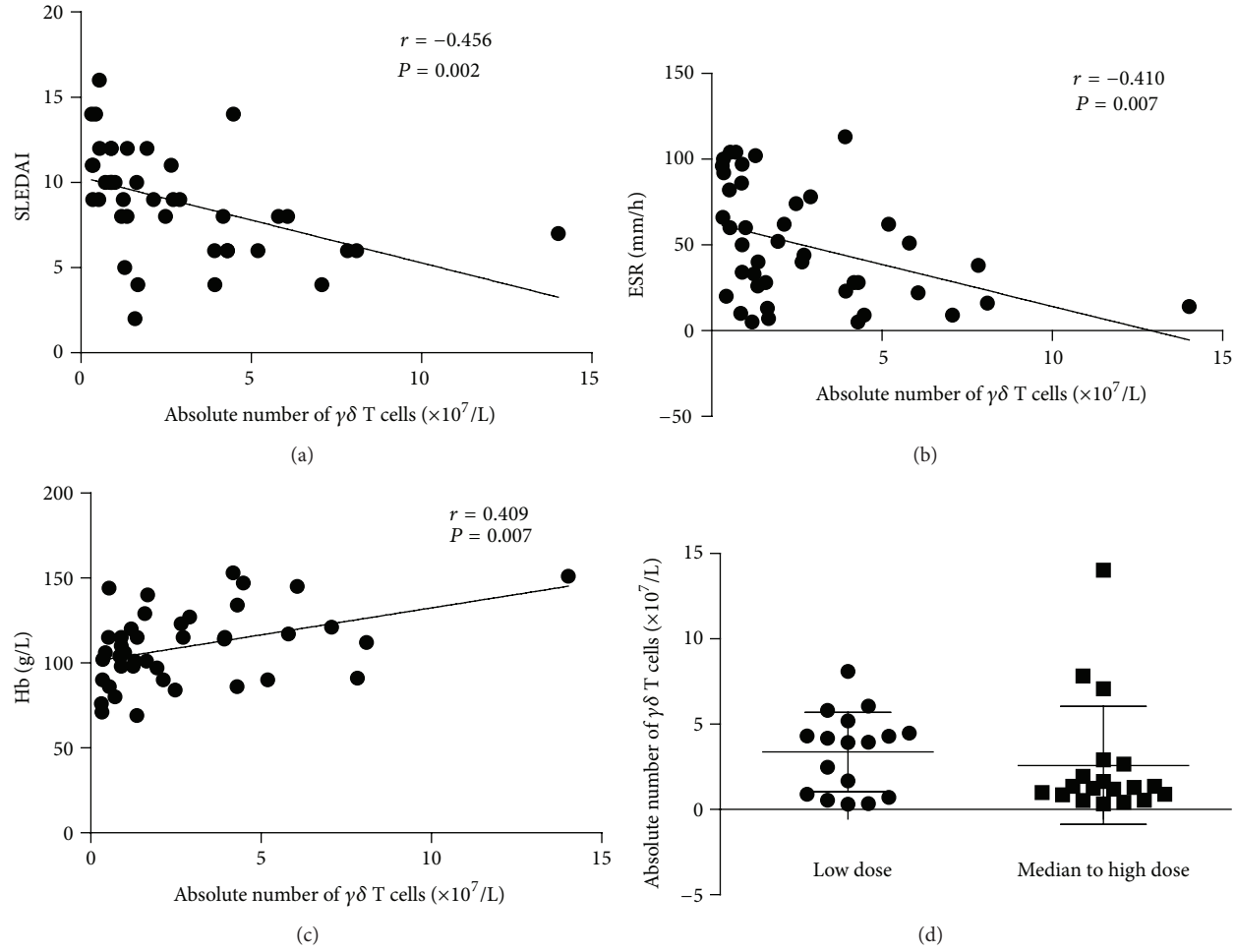

Figure 2: Associations of peripheral $\gamma\delta$ T cells with SLEDAI, ESR, Hb, and prednisone dosage in SLE patients. (a) Correlation of the absolute number of peripheral $\gamma\delta$ T cells with SLEDAI; (b) correlation of the absolute number of peripheral $\gamma\delta$ T cells with ESR; (c) correlation of the absolute number of peripheral $\gamma\delta$ T cells with serum hemoglobin level; (d), comparison of the percentage and absolute number of peripheral $\gamma\delta$ T cells with different doses of prednisone.

patients with or without prednisone treatment ($(3.86 \pm 2.38) \times 10^7$/L ($n = 6$) versus $(2.95 \pm 2.96) \times 10^7$/L ($n = 36$), $P > 0.05$).

3.3. Increased Apoptosis and Reduced Proliferation May Account for Decreased Number of Peripheral γδ T Cells in Active SLE Patients. To delineate the underlying mechanisms for the decrease of peripheral $\gamma\delta$ T cells in active SLE patients, we analyzed the apoptosis and proliferation status of these cells. AV/PI double staining flow cytometry showed that the apoptotic rate of peripheral $\gamma\delta$ T cells in 6 active SLE patients was increased compared with that in 6 healthy controls ($17.03 \pm 8.71\%$ versus $6.67 \pm 1.18\%$, $P < 0.05$, Figures 3(a)–3(d)). The apoptotic rate of $\gamma\delta$ T cells was significantly higher than that of total $CD3^+$ T cells in the peripheral blood of active SLE patients ($17.03 \pm 8.71\%$ versus $11.30 \pm 7.43\%$, $P < 0.05$, Figure 3(e)), while no significant difference was found in healthy controls ($6.67 \pm 1.18\%$ versus $6.22 \pm 1.78\%$, $P > 0.05$, Figure 3(e)).

$\gamma\delta$ T cell proliferation was determined by CFSE method using PBMC from 5 active SLE patients and 5 healthy controls after in vitro stimulation of immobilized anti-human $\gamma\delta$ TCR for 7 days. As shown in Figure 4, the proliferation rate of $\gamma\delta$ T cell in active SLE patients was significantly reduced compared with that in healthy controls ($54.43 \pm 11.32\%$ versus $15.15 \pm 11.90\%$, $P < 0.01$, Figure 4).

3.4. Peripheral γδ T Cells from SLE Patients Expressed Altered Intracellular Cytokine Profile. We examined intracellular cytokine expressions in 20 active SLE patients and 10 healthy controls by flow cytometry. As shown in Figure 5, the percentages of $\gamma\delta$ T cells that express intracellular IFN-γ, IL-4, IL-10, and TGF-β in SLE patients ($33.19 \pm 20.20\%$, $1.04 \pm 0.93\%$, $1.91 \pm 0.98\%$, and $2.20 \pm 1.97\%$, resp.) were significantly higher than those in healthy controls ($5.87 \pm 4.63\%$, $0.30 \pm 0.34\%$, $0.18 \pm 0.31\%$, and $0.21 \pm 0.22\%$, all $P < 0.01$, Figure 5). However, there was no significant difference of the percentage of IL-17^+ $\gamma\delta$ T cells and $Foxp3^+$ $\gamma\delta$ T cells between SLE patients and healthy controls ($0.14 \pm 0.24\%$ versus $0.18 \pm 0.31\%$, and $0.44 \pm 0.85\%$ versus $0.49 \pm 0.44\%$, resp., $P > 0.05$).

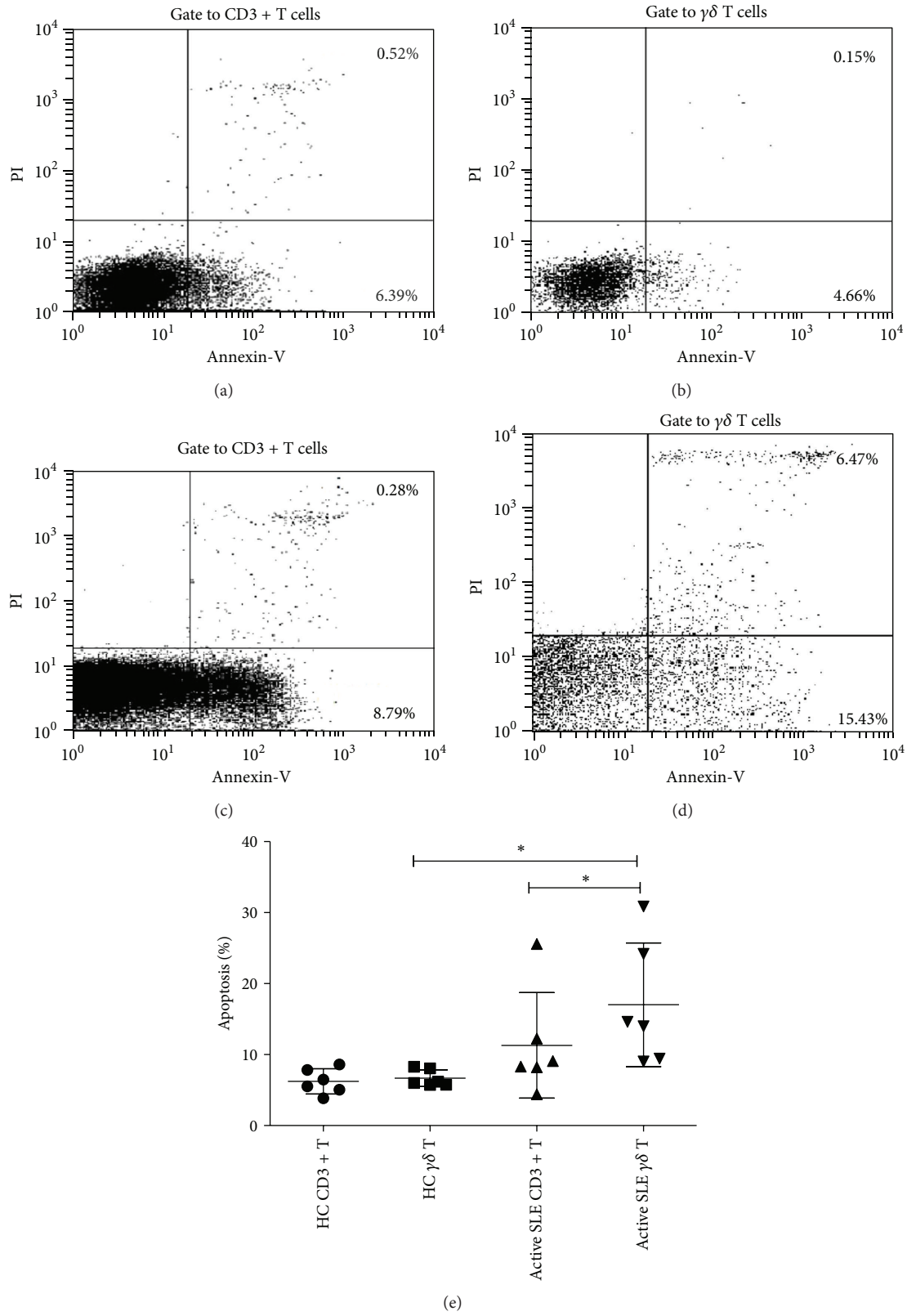

FIGURE 3: Increased apoptosis of peripheral $\gamma\delta$ T cells in active SLE patients. (a) and (b) Representative flow cytometric plot for apoptosis of total $CD3^+$T cells and $\gamma\delta$ T cells in one healthy control; (c) and (d) flow cytometric plot for apoptosis of total $CD3^+$T cells and $\gamma\delta$ T cells in one SLE patient; (e) comparison of apoptotic rate of $CD3^+$T and $\gamma\delta$ T cells between healthy controls and active SLE patients ($n = 6$, $^*P < 0.05$).

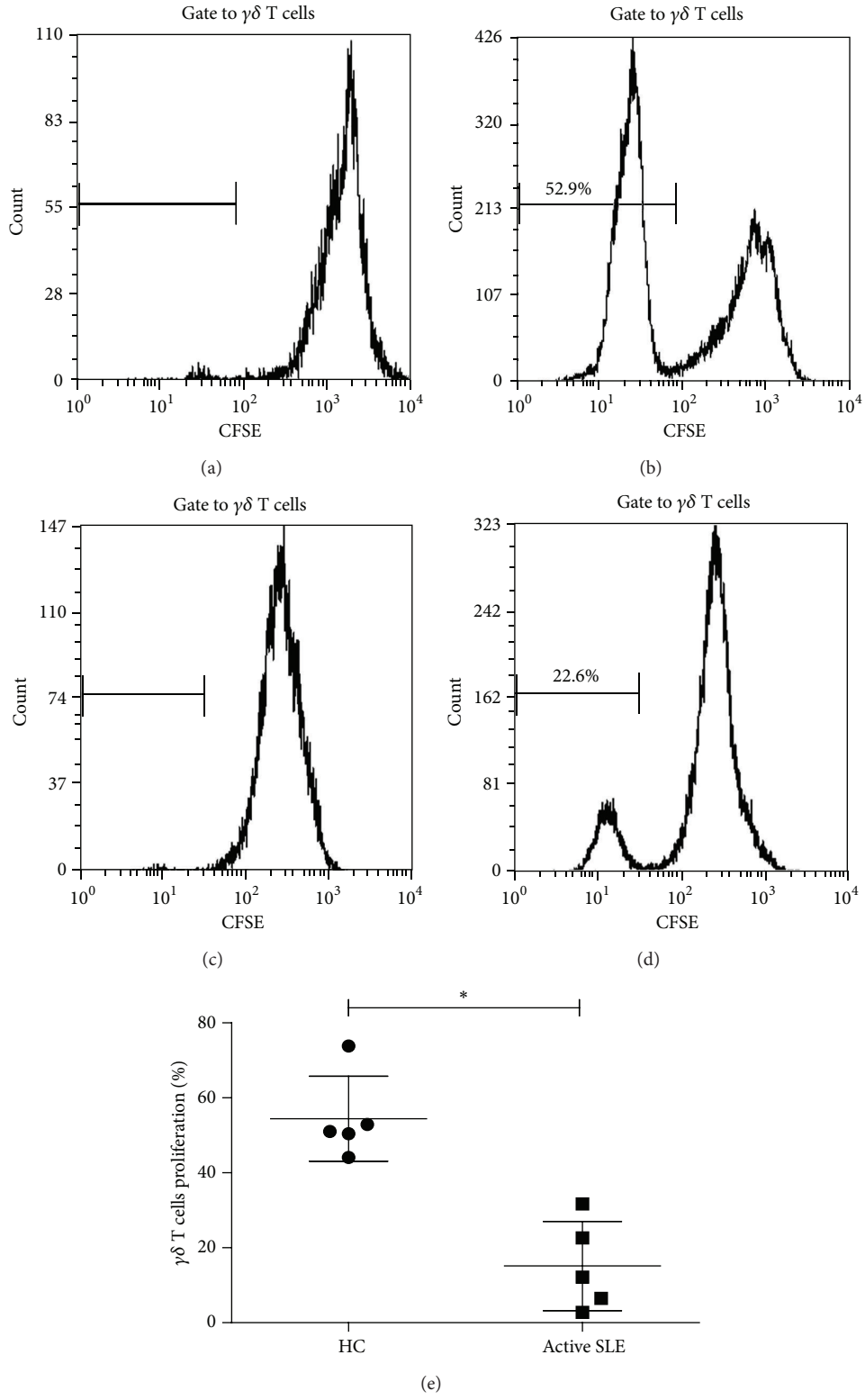

Figure 4: Reduced γδ T cell proliferation in active SLE patients. (a) and (b) Proliferation of γδ T cells in one healthy control cultured without or with stimulation of rhIL-2 (200 U/mL) and immobilized anti-human γδ TCR (1 ug/mL) for 7 days. (c) and (d) Proliferation of γδ T cells in a SLE patient cultured without or with stimulation. (e) Comparison of γδ T cell proliferation under the stimulation of rhIL-2 and immobilized anti-human γδ TCR between active SLE patients and healthy controls ($n = 5$, $^{*}P < 0.01$).

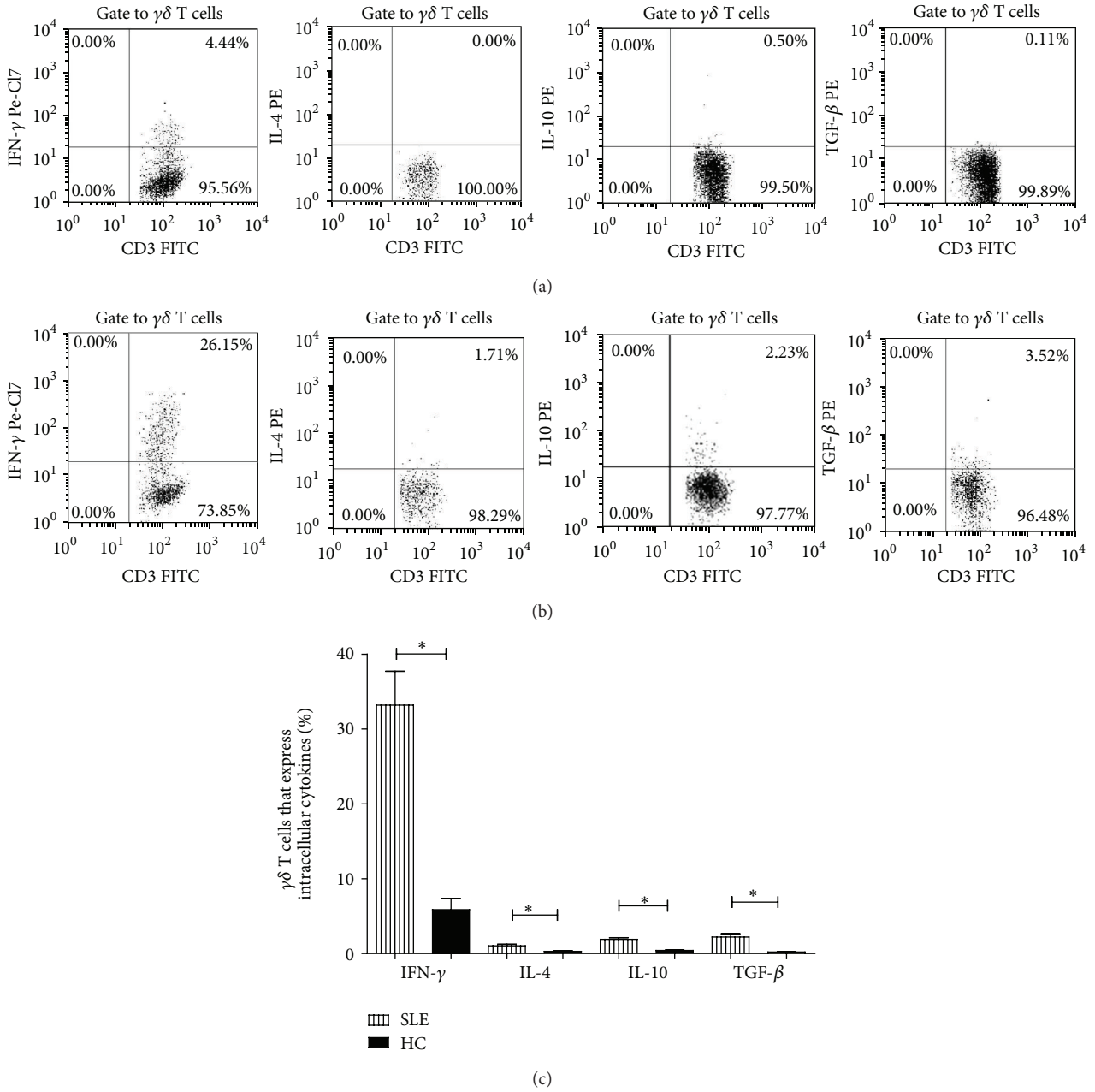

Figure 5: Expression of intracellular cytokines in peripheral $\gamma\delta$ T cells from SLE patients ($n = 20$, $^*P < 0.01$). (a) Representative flow cytometric plots of $\gamma\delta$ T cells that express various intracellular cytokines from one healthy control and SLE patient, respectively. The data of IL-17$^+$ and Foxp3$^+$ $\gamma\delta$ T cells, which were nearly undetectable, were not shown. (b) Comparison of the percentages of $\gamma\delta$ T cells expressing IFN-γ, IL-4, IL-10, or TGF-β in SLE patients and healthy controls.

4. Discussion

In recent years, more and more attention has been paid upon the role of $\gamma\delta$ T cells in the pathogenesis of autoimmune diseases including SLE [7, 14]. Our study demonstrated that the number of $\gamma\delta$ T cells in the peripheral blood of SLE patients was decreased and negatively correlated to disease activity. SLE $\gamma\delta$ T cells displayed high apoptotic rate, low proliferation ability, and abnormal cytokine profiles as compared to those of healthy controls.

Though some studies reported an elevated proportion of peripheral $\gamma\delta$ T cells in untreated SLE [15], the majority of previous reports showed that the percentage and absolute number of $\gamma\delta$ T cells were reduced in the peripheral blood of SLE patients [14, 16], which was negatively correlated with levels of ESR and C-reactive protein (CRP). Our study confirmed that the percentage of peripheral $\gamma\delta$ T cells in SLE patients decreased significantly compared with that in healthy controls and was more prominent in those with active disease. The reduced number of $\gamma\delta$ T cells was negatively

correlated with SLEDAI score as well as ESR, suggesting that downregulation of peripheral $\gamma\delta$ T cells might play a role in the aggravation of SLE disease.

The underlying mechanism for the decreased number of peripheral $\gamma\delta$ T cells in SLE patients remains elusive. It has been demonstrated that in patients with chronic cutaneous lupus, $\gamma\delta$ T cells were enriched in the skin lesions, whereas the percentage of peripheral $\gamma\delta$ T cells was not changed. Robak et al. also found that the frequency of $\gamma\delta$ T cells was significantly higher in the normal cutaneous tissue of SLE patients compared with that in healthy controls, and it was reduced after treatment with corticosteroids, which was supported by studies of Spinozzi et al. [15] who had shown that addition of dexamethasone to cultured $\gamma\delta$ T cells from the peripheral blood of SLE patients could reduce the number of these cells. These results indicated that the reduction in the number of $\gamma\delta$ T cells could be ascribed to their migration to the skin tissue under the influence of corticosteroids. In this study, we compared the number of peripheral $\gamma\delta$ T cells from SLE patients with or without skin lesions that of and those treated with different doses of prednisone. There was no significant difference between these groups, suggesting that skin-toward migration and corticosteroid application could not explain the reduction of peripheral $\gamma\delta$ T cells in our SLE patients.

Our data showed that the apoptotic rate of $\gamma\delta$ T cells was higher in SLE patients than in healthy controls, and the frequency of apoptotic $\gamma\delta$ T cells was higher than that of total $CD3^+$ T cells in SLE patients, indicating that the decreased number of $\gamma\delta$ T cells in the peripheral blood of SLE patients may be partly due to the upregulated apoptosis of these cells. Besides, in vitro culture of peripheral $\gamma\delta$ T cells from active SLE patients showed reduced proliferation than that from healthy controls under the stimulation of rhIL-2 and immobilized anti-$\gamma\delta$ TCR, suggesting that the impaired proliferative capacity may also contribute to the decreased number of peripheral $\gamma\delta$ T cells in SLE patients.

Apart from abnormal number, $\gamma\delta$ T cells from SLE patients were also shown to have some functional defects. In this study, we investigated the intracellular cytokine expression of peripheral $\gamma\delta$ T cells from SLE patients, including IL-4, IL-10, IL-17, IFN-γ, and TGF-β. Similar to $CD4^+\alpha\beta$ T cells, $\gamma\delta$ T cells can produce a variety of cytokines in response to different stimulating signals. Cytokines secreted by $\gamma\delta$ T cells under different microenvironments could influence the immune processes in some diseases, which might play crucial roles in the development of autoimmune diseases [7, 17–20]. Our study showed that in the peripheral blood of SLE patients, the percentages of $\gamma\delta$ T cells that expressed intracellular IFN-γ, IL-4, IL-10, and TGF-β were all significantly increased, which was possibly due to the immunological disorder of SLE and in turn might aggravate lupus disease. We further calculated absolute IFN-γ expressing cells in samples but found that there was no difference between SLE patients and healthy controls ($(0.90 \pm 1.27) \times 10^7$/L ($n = 20$) versus $(0.58 \pm 0.53) \times 10^7$/L ($n = 10$), $P > 0.05$). To which extent these different subsets of $\gamma\delta$ T cells exert their pathogenic or protective roles in SLE still needs in-depth investigations in vitro and in vivo. Unexpectedly, although the expression of TGF-β was elevated, we did not find significantly increased level of Foxp3 in peripheral $\gamma\delta$ T cells from SLE patients, suggesting that $\gamma\delta$ T cells might not exert their immunomodulatory effect through the Foxp3 pathway.

In conclusion, this study demonstrated that the percentage and absolute number of $\gamma\delta$ T cells in the peripheral blood of SLE patients decreased substantially compared with those of healthy controls and was conversely correlated with disease activity. The reduction of $\gamma\delta$ T cells might be attributed to increased apoptosis and impaired proliferative capacity of these cells. Expression of pro- and anti-inflammatory cytokines, including IL-10, IL-4, IFN-γ, and TGF-β, was significantly increased in the peripheral $\gamma\delta$ T cells from SLE patients, suggesting that there could be different subsets of $\gamma\delta$ T cells functioning in the pathogenesis of SLE.

Conflict of Interests

There is no conflict of interests in FCS express V3 analysis software and SPSS version 17.0 software.

Authors' Contribution

Zhimin Lu and Dinglei Su contributed equally to this work.

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Review Article

Epstein-Barr Virus in Systemic Autoimmune Diseases

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Systemic autoimmune diseases (SADs) are a group of connective tissue diseases with diverse, yet overlapping, symptoms and autoantibody development. The etiology behind SADs is not fully elucidated, but a number of genetic and environmental factors are known to influence the incidence of SADs. Recent findings link dysregulation of Epstein-Barr virus (EBV) with SAD development. EBV causes a persistent infection with a tight latency programme in memory B-cells, which enables evasion of the immune defence. A number of immune escape mechanisms and immune-modulating proteins have been described for EBV. These immune modulating functions make EBV a good candidate for initiation of autoimmune diseases and exacerbation of disease progression. This review focuses on systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS) and sum up the existing data linking EBV with these diseases including elevated titres of EBV antibodies, reduced T-cell defence against EBV, and elevated EBV viral load. Together, these data suggest that uncontrolled EBV infection can develop diverse autoreactivities in genetic susceptible individuals with different manifestations depending on the genetic background and the site of reactivation.

1. Systemic Autoimmune Diseases

Systemic autoimmune diseases (SADs), also called rheumatic connective tissue diseases, include rheumatoid arthritis (RA), Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), systemic scleroderma (SSc), and dermatomyositis/polymyositis (DM/PM). SADs are characterized by overlapping clinical symptoms and characteristic autoantibodies (Table 1). Some of the most extensively studied SADs are SLE, RA, and SS, and this review will focus on these.

The etiology of SADs is believed to be multifactorial with both genetic and environmental factors contributing to the disease development. Concordance has been observed in monozygotic twins, and specific genes including some coding for certain major histocompatibility complex (MHC) II alleles have been shown to be associated with development of these diseases [1–5]. The major environmental risk factors for SAD development are infections, including Epstein-Barr virus (EBV) infection which is suspected to have a central role in the pathogenesis of SADs as presented in the later sections of this review. Furthermore, EBV has for decades been associated with induction of various cancers, including lymphoid malignancies (e.g., Burkitt's lymphoma [6]) and epithelial cell malignancies (e.g., nasopharyngeal carcinoma [7]).

1.1. SLE. SLE is a rare autoimmune disease with a prevalence of 0.09% and an incidence of 1–10 new cases per 100.000 per year, and nine out of 10 patients are women [8–12]. Typical symptoms involve the butterfly rash at the malar region of the face, photosensitivity, oral- and nasopharyngeal ulcers, arthritis, renal and hematologic disorders, and autoantibodies against nuclear components. The clinical presentation of SLE is influenced by a variety of factors including ethnicity, gender, age, and age of onset [8]. The typical course of the disease is demonstrated by periods of disease flares alternating with remission.

Various immune-deficiencies have been identified in SLE patients. Abnormalities in the complement cascades are observed in some SLE patients. C1q and C4 deficiencies serve as severe risk factors for development of SLE with a risk of developing SLE at 93% and 75%, respectively. C1q deficiency can also be acquired as a result of production of

Table 1: Prevalence (%) of autoantibodies in RA, SS, and SLE.

	CCP	RF	Ro52	Ro60 (SSA)	La (SSB)	dsDNA	ANA	References
RA	50–80	70–80	5–10	5–10	0–5	0–10	30–50	[20–22]
SS	5–10	40–70	20–40	40–80	30–60	0–10	40–70	[21, 23, 24]
SLE	5–10	20–30	10–20	20–40	15–20	70–80	95–100	[9, 21, 25]

RA: rheumatoid arthritis; SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; CCP: cyclic citrullinated peptide; RF: rheumatoid factor; dsDNA: double-stranded DNA; ANA: nuclear antibodies.

C1q autoantibodies, which are detected in 40–50% of SLE patients. Deficiencies in the complement system result in decreased clearance of apoptotic material, which may initiate autoimmune responses and production of autoantibodies against cellular components [13–16]. Additionally, SLE is characterized as an immune complex disease comprising autoantibodies and their specific autoantigens. These will deposit in the subendothelium, when the concentration and size reach a critical level and cause inflammation and tissue damage [15, 17].

1.2. RA. RA is a common autoimmune disease with a prevalence of approximately 1% and an incidence of 5–50 per 100,000 per year with three times more female than male patients [18]. Common symptoms include arthritis, cardiovascular complications, metabolic syndrome, cognitive dysfunction, and depression. Furthermore, involvement of the lungs, kidneys, and skin are observed in RA patients [18, 19].

Another environmental risk factor for development of RA, besides infections, is smoking and other forms of pulmonary stress. Environmental stress may promote posttranslational modifications of proteins including citrullination via peptidylarginine deiminases [19]. Loss of tolerance will thereby result in autoantibodies against these citrullinated proteins (CCP antibodies) characteristic of RA patients (Table 1).

Several inflammatory processes are involved in the disease course. Most of all, the persistent synovial inflammation with infiltration of macrophages, T- and B-cells, immune complexes, and a variety of cytokines result in joint damage and cartilage destruction, ultimately leading to impaired movement and deformity of involved joints [18]. Furthermore, prolonged inflammation also leads to bone erosion by promoting osteoclast differentiation resulting in osteoporosis and bone fractures [19].

1.3. SS. SS is a rather common autoimmune disease with a prevalence of about 0.5% and an incidence of 3–6 per 100,000 per year with a female preponderance (nine out of 10 SS patients are women) [26, 27]. It may present as primary SS, but it can also be associated with other SADs including SLE and RA.

SS is characterized by disorders of exocrine glands (particularly salivary and lacrimal glands resulting in dry eyes and dryness of mouth) with presence of infiltrating lymphocytes, dysfunction of muscarinic receptors, chronic inflammation, and development of specific autoantibodies (Table 1). Furthermore, several extraglandular manifestations are observed in SS patients, including dry skin, pancreatitis, gastritis, arthritis, neurosensory deafness, serositis, pulmonary fibrosis, hypergammaglobulinemia, and involvement of kidneys and the nervous system [27].

Presumably, the initial pathogenic steps in the development of SS involve changes in the glandular epithelial cells, including cell death giving rise to upregulation of adhesion molecules and chemokines, which stimulate lymphocyte migration to the glands resulting in lymphocyte extravasation, infiltration, and glandular destruction [26]. Actually, E-cadherin, an epithelial cell adhesion molecule, has been demonstrated to be increased in patients with SS suggesting enhanced adhesion of lymphocytes to epithelial cells tissue [28]. The systemic manifestations in SS presumably occur upon lymphocyte infiltrations in other tissues and also as a result of pathogenic autoantibodies.

2. EBV

EBV is a ubiquitous infectious agent, latently infecting approximately 95% of the world's population [29]. Primary infection with EBV mostly occurs during childhood and causes a mild, usually asymptomatic infection. However, primary infection in adolescence causes infectious mononucleosis (IM) in 30–70% of cases, where up to 20% of B-cells are infected with EBV [30, 31]. This age-related difference in disease progression has yet to be explained [32].

EBV is a DNA virus of the herpes family (human herpesvirus 4). It is comprised of a linear dsDNA genome enclosed by an icosahedral capsid, which is surrounded by the tegument and a host cell membrane-derived envelope embedded with glycoproteins (gps) (Figure 1). EBV has a fairly large genome coding for 87 proteins, and the functions of 72 of these are so far elucidated [33].

EBV is transmitted in saliva and initially infects epithelial cells in the oropharynx and nasopharynx. Subsequently, EBV enters the underlying tissues and infects B-cells [34, 35]. After primary lytic infection, EBV persists in immortalized resting memory B-cells for the rest of the individual's life and can shift between an active lytic cycle and a latent state, from which it occasionally reactivates [36]. This ability of the virus to reactivate makes it a constant challenge to the host.

In the latent state, the EBV genomic DNA will undergo circularization and replicate together with the host's chromosomal DNA, which results in a restricted expression of viral genes and conceals the virus from the host's immune system [34, 35]. During the latent state, a maximum of nine genes are expressed including the EBV nuclear antigens (EBNA1, -2, -3A, -3B, and -3C), the leader protein (LP),

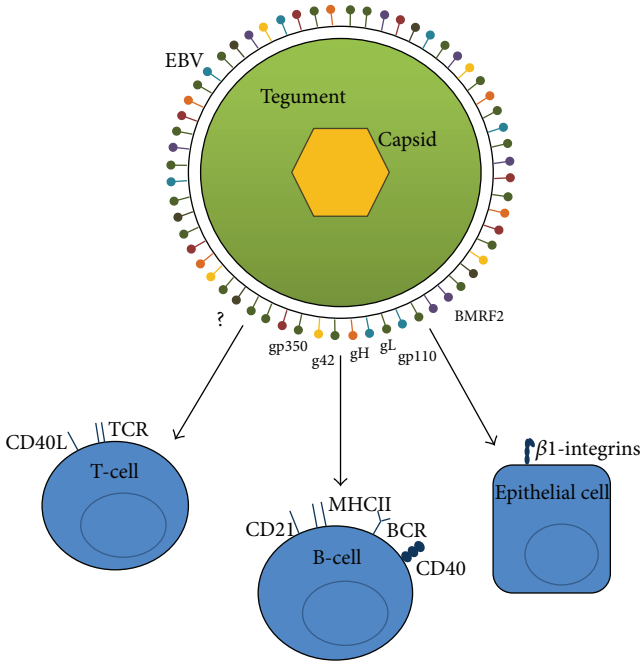

Figure 1: Epstein-Barr virus structure and infection of cells. Epstein-Barr virus (EBV) is comprised of a dsDNA genome inside an icosahedral capsid which is surrounded by the tegument and enclosed by a host cell membrane-derived envelope. During infection with EBV, different envelope glycoproteins (gps) (shown in different colors) induce viral entry. During viral entry of B-cells, viral gp350 binds to type 2 complement receptor (CD21) on B-cells, and via a complex of gp42, gH, and gL, fusion of the cell membrane and the viral envelope is induced through (major histocompatibility complex) MHC II on the B-cell. During viral entry of epithelial cells, viral BMRF2 binds to β1-integrins on the epithelial cell, and fusion of the membranes is facilitated by a complex of gH and gL. gp110 improves the efficiency of EBV to infect B-cells and epithelial cells. EBV can also infect T-cells; however, the mechanism of viral entry is unknown (?). BCR: B-cell receptor, TCR: T-cell receptor.

and the latent membrane proteins (LMP1, -2A, and -2B) [36, 37] (Table 2). EBNA1 is the only protein required for maintenance of the viral genome serving as a replication factor. When B-cells are latently infected for longer periods of time, EBV will only express EBNA1 [34–36]. EBNA2 is an important transcription factor during latency as it controls the expression of all other latent viral genes [36]. LMP1 and LMP2A rescue the infected B-cells from apoptosis, as they deliver the signal that normally comes from the CD40 signal transduction pathway initiated by CD4+ T-cell help and provide the signal normally generated by antigen binding of the B-cell receptor, respectively [37, 38].

The exact triggers for lytic cycle reactivation are unknown, but the process is a dynamic interaction between the host's immune response to EBV and the infection state. Activation of the promoter for the early lytic genes and, thereby, initiation of lytic replication are triggered by the differentiation of infected B-cells into plasma cells [34, 36, 40, 41].

Table 2: Selection of Epstein-Barr virus proteins and their functions [33, 39].

Function	Protein
Latent state nuclear antigens	EBNA1
	EBNA-LP
	EBNA2
	EBNA3A
	EBNA3B
	EBNA3C
Glycoproteins involved in viral entry	gp350
	gp42
	BMRF2
	gH
	gL
	gp110
Envelope proteins	gN
	gp150
	BILF2
	BILF1
	BDLF2
Initiation of lytic replication	BZLF1
	BRLF1
	EA/D
	BSLF1
	BBLF4
	BBLF2/3
	BALF5
	BALF2
Immune evasion	Viral IL10
	BARF1
	LF2
	BNLF2a
	BMLF1/BSLF2
Antiapoptotic	EA/R
	BALF1
	LMP1
	LMP2

During lytic cycle of infection, EBV expresses numerous proteins involved in different viral activities. In the induction of lytic replication, two transcription factors, BZLF1 and BRLF1, activate early viral promoters required for generation of the initiation complex consisting of six viral proteins (Table 2): the viral DNA polymerase (BALF5), the viral DNA polymerase accessory protein, early antigen diffuse (EA/D), a single-stranded DNA-binding protein (BALF2), the primase (BSLF1), the helicase (BBLF4) and the helicase/primase-associated protein (BBLF2/3) [42–47]. The binding of BZLF1 and the gathering of the initiation complex at the lytic origin of replication, *oriLyt*, result in multiple viral genome replication cycles with a 100- to 1000-fold amplification [35, 46] and expression of lytic genes [33]. After synthesis of viral DNA, various viral proteins induce packaging and encapsidation of the viral genome, which is subsequently

released from the nucleus to the cytoplasm of the infected cell. In the tegument, various viral enzymes induce assembly, envelopment, and glycosylation of the virion. Ultimately, new infectious virions are produced and shed from the cell. These can infect other cells and can also be transmitted to a new host [36].

New virions primarily infect B-cells and epithelial cells, but various other cell types, including T-cells and natural killer cells, can also be infected [48–50]. During viral entry of B-cells, viral gp350 binds to the B-cell type 2 complement receptor (CD21) [34, 35]. A complex of three gps (gp42, gH, and gL) induces fusion of the viral envelope with the cell membrane by binding to MHC II. Viral entry of epithelial cells is induced via binding of viral BMRF2 to β1 integrins and similarly to fusion with the B-cell membrane, a complex of gH and gL facilitates fusion of the viral envelope. Furthermore, gp110 improves the efficiency of the virus to infect both B-cells and epithelial cells [51] (Figure 1) (Table 2). The mechanism of viral entry in T-cells is unknown. However, it could be speculated that some of the envelope proteins (Table 2) with unknown function may be implicated in viral entry in T-cells and possibly other immune cells.

Several EBV proteins are involved in immune evasion (Table 2) mainly by inhibiting the interferon (IFN) pathways and T-cell immunity. An example is the viral interleukin(IL)10 homologue, which, like human IL10, inhibits the synthesis of IFNγ and suppresses CD8+ cytotoxic T-cell responses and the upregulation of MHC I expression [52]. Furthermore, viral antiapoptotic proteins are expressed during lytic cycle of infection including early antigen restricted (EA/R), which is a viral Bcl2 homologue that protects both infected B-cells and epithelial cells from apoptosis [53].

3. EBV in SADs

3.1. EBV in SLE. Many studies have linked EBV to the development of SLE. SLE patients have been shown to have an abnormally high viral load in the peripheral blood mononuclear cells (PBMCs) compared to healthy controls with 10–40-fold increase [54–58]. The viral load was found to be associated with disease activity and to be independent of intake of immunosuppressive medication. Furthermore, an elevated level of EBV DNA was found in serum from 42% of SLE patients compared to only 3% of healthy controls [56]. The findings on increased EBV load suggest active EBV lytic replication in SLE patients. As the viral load was associated with disease activity, it could be speculated that the reactivation of EBV is associated with development of SLE and flares.

Usually, little or no mRNA expression by EBV is observed in normal immune competent carriers of EBV. However, several groups have demonstrated that SLE patients have abnormally high expression of several viral mRNAs (coding for BZLF1, gp350, viral IL10, LMP1, LMP2, and EBNA1) [54, 59]. High expression of BZLF1 could imply reactivation of EBV, and increased gp350 could be speculated to result in an amplified number of B-cells being infected with EBV. Furthermore, increased expression of viral IL10 may give rise to enhanced immune evasion from the cell-mediated part of the immune system. In addition, an abnormal EBV latent state is also indicated by these results with improved survival of infected cells via enhanced expression of the LMP's [54, 59].

Much serologic evidence of a connection between EBV infection and SLE has been demonstrated. Antibodies to EBNA1, viral capsid antigen (VCA), and EA in sera from SLE patients have been examined. Most studies find no difference between SLE patients and healthy controls in the prevalence of IgG and IgM antibodies to either EBNA1 and VCA [60–63], but studies on pediatric SLE patients and one study on adults show that all SLE patients are seropositive for these antibodies compared to two-thirds of healthy controls [29, 64, 65]. Furthermore, elevated titers of IgG antibodies to EA/D, EA/R, and BALF2 have been observed in about half of SLE patients compared to only 8–17% of healthy controls [60, 62, 63, 66, 67]. Additionally, high levels of IgA antibodies to EA/D have been found in 58% of SLE patients and not in healthy controls [68, 69]. These results could not be explained by immunosuppressive medication, indicating that the antibodies are not produced upon reactivation of EBV due to an iatrogenically suppressed immune system. Presumably, these results reflect the host's attempt to control reactivation or reinfection of EBV in epithelial cells [68].

EBV infection is mainly controlled by cell-mediated immunity. However, EBV-specific cytotoxic T-cell reactivity has been observed to be reduced in SLE patients resulting in poor control of the EBV infection. Less CD8+ cytotoxic T-cells were found to produce IFNγ upon stimulation with EBV in the SLE patients compared to healthy controls, which must be a consequence of either defective or fewer EBV-specific cytotoxic T-cells [55, 70, 71].

Thus, SLE patients have an elevated viral load, increased EBV mRNA expression, elevated levels of EBV-directed antibodies, and decreased EBV-directed cell-mediated immunity compared to healthy controls, indicating poor control of EBV with frequent reactivation.

3.2. EBV in RA. EBV has for long been suspected to have a role in the pathogenesis of RA. By the use of several methods including in situ hybridization and PCR, presence of EBV DNA/RNA has been demonstrated in PBMCs, saliva, synovial fluid, and synovial membranes of RA patients [72–76]. Furthermore, 10-fold higher frequencies of EBV-infected B-cells have been observed in RA patients compared to healthy controls [77]. Interestingly, EBV DNA was found in many of the plasma cells producing CCP antibodies localized in synovial tissues of RA patients [78]. These results indicate a widespread lytic EBV infection in RA patients, that also localize in the joints, suggesting a role for EBV-infected cells in the synovial inflammation characteristic for RA patients [78].

In addition, studies on EBV antibodies have shown a humoral response to both latent and lytic EBV antigens with elevated titers of antibodies against EBNA1, VCA, and EA/R in both sera and synovial fluids from RA patients compared to healthy controls [72, 79–81].

Investigations on EBV-specific T-cells in the peripheral blood of RA patients have revealed a defective IFNγ

response to EBV proteins compared to healthy controls [82]. A study regarding gp110-specific T-cells in the peripheral blood showed that T-cells from RA patients had a decreased response to gp110 compared to healthy controls, and this was associated with disease activity [83]. As gp110 is important in viral entry during infection of B-cells and epithelial cells, a decreased gp110-specific T-cell response could be speculated to reduce the control of EBV and also enhance spreading of the EBV infection in RA patients. Contrarily, CD8+ cytotoxic T-cells specific for the two lytic cycle EBV antigens, BZLF1 and BMLF1, have been detected in synovial fluid and synovial membranes of RA patients, indicating a contribution of infiltrated cytotoxic T-cells specific for EBV lytic cycle antigens in joint inflammation [84, 85].

Thus, research has revealed increased viral load, high titers of EBV-directed antibodies, and decreased cell-mediated control of EBV in RA patients compared to healthy controls and suggested a role for infiltrated EBV-specific T-cells in synovial inflammation of RA patients.

3.3. EBV in SS. EBV infection has also been associated with SS, with findings of increased viral load [86–90] and EBV-directed antibodies in SS patients [91–94]. Furthermore, SS patients are known to have an increased risk of development of EBV-associated lymphomas, additionally indicating this association [95]. About 5% of SS patients will develop a lymphoid malignancy, in most cases (mucosa-associated lymphoid tissue) MALT lymphoma in the salivary gland or non-Hodgkin's lymphoma [95].

One study has shown that saliva from SS patients is able to activate EBV. Eight out of 12 SS saliva samples were found to have an activating effect on the *BZLF1* promoter in EBV-negative *BZLF1*-transfected salivary gland cells, indicating a possible frequent reactivation of EBV in the oropharynx of SS patients [96].

High loads of EBV DNA have been observed in saliva from SS patients and in both infiltrated B-cells and epithelial cells in salivary glands from SS patients [86, 87, 89, 90]. Furthermore, by the use of a monoclonal antibody directed against the lytic cycle antigen EA/D, a cytoplasmic staining of epithelial cells in salivary glands has been observed in 57% (eight of 14) of SS patients compared to none of the healthy controls [86]. These results suggest EBV reactivation in the epithelial cells in salivary glands of SS patients, which could initiate an immune response that damages the salivary glands of SS patients.

Moreover, EBV DNA has been observed in the lacrimal glands of SS patients and EBV latent and lytic proteins were detected by the use of immunohistochemistry in areas with B-cells and epithelial cells in lacrimal gland tissue from SS patients and not in the healthy controls [88]. Thus, EBV may also play a role in the lacrimal gland disorders characteristic of SS patients.

Studies have shown elevated levels of antibodies against EBNA, VCA, and EA in serum from SS patients [91, 92, 94]. One study demonstrated IgG antibodies directed against EA/D in 36% (36 of 100) of SS patients compared to only 4.5% of healthy controls. The presence of these antibodies was not associated with intake of immunosuppressant medication [93].

Thus, increased viral load and EBV proteins have been found in salivary and lacrimal glands of SS patients indicating active infection, and elevated levels of EBV-directed antibodies have been found in the circulation.

4. Genetic Factors and Possible Mechanisms Associated with Induction of Autoimmunity

Much investigation has suggested an etiologic role for active and uncontrolled EBV infection in development of the SADs in genetically predisposed individuals. This is demonstrated by defective EBV-specific T-cells, increased viral loads and elevated expression of lytic cycle proteins, and high levels of antibodies against EBV in SLE, RA, and SS patients [29, 54–58, 60–68, 70–83, 86–94]. These findings suggest widespread infection and frequent EBV reactivation in SLE, RA, and SS patients.

EBV is a good candidate for a causal agent in SADs. EBV has the ability to persist in the host as a latent infection that occasionally reactivates, which presumably contributes to the disease flares observed in the chronic SADs. EBV-induced IM has similar symptoms and clinical manifestations as the individual SADs, including presence of rheumatoid factor and other autoantibodies [32, 97–99], and primary acute EBV infection is also known to induce production of nuclear autoantibodies characteristic of SADs [100]. Furthermore, inoculated EBV infection in humanized mice has been demonstrated to generate RA-resembling arthritis [101].

Several mechanisms have been associated with the induction of autoimmunity by EBV [102]. EBV infection influences the host's immune system both directly through infection of various lymphocytes (for instance will the infection of B-cells possibly result in proliferation, enhanced antibody production, and formation of immune complexes [102]) and indirectly by expression of numerous immune-modulating proteins [36–38, 52, 53, 102]. EBV proteins involved in immune evasion and suppression of apoptosis of transformed infected lymphocytes are likely to result in loss of tolerance and development of autoimmunity [37, 38, 52, 53].

EBV is able to stimulate the innate immune system via EBV-encoded small RNA (EBER) in complex with La (SSB) through Toll-like receptor 3 and thereby induce production of inflammatory cytokines [103]. Thus, in this way, EBV might enhance the autoreactivity against the ribonucleoprotein La (SSB) often found in SS and SLE. Furthermore, bystander activation and expansion of autoreactive T-cells are known to occur due to the virus-induced severe local inflammation and intense local cytokine production [102].

An additional mechanism by which EBV may contribute to loss of tolerance and development of autoimmunity is molecular mimicry [102]. EBNA1 has been shown to cross-react with the autoantigen Ro (SSA) resulting in cross-reactive antibodies followed by epitope spreading [59, 65, 93, 104, 105].

EBV could be involved in SLE, RA, and SS through both common and different genetic or acquired immune-deficiencies connecting EBV to these overlapping yet

different diseases. Common gene variants known to be involved in the pathogenesis of all three diseases include components of cytokine pathways (e.g., *IRF5*, *STAT4,* and *TNFSF4*) [2, 3, 19, 106–108] presumably contributing to the development of systemic autoimmunity. In addition, several individual gene variants are associated with the pathogenesis of the specific diseases. Especially gene variants involved in the complement system (including *ITGAM*) are specific for SLE patients [3, 109], and gene variants involving the muscarinic receptors (*CHRM3*) are specific for SS patients [110]. Gene variants specific for RA patients include *PADI4* variants coding for the enzyme that catalyzes the citrullination of arginine residues of proteins [19]. Thus, genetic (and epigenetic) variations may contribute to specific immune-deficiencies and, thereby, altered immune response to EBV and altered control of EBV infection [2, 3, 19, 106–110]. The constant interplay between EBV reactivation and the host's immune response probably results in individual disease patterns and clinical manifestations according to the genetic background, site of reactivation or reinfection and type of infected cell [3, 19, 36, 40, 48, 110].

In SS patients, studies show that the EBV replication is mainly localized in epithelial cells and infiltrated B-cells of the salivary and lacrimal glands [86–90, 96]. In RA patients, the EBV-infected cells in the synovial joints are shown to undergo frequent reactivation including EBV-infected plasma cells producing CCP autoantibodies [72, 74–76, 78, 84, 85]. The EBV reactivation results in production of lytic cycle proteins giving rise to host immune responses that presumably contribute to the inflammation and destruction of exocrine glands and synovial joints in SS and RA patients, respectively [72, 74–76, 78, 84–90, 96]. In SLE patients, EBV reactivation of epithelial cells could be involved in the symptoms of skin and mucosa [68]. Furthermore, a systemic EBV reactivation of both B-cells and epithelial (and possibly other cell types) may occur, giving rise to the various overlapping systemic manifestations observed in the three SADs. Reactivation of EBV and thereby an increased number of EBV-infected cells presumably result in increased amounts of cellular waste and thus stimulation of autoreactive B-cells and consequently production of autoantibodies resulting in disease flares. For each reactivation, the amounts of EBV immune evasion and antiapoptotic molecules expand, resulting in a vicious circle of increased disease activity [35, 46].

5. Conclusion

In conclusion, EBV is demonstrated to have a role as an environmental trigger in the development of SADs. Individual genetically determined and acquired differences in innate and adaptive immunity and the constant interplay between the host's immune response and EBV immune-modulating proteins may result in individual disease patterns, which are overlapping, but clinically may be classified as SLE, RA, and SS. It is also possible that every time the control of EBV diminishes, EBV reactivates and reinfects more cells of different types in different locations of the body, resulting in specific manifestations and progression of disease flares [3, 19, 36, 38, 48, 52, 53, 110].

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Review Article

The Use of ^{18}F-FDG-PET/CT for Diagnosis and Treatment Monitoring of Inflammatory and Infectious Diseases

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FDG-PET, combined with CT, is nowadays getting more and more relevant for the diagnosis of several infectious and inflammatory diseases and particularly for therapy monitoring. Thus, this paper gives special attention to the role of FDG-PET/CT in the diagnosis and therapy monitoring of infectious and inflammatory diseases. Enough evidence in the literature already exists about the usefulness of FDG-PET/CT in the diagnosis, management, and followup of patients with sarcoidosis, spondylodiscitis, and vasculitis. For other diseases, such as inflammatory bowel diseases, rheumatoid arthritis, autoimmune pancreatitis, and fungal infections, hard evidence is lacking, but studies also point out that FDG-PET/CT could be useful. It is of invaluable importance to have large prospective multicenter studies in this field to provide clear answers, not only for the status of nuclear medicine in general but also to reduce high costs of treatment.

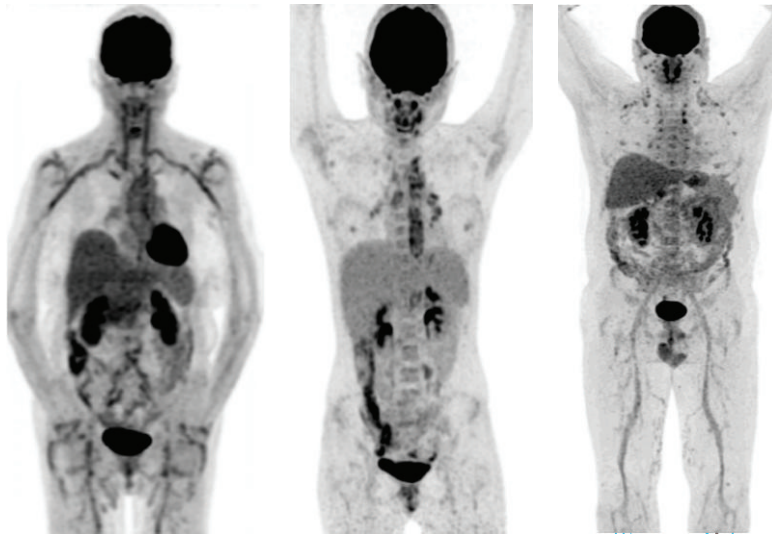
1. Introduction

In recent years, the use of nuclear medicine to characterize and diagnose infectious and inflammatory diseases is rapidly increasing. Several SPECT and PET radiopharmaceuticals have been developed and applied in this field, radiolabelled white blood cells being the centerpiece [1]. However, [^{18}F]-FDG-PET combined with low dose or diagnostic computed tomography (CT) is gaining interest in the diagnosis of many infectious and inflammatory diseases and is already the gold standard for some indications.

The accumulation of FDG in inflammatory and infectious diseases is based on the high uptake in activated granulocytes. This accumulation is based on the fact that these cells use glucose as an energy source only after activation during the metabolic burst. Transport of FDG across the cellular membrane is mediated by the glucose transporter (GLUT) proteins, which are also to a higher amount present on the cell membrane of inflammatory and infectious cells [2].

For therapy followup, the indication of FDG-PET is less clear. The European Medicines Agency (EMEA) Committee for Medicinal Products for Human Use (CHMP) only mentions the use of FDG for the therapeutic followup of unresectable alveolar echinococcosis, in which it may be used in the search for active localizations of the parasite during medical treatment and after treatment discontinuation [3]. Despite this lack of attention, we think that FDG-PET/CT is not only valuable for therapy monitoring in some infectious and inflammatory diseases but could even play a pivotal role in their management, leading to better drug dosage, proof of the usefulness of the treatment, and early modification of the therapeutic strategy.

The literature for this review was collected with PubMed and Cochrane search using the combination of "FDG," "therapy," "therapy evaluation," and the specific term for each inflammatory or infectious disease. The reference lists of selected articles were checked for additional valuable literature. This paper summarizes most papers published so



(a) (b) (c)

Figure 1: FDG-PET examples of vasculitis: (a) giant cell arteritis and polymyalgia rheumatica: high FDG uptake in the large vessels (aorta, subclavian arteries, carotid arteries, iliac arteries, and femoral arteries) accompanied by high uptake in the large joints (shoulders and hips), (b) Takayasu's arteritis: high FDG uptake located more centrally (aorta and main branches in the thoracic region) and in this case uptake in reactive lymph nodes in mediastinum and hili (confirmed by biopsy), and (c) polyarteritis nodosa and polychondritis: high uptake in the medium- and small-sized arteries (best visible in the legs) accompanied by uptake in the nose, the ears, and the costochondral regions.

far in this field and tries to define the role of FDG-PET/CT for therapy monitoring in various infectious and inflammatory diseases.

2. Vasculitis

2.1. FDG-PET/CT and Classification of Vasculitis. Systemic vasculitis is a multisystem disease characterized by inflammation with infiltration of leukocytes into the blood vessels. Classification of vasculitis is still unsatisfactory and controversial. Existing classifications—the American College of Rheumatology criteria [4, 5], the Chapel Hill Consensus Conference definitions [6], and the adapted Zeek classification system [7]—are useful but limited [8]. For nuclear medicine, the Zeek system is most useful since it reflects dominant vessel size, in association with antineutrophil cytoplasmic antibodies (ANCAs).

For diagnosis, CT is useful for showing mural changes, including wall thickening, calcification, and mural thrombi. CT angiography (CTA) provides the possibility to reveal luminal changes, such as stenosis, occlusion, dilatation, and aneurysms [9]. MRI is probably the best method to evaluate and reveal structural vascular abnormalities (aneurysms, stenoses) but does not identify inflammation in structural normal blood vessels [10]. Because of the limited spatial resolution of the PET/CT camera, nuclear medicine is most of the times able to visualize only inflammation of the aorta and the larger arteries. However, depending on the spatial resolution of the used camera, it is possible to find FDG uptake also in smaller arteries, as other studies mentioned [11–13]. Furthermore, FDG-PET/CT may be able to differentiate between giant cell arteritis (GCA), Takayasu's arteritis (TA), and polyarteritis nodosa (PAN).

GCA, also called temporal arteritis, is a granulomatous inflammation of the aorta and its main branches, most of the times occurring in patients older than 50 years. The extracranial branches are also involved, especially the temporal artery. Normally, involvement of the temporal artery is difficult to see on FDG-PET due to its small diameter, but involvement of the aorta, subclavian, carotid, and iliac arteries is enough to settle the diagnosis GCA based on FDG-PET findings. GCA is often associated with polymyalgia rheumatica (PMR), an inflammatory disease around the joints, causing pain and stiffness (Figure 1(a)).

Takayasu's arteritis is more centrally located (therefore also known as aortic arch syndrome) and mainly affects the aorta and the main branches in the thoracic region (carotid arteries, brachiocephalic trunk, subclavian arteries, and the pulmonary arteries). In the majority, it affects young or middle-aged woman. An example is shown in Figure 1(b). In this 14-year-old girl, Takayasu's arteritis was confirmed by biopsy, together with inflammatory lymph nodes in the mediastinum around the inflamed arteries.

PAN is a vasculitis of the medium and small-sized arteries, which become swollen and damaged by immune cells. Most cases occur at middle age, but it can affect nearly everyone. PAN may be associated with polychondritis [14], as shown in Figure 1(c). On FDG-PET, uptake may be seen in the smaller arteries, best visible in the extremities.

When choosing between FDG-PET/CT and MRI, it is important to consider both study-specific and patient-specific

factors. For FDG-PET/CT, some questions still remain to be answered, for example, the usefulness of this technique in patients taking corticosteroids and immunosuppressives [15].

2.2. The Role of FDG-PET/CT in Diagnosing and Evaluating Therapy in Vasculitis. In the setting of diagnosing vasculitis, FDG-PET/CT was proven valid and utility of FDG-PET/CT was found in:

(i) the initial diagnosis of patients suspected of having a vasculitis, and particularly in patients that presented with nonspecific symptoms. In GCA, sensitivities ranging from 77 to 92% and specificities from 89 to 100% were reported [16]. In TA, results were even better (sensitivity 92%, specificity 100%) [17];

(ii) the identification of areas of increased FDG uptake as a target site in which a biopsy should best be taken to obtain a definite diagnosis [18, 19];

(iii) the evaluation of the extent of the disease and involvement of extracranial sites, with more vascular involvement found by FDG-PET/CT compared to MRI imaging and traditional angiography [20, 21]. A correlation was also found with inflammatory markers as C-reactive protein and erythrocyte sedimentation rate [21].

Many case reports are published that mention a role of FDG-PET/CT in guiding treatment strategy and evaluating therapy response. However, only a small number of large patient studies exist.

Blockmans et al. performed FDG-PET at baseline and after 3 and 6 months of therapy with corticosteroids in 35 patients with suspected GCA. The FDG-PET at baseline was positive in 29 patients, leading to a sensitivity of 83%. The total vascular score ((TVS) a scoring system of 0 to 3 points in 7 vascular regions using the Meller visual grading scale) decreased from 7.9 at baseline to 2.4 after 3 months of therapy. No further decrease was found at 6 months. However, in long-term followup, 18 relapses were found, with the TVS at baseline of this group of relapsing patients not differing from the whole patient group. The authors concluded that FDG-PET is sensitive for detecting GCA and that therapy evaluation after 3 months is possible without added value of scanning on a later time point. Relapse of GCA could not be predicted by FDG-PET [22].

Bertagna et al. performed FDG-PET/CT before and at least 4 months after corticosteroid therapy in 9 patients with proven vasculitis. Eight patients became negative after therapy (aorta/liver ratio < 1 or SUVmax < 2), which was concordant with clinical and biochemical findings. One patient remained positive on FDG-PET, which was also confirmed clinically and biochemically. Using FDG-PET/CT, instead of FDG-PET alone, allowed the authors to precisely identify the anatomical sites of disease, which was found particularly useful after therapy to identify possible weak persistence of disease. They concluded FDG-PET/CT being a useful, accurate tool for establishing the diagnosis of large-vessel vasculitis, for evaluating disease extension, and for monitoring therapy in conjunction with clinical and biochemical findings [10].

More recently, Henes et al. retrospectively studied 10 patients with proven vasculitis that were treated with cyclophosphamide, because they were not responding to corticosteroids and/or had organ or limb threatening vascular stenosis. FDG-PET/CT was performed before and during (6 out of 10 cycles mostly) therapy and the visual grading score of Meller was used. Before therapy, the grading score was 3 in 8 patients and 2 in 2 patients. During therapy, all patients had a grading score equal to or lower than 2. Nine out of 10 patients showed clinically a complete remission after 10 cycles of cyclophosphamide. The authors concluded that cyclophosphamide was an effective therapy in patients and that FDG-PET/CT might serve in future as an additional tool to continue successful treatment or stop the unsuccessful [23].

FDG-PET/CT was also found to have impact on the clinical management in a significant proportion of 30 patients with suspected GCA, both in patients without and already with immunosuppressive medication. The addition of FDG-PET increased the number of indicated biopsies and changed treatment recommendation in 27% of patients [24].

3. Sarcoidosis

3.1. FDG-PET/CT and Classification of Sarcoidosis. Sarcoidosis is a multisystem granulomatous disease that affects predominantly the lungs and associated lymph nodes but may involve virtually any organ. Despite the progress in imaging techniques, only one imaging classification system exists that was already described more than 4 decades ago [25]. This system classifies chest radiographic findings as stage 0 (normal radiography), stage I (bilateral hilar lymphadenopathy), stage II (stage I and parenchymal infiltration), stage III (parenchymal infiltration without hilar lymphadenopathy), and stage IV (parenchymal infiltration with pulmonary fibrosis) [26]. Of course, this classification system is obsolete, because of the currently existing imaging modalities.

For conventional imaging, high resolution computed tomography (HRCT) is now widely accepted as the radiographic imaging reference standard in the evaluation of sarcoidosis and other diffuse infiltrative lung diseases [27]. HRCT is superior to conventional CT in delineating the distribution and pattern of pulmonary interstitial lesions [26] and has typical findings for sarcoidosis with small nodules in perilymphatic distribution or along fissures but also with alveolar consolidation with air bronchograms, cavitation, and fibrosis when there is lung involvement. Sensitivities reported for HRCT in diffuse infiltrative lung diseases are high (>90%) [28]; however, it is often difficult to differentiate sarcoidosis from other interstitial diseases, and biopsy is still required.

FDG-PET provides valuable information in this disease as FDG is highly taken up by the granulomas. Moreover, whole body imaging is possible with FDG-PET. Different presentations of sarcoidosis on FDG-PET exist. Keijsers et al. categorized FDG-PET patterns in sarcoidosis patients based on the presence and extent of organ involvement. The involvement of thoracic lymph nodes and lung parenchyma

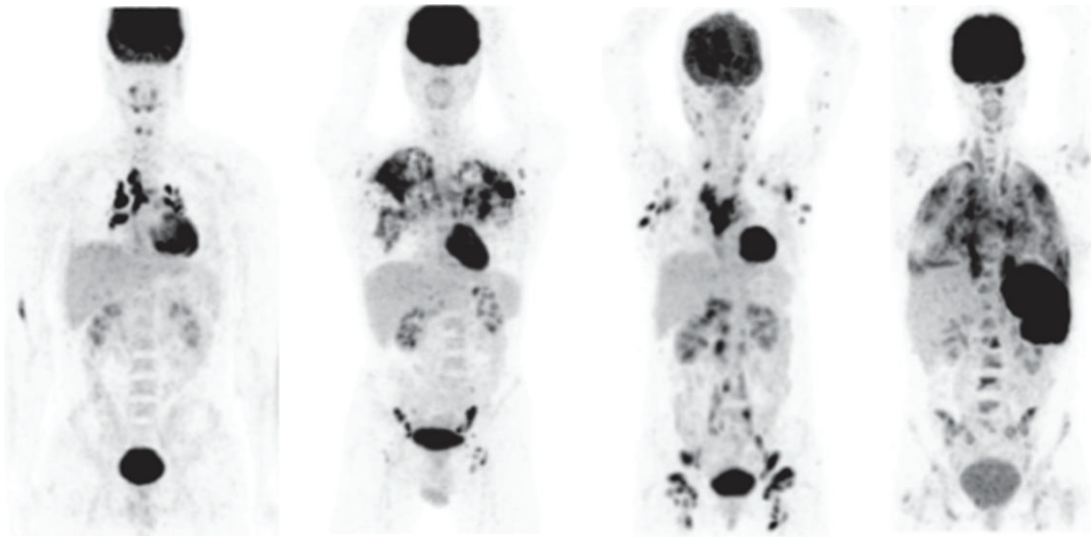


Figure 2: FDG-PET classification of sarcoidosis. Type I: thoracic lymph node involvement (in this image: mediastinal and hilar regions), type II: involvement of the lung parenchyma, type III: diffuse lymph node involvement (in this image all lymph node regions of the body are involved), and type IV: organ involvement (in this image involvement of the spleen and bones) (images courtesy by R. Keijsers).

was considered as the presence of extrathoracic disease (Figure 2). This system could have added value for prognosis and stratification as parenchymal disease, splenomegaly, and involvement of more than three organ systems is associated with a poor prognosis [29, 30].

For diagnosis, both FDG-PET and HRCT are necessary. HRCT is required because of its typical findings for sarcoidosis, as mentioned earlier, and because FDG-PET is not very specific: the uptake pattern in sarcoidosis can mimic malignancy or lymphoma disease. Despite this low specificity, FDG-PET has been extensively studied on a relatively large number of patients, and many publications support the use of FDG-PET for diagnosis, although in combination with (HR) CT.

FDG-PET has a high sensitivity for diagnosing sarcoidosis, and provides valuable information to evaluate pulmonary and extrapulmonary sarcoidosis [2]. Whole body FDG-PET imaging may uncover an occult diagnostic site or multiple organ involvement [31] and is also useful in cardiac [32] and cerebral [33] sarcoidosis. Compared with the old "gold standard" tracer for sarcoidosis, ^{67}Ga-citrate SPECT, FDG-PET was found more suitable for imaging the mediastinum, the hilar lymph nodes, the posterior regions of the lungs, and nonthoracic lesions [34, 35]. The metabolic activity measured with FDG-PET reflects the disease activity in sarcoidosis in quantitative terms and the SUVmax correlates with histopathological results from bronchoalveolar lavage [36]. Diffuse parenchymal uptake of FDG predicts a future deterioration—when untreated—of the diffusion capacity of the lung and absence of activity in the lung parenchyma could justify a wait-and-see policy [37]. Furthermore, FDG-PET may show some specific features that could help in the diagnosis, for example, thick linear FDG uptake in the lower legs in muscular sarcoidosis (the so-called "tiger man" sign) [38].

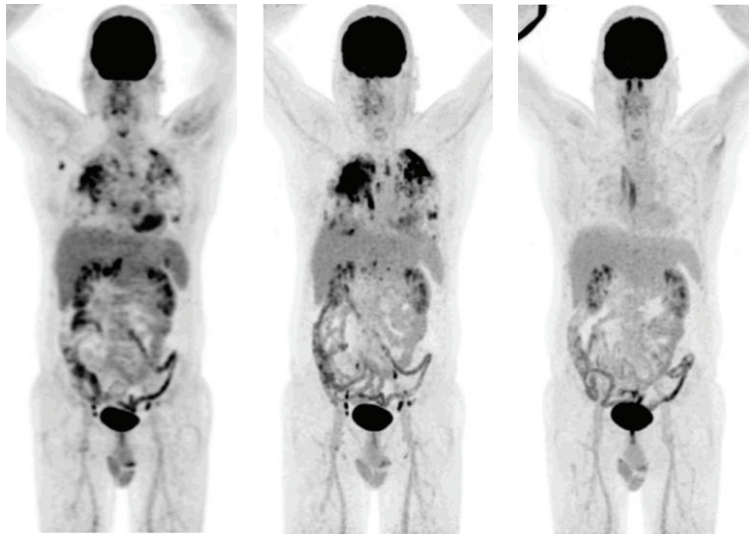
3.2. FDG-PET/CT and Therapy Evaluation. For treatment of sarcoidosis, several options are possible: treatment with corticosteroids in various doses, antimalarial drugs such as hydroxychloroquine, cytotoxic drugs, and also with cytokine modulating drugs, such as infliximab or adalimumab, both being antitumor necrosis factor alpha (anti-TNFα) antibodies.

Again, there is arguable evidence in the literature: FDG-PET/CT is a valuable imaging tool in assessing treatment efficacy in patients with sarcoidosis and deciding whether to switch to an alternate therapeutic regimen [31, 39–42] (Figure 3). Almost all patients in these studies were treated with corticosteroids, which resulted in a decrease in uptake on FDG-PET but also in clinical and biochemical improvement.

One study performed FDG-PET/CT before and after 6 cycles of infliximab therapy in 12 patients with refractory sarcoidosis (not reacting on corticosteroid therapy). Clinical improvement was seen in all patients, although minor response in one. FDG-PET improvement or normalization was seen in 11 of the patients with an overall decrease in SUVmax of 55%. However, the patient with a minor clinical response showed a 34% increase in FDG uptake [43]. We may conclude from this study that FDG-PET/CT is not only useful to assess the efficacy of corticosteroid therapy but may also be useful in other therapies, such as infliximab.

4. Autoimmune Diseases

4.1. Rheumatoid Arthritis. Rheumatoid arthritis (RA) is an autoimmune disease, which is associated with systemic and chronic inflammation of the joints, resulting in synovitis and pannus formation, both leading to increased FDG uptake.



(a) (b) (c)

Figure 3: An example of the value of FDG-PET/CT in a patient with sarcoidosis: (a) baseline scan, (b) scan after 3 months of corticosteroid therapy with progression of lung infiltration, and (c) scan after 3 months of treatment with corticosteroids and methotrexate together, resulting in complete remission (the linear uptake is located in a muscle in the back and considered physiological muscle uptake).

Several clinical studies evaluated the role of FDG-PET in patients with RA [44–46]. The degree of FDG uptake in affected joints reflects the disease activity of RA [47] and correlates with clinical parameters, including the disease activity score (DAS), swelling and tenderness, ultrasonography (US) findings for synovitis and synovial thickening, power Doppler studies for neovascularization, ESR, and CRP [48]. FDG-PET was eligible to identify joints with active RA with higher sensitivity than clinical symptoms [47]. The quantification of metabolic changes in joint inflammation with FDG-PET was comparable to volumetric changes visualized with MRI. However, both MRI and FDG-PET were not associated with treatment outcome [48].

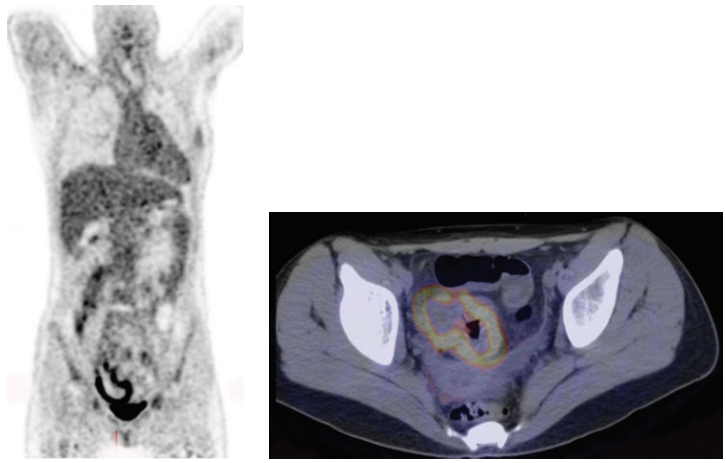
For therapy evaluation, studies are scarce. Beckers et al. assessed 16 patients with active RA in the knee joint using FDG-PET, dynamic MRI, and US at baseline and four weeks after the initiation of anti-TNFα treatment. Significant differences in the MRI and US findings were observed between the FDG-PET positive and FDG-PET negative patients. Changes in the SUV after four weeks were correlated with changes in the MRI parameters, but not with the changes in synovial thickness observed by US [49]. This suggests metabolic changes are preceding morphological changes in patients with RA.

Goerres et al. used a visual assessment total joint score, that is, the sum of all scores based on FDG uptake intensity between zero and four in 28 joints, in seven patients with active RA before and after infliximab treatment. The reduction of FDG joint uptake in the follow-up scans correlated significantly with clinical evaluation of disease activity [50].

Recently, an association was found between changes in FDG joint uptake between baseline and after two weeks of infliximab treatment and the clinical outcome on long term. Changes in the mean SUV between the baseline scan and the scan after two weeks of treatment correlated significantly with the DAS at 14 and 22 weeks and contributed significantly to the prediction of DAS at these time points. So, early changes in FDG uptake in joints during infliximab treatment may predict clinical outcome [51].

These current collected data together deliver not enough evidence to support the use of FDG-PET for the routine use in patients with RA. In the recently published EULAR recommendation for the use of imaging of the joints in the clinical management of RA, FDG-PET was not recommended as an imaging tool, neither for diagnosis nor for therapy evaluation [52]. To better define the role of FDG-PET in patients with RA, larger patient studies are warranted to understand the clinical usefulness of this technique in this setting.

4.2. Inflammatory Bowel Diseases. Inflammatory bowel diseases (IBD) are represented mainly by ulcerative colitis (UC) and Crohn's disease (CD) and characterized by a chronic, uncontrolled inflammation of the intestinal mucosa. Reported studies in the literature about the use of FDG-PET in IBD—although few in number—concluded that this imaging modality holds potential in evaluating disease activity and providing an objective assessment of the severity of bowel inflammation. Despite these findings, overall not enough literature has been published to support a role for FDG-PET for diagnostic purposes [40, 53]. FDG-PET correlates globally well with clinical activity scores and may be useful when conventional imaging fails to yield a conclusive diagnosis [54]. Despite MRI being the technique of choice in children [55, 56], FDG-PET was found especially suitable for the assessment of IBD in children, where it detected inflamed gut segments with high sensitivity and specificity [57] and could be useful as a noninvasive tool in the followup of children with known chronic IBD, where a yearly invasive colonoscopy is not desirable [58] An example is shown in Figure 4. In another study, the clinical utility of FDG-PET/CT



(a) (b)

Figure 4: A 14-year-old girl known with Crohn's disease. FDG-PET/CT (left: MIP image of the FDG-PET, right: fused FDG-PET/CT transaxial slice) showed inflammation of the caecum.

was compared to the standard workup in patients with known or suspected IBD and found very useful, not only in diagnosis but also in therapy management. In this study, unnecessary disease escalation or initiation of IBD therapy was avoided based on the PET/CT results [59].

Despite all these positive results, a major limitation of the use of FDG in IBD is that in a lot of patients gradual physiological uptake in the bowel can be seen, especially in the large bowel, which may create problems in diagnosing IBD in colonic segments. Bowel movements during the scan acquisition can blur the images. Furthermore, diabetic patients who take antidiabetic drugs (e.g., metformin) may show intense uptake in the large bowel. To solve these problems, maybe FDG-PET/CT colonography offers a novel technique for the assessment of extent and activity of IBD. In this technique, the colon is inflated with oral ingestion of polyethylene glycol before acquiring images. In a pilot study in 15 patients, a good correlation was found between PET activity grades after PET/CT colonography and the endoscopic grade of inflammation [60].

Aside from the diagnostic use of FDG, its main utility in IBD can be the early evaluation of treatment success. Indeed FDG represents the whole inflammatory burden of the gut, and an early posttherapy scan (within weeks of beginning of therapy) compared with a pretherapy scan could allow the evaluation of therapy efficacy. To this aim, the only published study is in a small group of 5 patients. FDG-PET/CT was performed in these patients before and after successful medical therapy in patients with moderately active IBD. Five bowel segments were scored on a 0–3 scale (0 = no uptake or uptake lower than liver, 1 = equal to liver, 2 = greater than liver, and 3 = three times liver uptake or higher) for the appropriate FDG-PET assessment. The total score of all segments was 32 before treatment and 14 after treatment. Of 11 pretreatment active segments (score 2 or 3), nine (82%) segments either became inactive or displayed decreased activity, while two showed no change. These findings correlated with clinical symptoms [61]. One major limitation of this study, however, was the time point of the FDG-PET/CT after therapy, this ranged from 77 to 807 days after the baseline scan.

Taken together, all these findings about the use of FDG-PET/CT in IBD demonstrate that currently there are no large patient studies to support the use of this imaging technique for diagnosis and therapy evaluation. This was confirmed in a recently published meta-analysis [62]. However, considering the potential in evaluating disease activity, FDG-PET/CT may have a role to evaluate therapy in IBD in future.

4.3. Autoimmune Thyroiditis. Several forms of autoimmune thyroiditis (AIT) exist, the most important being Riedel's thyroiditis, characterized by a replacement of the normal thyroid parenchyma by a dense fibrosis that invades adjacent structures of the neck and may extend beyond the thyroid capsule, and Hashimoto's thyroiditis (also called lymphocytic thyroiditis), in which the thyroid gland is gradually destroyed by a variety of cell- and antibody-mediated immune processes. AIT can result—on short or longer term—in hypothyroidism.

Normally, uptake of FDG in thyroid tissue is low or absent and unexpected findings in the thyroid gland fall into 2 categories: focal or diffusely increased uptake of FDG. Diffusely increased uptake of FDG in the thyroid is thought to be associated with AIT or hypothyroidism [63] and is mentioned in several case reports [64–66]. In contrast, other authors mention that only 9.5% of PET scans in patients with hypothyroidism as a result of Hashimoto's thyroiditis display diffuse thyroid activity [67].

For the use of FDG-PET as an aid in the followup in Riedel's thyroiditis, Kotilainen et al. describe a 60% decrease in the FDG uptake in the thyroid in the follow-up PET after two weeks of treatment with corticosteroids, indicating that FDG metabolic activity can also be used to assess a patient's response to therapy in Riedel's thyroiditis [68].

One important large retrospective study mentioned different findings. Of 4,732 investigated FDG-PET/CT scans, 138 (2.9%) showed diffuse thyroid uptake. In 47%, a prior diagnosis of hypothyroidism or AIT was found, of whom the majority received thyroxin therapy. In a control group without thyroid uptake, 10% had a prior diagnosis of hypothyroidism and received therapy for that. Of the remaining patients with diffuse thyroid uptake, 32 were examined for thyroid diseases after the findings on the FDG-PET, of which 19 were found to have AIT or hypothyroidism. So, diffusely increased FDG uptake in the thyroid is associated with AIT, but uptake seems not to be affected by hormonal therapy. Furthermore, no correlation was found between SUV and the degree of hypothyroidism [63].

At the moment, there is no special role for FDG-PET/CT, neither in diagnosing AIT nor in evaluating treatment efficacy.

4.4. Autoimmune Pancreatitis. Autoimmune pancreatitis (AIP) is a subset of pancreatitis characterized by enlargement of the pancreatic parenchyma and irregular narrowing of the main pancreatic duct, caused by an autoimmune inflammatory process with prominent lymphoplasmacytic infiltration and fibrosis of the pancreas [69].

FDG-PET/CT imaging findings in patients with AIP have been explored in a small number of studies. FDG-PET was found to be useful for detecting AIP and associated extra pancreatic autoimmune lesions and also for monitoring disease activity [70–73]. These studies were performed in small numbers of patients, so larger patient studies are definitely warranted.

Another important message is the ability of FDG-PET/CT to differentiate between AIP and pancreatic cancer. Reported sensitivities for pancreatic cancer vary between 72 and 96%, the latter result achieved together with contrast-enhanced CT [74, 75]. For AIP, published studies are too small to calculate sensitivity and specificity. Both conditions normally accumulate FDG, so FDG-PET/CT cannot always discriminate between the two conditions. However, by looking carefully at the pattern of FDG accumulation, accompanying other autoimmune diseases, and the change in FDG uptake after steroid treatment, it may be possible to differentiate between both diseases on the short term. At baseline, FDG-PET shows more diffuse uptake in the pancreas in AIP compared to the more focally located lesions in pancreatic cancer. The detection of other autoimmune diseases, such as uptake in the salivary glands (sclerosing sialadenitis), in the thyroid (AIT) and in the bile ducts (cholangitis), also points towards the diagnosis AIP. Furthermore, a decrease in pancreatic uptake after a short period of steroid therapy (two weeks) may be useful for discriminating AIP from pancreatic cancer [76, 77].

Larger patient studies are necessary to clarify the usefulness of FDG-PET/CT in diagnosis and therapy monitoring. However, when having a diagnostic dilemma between AIP and pancreatic cancer, or when biopsy is not feasible, the FDG uptake pattern and decrease of FDG uptake on the follow-up scan after a short period of therapy, may help to solve this clinical problem.

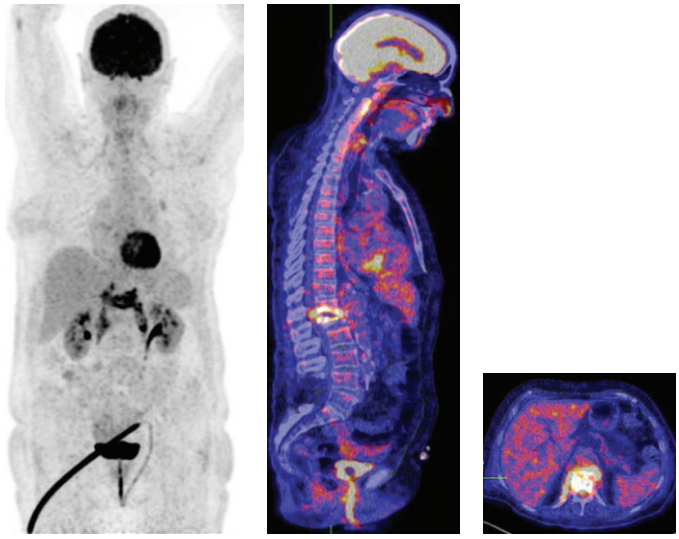
5. Osteomyelitis

The quick identification and precise localization of osteomyelitis (OM) is critical for early initiation of antimicrobial and/or surgical treatment and has a significant impact on patient outcome [78]. FDG-PET/CT has been evaluated in patients with primary OM extensively, has been shown to offer good sensitivity (>95%) and specificities above 87% [79], and was found superior to MRI [80] and other nuclear medicine imaging modalities [81]. However, used in combination with conventional methods, FDG-PET/CT may have limited additional value in the diagnosis of primary OM. In contrast, FDG-PET/CT may play an important role in patients with chronic OM, especially in those patients with previously documented OM and suspected recurrence, or presenting with symptoms of OM for more than six weeks (chronic OM) [3]. In children with suspected OM, dissemination in multiple bones has to be kept in mind, for which FDG-PET/CT would be suitable. However, to avoid radiation exposure, pediatricians tend to perform MRI rather than FDG-PET/CT in cases of suspected OM [82].

For therapy evaluation in patients with OM, the literature results are hopeful. In a recent retrospective study, FDG-PET/CT had a strong impact on the clinical management (initiation or prolongation of antibiotic therapy or recourse to surgical intervention) in 52% of patients with an infection [83]. Gemmel et al. highlighted the clinical role of FDG-PET/CT in the diagnosis of spinal infections, especially in patients with contraindications to MRI, and in evaluation of the postoperative spine [84]. Worth to mention is that in MRI inflammatory changes can be seen long after the disappearance of the infection. For this indication, FDG-PET is probably superior to MRI. In children, FDG-PET/CT was found superior in distinguishing between infection and reparative activity within the musculoskeletal system after treatment for acute OM, and termination of antibiotic treatment for children after acute OM seems justified when laboratory parameters and clinical parameters are normal, and FDG-PET/CT is unsuspicious [85].

Despite these results, major limitations exist for the use of FDG-PET/CT in some infectious bone diseases. In general, in patients with infections or inflammation, the bone marrow, at various levels, can show increased uptake. In prosthetic joint infections (PJI), the problem is the generation of artifacts, characterized by artificial FDG uptake adjacent to prostheses, because of the inherent problem of partial volume mapping and overcorrection. In PJI, white blood cell scintigraphy is still the first choice [86]. Further on, nonspecific FDG uptake may be seen in healing tissues, up to 6 months after surgical intervention [87]. In the diabetic foot, FDG-PET/CT was found to have a low diagnostic accuracy for OM and cannot replace white blood cell scintigraphy [88].

At this moment, evidence-based indications for FDG-PET/CT are primary peripheral bone OM (not postoperative, not in diabetic foot) and suspected spinal infection (spondylodiscitis or vertebral OM, not postoperative; see Figure 5) [89]. In the future, one can expect that FDG-PET/CT will also be used to monitor treatment efficacy, and



(a) (b) (c)

Figure 5: FDG-PET of a patient with spondylodiscitis and involvement of the psoas muscles. (a) Coronal MIP view, (b) fused PET/CT sagittal view, and (c) fused PET/CT transaxial view.

maybe this technique can provide criteria to decide when the treatment can be stopped safely.

6. Fungal Infections

Fungal infections can develop in patients who are taking antibiotics for a long time period (due to an altered balance of microorganisms in the body and an overgrowth of fungus) and in patients with a suppressed immune system, for example, HIV/AIDS, steroid treatment, and chemotherapy.

In daily practice, FDG-PET/CT has been used already for many years in various fungal infections, such as aspergillosis, candidiasis, histoplasmosis, coccidioidomycosis, cryptococcosis, and *Pneumocystis jiroveci* [90–96]. However, systemic investigations of use of FDG-PET/CT in patients with fungal infections are virtually absent. In the majority, these infections were found by coincidence in patients that were scanned for other reasons. The largest study in diagnosing fungal infections was published by Chamilos et al., who reported their own experiences in 13 patients, together with the results of nine case reports in the literature. Most patients had an underlying malignancy (73%), primarily of hematological origin, and seven were allogeneic hematopoietic stem cell transplant recipients. FDG-PET frequently found occult lesions that were not found with other imaging techniques, and the results helped to determine treatment length in eight of these 16 patients. Overall, FDG-PET was helpful in 60% of the patients [97].

The role of FDG-PET in monitoring therapeutic efficacy has also been described in the setting of invasive aspergillosis, chronic disseminated candidiasis, candidal lung abscess following antifungal therapy, and *Pneumocystis carinii* pneumonia, all with good results [98–100].

In our opinion, FDG-PET/CT offers a unique possibility in the monitoring of therapy efficacy in patients with fungal infections. Antifungal therapy is extensive and must be prolonged for a long time, sometimes even for months. FDG-PET/CT could help to decide whether therapy should be continued, stopped, or switched. An example is given in Figure 6.

7. Guidelines

Recently, combined guidelines from the European Association of Nuclear Medicine (EANM) and the Society of Nuclear Medicine and Molecular Imaging (SNMMI) were published for the use of FDG-PET in inflammation and infection [89]. Based on cumulated reported accuracies, >85% of these guidelines state that the major indications for the use of FDG-PET/CT in infection and inflammation are sarcoidosis, peripheral bone osteomyelitis, spondylodiscitis, evaluation of fever of unknown origin, and the primary evaluation of vasculitis. Other well-described applications, however, at this time-point without sufficient evidence-based indications, are the evaluation of potentially infected liver and kidney cysts, suspected infection of intravascular devices, AIDS-associated opportunistic infections, and the assessment of metabolic activity in tuberculosis lesions. For inflammatory bowel diseases, it is unclear if FDG-PET/CT offers advantages over other imaging techniques. The guidelines do not describe the role of FDG-PET/CT for the other described applications in this paper (rheumatoid arthritis, autoimmune thyroiditis, autoimmune pancreatitis, and fungal infections). To our opinion, however not stated in the literature yet, FDG-PET/CT could also have additional value in rheumatoid arthritis and fungal infections.

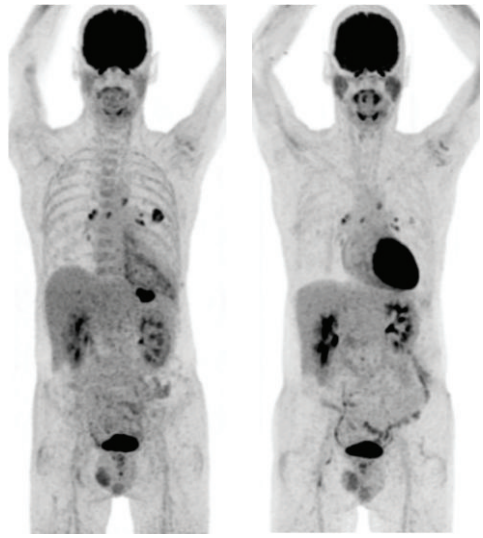
8. Comparison with Other Tracers

In this paper, we focused only on FDG-PET. To emphasize, one should keep in mind that FDG merely detects glucose metabolism and is therefore not able to discriminate

Table 1: Applications of FDG-PET for diagnosis and therapy evaluation in various inflammatory and infectious diseases.

Inflammatory/infectious disease	Evidence for clinical usefulness	Developing evidence for clinical usefulness	Need for multicentre studies	Class of recommendation level of evidence	Comments
Vasculitis					
Diagnosis	+			I-A	Differentiation possible between GCA, TA, and PAN*
Therapy evaluation	+			I-C	Best time point at 3 months
Sarcoidosis					
Diagnosis	+			I-A	High sensitivity, low specificity
Therapy evaluation	+			I-C	To evaluate steroid therapy; maybe also for other therapies
Rheumatoid arthritis					
Diagnosis		+	+	IIa-C	
Therapy evaluation		+	+	IIa-C	
Inflammatory bowel diseases					Especially in children
Diagnosis	+			I-C	
Therapy evaluation		+	+	IIa-C	
Autoimmune thyroiditis					
Diagnosis	−			III-B	
Therapy evaluation	−			III-B	
Autoimmune pancreatitis					
Diagnosis		+	+	IIa-C	Maybe possible to differentiate between AIP and pancreatic cancer
Therapy evaluation		+	+	IIb-C	
Osteomyelitis					
Diagnosis	+			I-A	Only in chronic osteomyelitis
Therapy evaluation		+	+	IIa-C	
Spondylodiscitis					
Diagnosis	+			I-A	
Therapy evaluation		+	+	IIa-C	
Prosthetic joint infections					
Diagnosis	−		+	IIb-C	White blood cell scintigraphy still the first choice
Therapy evaluation	−		+	IIb-C	
Diabetic foot					
Diagnosis	−		+	IIb-C	White blood cell scintigraphy still the first choice
Therapy evaluation	−		+	IIb-C	
Echinococcosis					
Diagnosis	+			I-C	
Therapy evaluation	+			I-C	
Fungal infections					
Diagnosis	+			I-C	
Therapy evaluation		+	+	IIa-C	Maybe helpful to reduce high costs of antifungal therapy

*GCA: giant cell arteritis; TA: Takayasu's arteritis; PAN: polyarteritis nodosa.



(a) (b)

Figure 6: FDG-PET of a neutropenic patient (due to leukemia) with a fungal infection (aspergillosis) before (left image) and during antifungal therapy (right image) resulting in a decrease in FDG uptake in the lung lesions. Because on the last FDG-PET scan (right image) there is still no complete remission, the patient is still treated with antifungal drugs.

between infection, inflammation, and neoplastic disease. Consequently, there is lack of specificity in some indications, especially in sarcoidosis. Many other, more specific tracers, targeting specific cells and molecules involved in a specific disease, are developed and investigated. These objective biomarkers are used for histological characterization of inflammatory lesions (specific receptor expression), for selection of patients for receptor-targeted therapy (overexpression of a specific receptor), and for therapy response prediction and followup (intensity of receptor expression and its modulation). Other articles are available that provide an overview of all the tracers used for inflammatory and infectious diseases [2].

9. Conclusions

This paper provides an overview of the use of FDG-PET/CT in various infectious and inflammatory diseases, not only in the setting of diagnosis but also in the evaluation of treatment efficacy (see Table 1). For vasculitis, sarcoidosis, and spondylodiscitis, scientific evidence indicates that FDG-PET/CT is useful for diagnosis and for therapy evaluation. For other diseases, such as inflammatory bowel diseases, rheumatoid arthritis, autoimmune pancreatitis, osteomyelitis, prosthetic joint infection, and fungal infections, a strong evidence is lacking. Only for autoimmune thyroiditis there is lack of evidence, and therefore, FDG-PET/CT should not be used in this setting.

In any case where FDG can be used for therapy followup, a pretherapy scan is of relevance to compare it with the posttherapy scan.

As it is a repeating problem in nuclear medicine, that no large patient studies are available in the literature to give final answers, we want to stress the importance to start prospective large multicenter studies to provide evidence-based answers. This is not only important in the era of our own imaging field but may also prove to be cost effective as anti-inflammatory therapies are in general expensive.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Determinants of Brachial-Ankle Pulse Wave Velocity in Chinese Patients with Rheumatoid Arthritis

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Objective. To investigate the relationship between Brachial-ankle pulse wave velocity (baPWV), and its associated risk factors in Chinese patients with RA. *Methods*. 138 Chinese RA patients and 150 healthy subjects were included. baPWV of all the participants was measured. RA related factors were determined, as well as traditional cardiovascular risk factors. *Results*. baPWV was significant higher in RA group (1705.44 ± 429.20 cm/s) compared to the healthy control group (1386.23 ± 411.09 cm/s) ($P < 0.001$). Compared with low baPWV group, high baPWV group patients were significantly older ($P = 0.008$) and taller ($P = 0.033$). Serum cholesterol ($P = 0.035$), triglycerides ($P = 0.004$), and LDL level ($P = 0.006$) were significantly higher in high baPWV group patients compared with low baPWV group patients. The baPWV of RA patients was positively correlated with age ($r = 0.439$, $P < 0.001$), and serum cholesterol level ($r = 0.231$, $P = 0.035$), serum triglycerides level ($r = 0.293$, $P < 0.001$), serum LDL level ($r = 0.323$, $P = 0.003$). Meanwhile, baPWV negatively correlated with the height of RA patients ($r = -0.253$, $P = 0.043$). Multivariate regression analysis showed that baPWV of RA group was independently associated with age and serum triglycerides level. *Conclusions*. The old age and high level of serum triglycerides may be the major determinants of arterial stiffness in Chinese RA patients.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory disease of progressive joint destruction. It is associated with increased cardiovascular morbidity and mortality, mostly due to an excess of cardiovascular disease [1–3]. Even after adjustment for traditional cardiovascular risk factors such as hypertension, diabetes mellitus, smoking, and hypercholesterolemia, there is a higher rate of atherosclerosis and cardiovascular events in patients with RA than in healthy subjects. Therefore, it is suggested that additional mechanisms are responsible for the excess cardiovascular risk observed in RA [4, 5].

Arterial stiffness has been shown to be an independent predictor of cardiovascular mortality and become a useful index in the prevention and early detection of cardiovascular disease [6, 7]. BaPWV which reflects the stiffness of both central and peripheral muscular arteries has been frequently used as a simple and noninvasive index for assessing arterial stiffness [8–10]. It is the useful tool for identifying a subgroup in the population that is at increased risk for cardiovascular events [11].

The relationship between RA-related factors, as well as traditional cardiovascular risk factors, and the arterial stiffness measured by baPWV has not previously been established in Chinese patients with RA. The aims of this study were to investigate the relationship between arterial stiffness and its associated risk factors in Chinese patients with RA.

2. Material and Methods

2.1. Patients. A total of 138 RA patients, with 111 of them being women, attending outpatient clinics at China-Japan Union Hospital of Jilin University, were enrolled in this study. All the patients met the American College of Rheumatology 1987 revised criteria for RA [12]. Patients with hypertension, diabetes, heart failure, renal failure (serum creatinine > 1.5 mg/dL), hepatic failure, heart valve disease, and previous

Table 1: Clinical characteristics of patients and controls.

Characteristic	RA (n = 138)	Controls (n = 150)	P
Age (years), mean ± SD	50.17 ± 12.49	49.61 ± 8.74	0.500
Female, n (%)	111 (80.43)	119 (79.33)	0.816
Weight (kg), mean ± SD	58.74 ± 10.26	59.11 ± 6.09	0.091
Height (cm), mean ± SD	161.77 ± 6.15	163.05 ± 9.12	0.253
BMI (kg/m^2), mean ± SD	22.41 ± 0.72	22.25 ± 0.09	0.549
SBP (mmHg), mean ± SD	116.57 ± 13.38	114.19 ± 12.58	0.137
DBP (mmHg), mean ± SD	75.59 ± 9.25	74.33 ± 8.26	0.382
Cholesterol (mmol/L), mean ± SD	4.82 ± 0.41	5.15 ± 0.79	0.097
Triglycerides (mmol/L), mean ± SD	1.96 ± 0.96	1.27 ± 0.96	0.025
HDL (mmol/L), mean ± SD	1.03 ± 0.27	1.05 ± 0.22	0.608
LDL (mmol/L), mean ± SD	2.78 ± 0.63	3.16 ± 0.71	0.079
Glucose (mmol/L), mean ± SD	5.46 ± 0.59	5.31 ± 0.57	0.774
baPWV (cm/s), mean ± SD	1705.44 ± 429.20	1386.23 ± 411.09	<0.001

Data are given as mean ± SD or number (and percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

Table 2: Disease-related factors in RA patients.

	All patients n = 138
Disease duration (months), mean (IQR)	24.00 (10.00–102.00)
RF-IgG positive, n (%)	80 (57.97)
RF-IgA positive, n (%)	65 (47.10)
RF-IgM positive, n (%)	108 (78.26)
Anti-CCP positive, n (%)	98 (71.01)
ESR (mm/h), mean ± SD	45.69 ± 21.26
CRP (mg/dL), mean (IQR)	14.90 (5.57–32.55)
DAS28, mean ± SD	5.08 ± 1.00
Currently using DMARDs, n (%)	9 (6.52)
Currently using NSAIDs, n (%)	74 (53.62)
Currently using corticosteroid, n (%)	0 (0)

Data are expressed as mean ± SD when normally distributed or as median (25–75%) when nonnormally distributed. IQR: interquartile range; RF: rheumatoid factor; anti-CCP: anticyclic citrullinated peptide; DAS28: disease activity score for 28 joint indices; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DMARDs: disease-modifying antirheumatic drugs; NSAIDs: nonsteroidal anti-inflammatory drugs.

myocardial infarction anamnesis and smokers were excluded from the study. 150 age- and sex-matched healthy volunteers were recruited as controls. The individuals of control group had no history of rheumatic disease. The study was approved by the ethics committee of China-Japan Union Hospital and is in accordance with the Helsinki Declaration.

Information was obtained from all subjects regarding traditional cardiovascular risk factors. Blood pressure (BP) and lipid level were measured. BP was obtained manually in supine position after at least 5 min of rest. The height and weight of the subjects were measured just before examining baPWV. The body mass index (BMI) was expressed as the weight in kilograms divided by squared height in meters (kg/m^2). Information on current medication use of patients and controls was obtained by questionnaire. The medical records of patients specially focused on disease-modifying antirheumatic drugs (DMARDs), nonsteroidal antiinflammatory drugs (NSAIDs), or corticosteroid usage for 6 months before the study.

2.2. Laboratory Analysis. Blood samples were collected after at least 8 hours of fasting. Serum glucose, lipid profile, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), anticyclic citrullinated peptide (anti-CCP), rheumatoid factor IgM (RF-IgM), RF-IgG, and RF-IgA were checked concurrently in study day.

2.3. Evaluation of RA Disease Activity. Disease activity was evaluated by the Disease Activity Score for 28 joints (DAS28). This utilizes the ESR, visual analogue score (VAS), and the number of tender and swollen joints, from a total of 28 joints assessed as in the following equation [13, 14]:

$$\begin{aligned}\text{DAS28} = {} & \sqrt{0.56 \times \text{number of tender joints}} \\ & + \sqrt{0.28 \times \text{number of swollen joints}} \\ & + [0.70 \times \ln(\text{ESR})] + (0.014 \times \text{VAS}).\end{aligned} \quad (1)$$

2.4. Measurement of baPWV. After the subjects had rested in a supine position for more than 5 min, the measurement of baPWV was conducted using a waveform analyzer (VP-2000, Colin Co Ltd, Komaki, Japan). Pulse waves were recorded automatically by sensors in the cuffs. The transmission times and distances between the cuffs of arms and legs were recorded, and the baPWV was outputted. The mean of the baPWVs in the left and right sides was used for the analysis. Patients were divided into two subgroups according to mean baPWV value of RA group: those above the mean baPWV value were in the high baPWV group, while those below the mean baPWV value were in the low baPWV group.

Table 3: Demographic and clinical characteristics of RA patients and comparisons between subgroups.

Variables	Low baPWV group ($n = 76$)	High baPWV group ($n = 62$)	P
Age (years), mean ± SD	46.67 ± 11.54	55.07 ± 11.86	0.008
Female, n (%)	60 (78.95)	51 (82.26)	0.626
Weight (kg), mean ± SD	59.25 ± 5.13	57.96 ± 9.55	0.325
Height (cm), mean ± SD	162.95 ± 6.26	160.69 ± 5.20	0.033
BMI (kg/m^2), mean ± SD	22.78 ± 2.96	22.27 ± 3.91	0.389
SBP (mmHg), mean ± SD	115.00 ± 13.74	117.04 ± 22.56	0.084
DBP (mmHg), mean ± SD	74.91 ± 9.55	76.07 ± 14.68	0.215
Cholesterol (mmol/L), mean ± SD	4.63 ± 0.79	5.13 ± 0.99	0.035
Triglycerides (mmol/L), mean ± SD	1.79 ± 0.75	2.28 ± 0.68	0.004
HDL (mmol/L), mean ± SD	1.05 ± 2.35	1.01 ± 0.29	0.538
LDL (mmol/L), mean ± SD	2.56 ± 0.65	3.09 ± 0.77	0.006
Glucose (mmol/L), mean ± SD	5.51 ± 0.56	5.34 ± 0.69	0.434
Disease duration (months), mean (IQR)	24 (8, 66)	48 (12, 132)	0.169
RF-IgG positive, n (%)	46 (60.53)	34 (54.84)	0.501
RF-IgA positive, n (%)	37 (48.68)	28 (45.16)	0.100
RF-IgM positive, n (%)	56 (73.68)	52 (83.87)	0.149
Anti-CCP positive, n (%)	55 (72.37)	43 (69.35)	0.698
ESR (mm/h), mean ± SD	42.56 ± 23.16	49.74 ± 17.11	0.172
CRP (mg/dL), mean (IQR)	13.90 (5.34, 28.55)	18.15 (6.24, 68.05)	0.447
DAS28, mean ± SD	4.87 ± 1.05	5.26 ± 0.75	0.113
Currently using DMARDs, n (%)	5 (6.58)	4 (6.45)	0.976
Currently using NSAIDs, n (%)	38 (50.00)	36 (58.06)	0.345

Data are given as mean ± SD when normally distributed, as median (25–75%) when nonnormally distributed, or as number (and percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; RF: rheumatoid factor; anti-CCP: anticyclic citrullinated peptide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS28: disease activity score for 28 joint indices; IQR: interquartile range; DMARDs: disease-modifying antirheumatic drugs; NSAIDs: nonsteroidal anti-inflammatory drugs.

2.5. Statistical Analysis. The statistical analysis was performed using the SPSS (SPSS ver 17.0 for Window, Chicago, IL, USA) software package. All data were expressed by mean ± SD for normal distributed values and median (interquartile range, IQR) for nonparametric values. Comparisons between the two groups were done by Student's t-test or χ^2 test. Nonparametric data were compared using Mann-Whitney U test. Univariate analysis was done to explore relationships between baPWV and other variables by Pearson correlation test for normally distributed data and Spearman Rank correlation test for non-parametric data. Stepwise multiple linear regression analysis was used to assess the independent determinants of increased baPWV. P level less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical Characteristics of Patients and Controls. Characteristics of patients and controls are given in Table 1. Among the traditional cardiovascular risk factors, only triglycerides levels were higher in patients with RA compared with controls ($P < 0.05$). RA patients had higher baPWV than controls ($P < 0.001$). The other factors had no difference between RA patients and controls.

3.2. Disease-Related Factors. The mean disease duration was 24 (10.00–102.00) months. 80 (57.97%) patients were RF-IgG positive, 65 (47.10%) were RF-IgA positive, 108 (78.26%) were RF-IgM positive, and 98 (71.01%) were anti-CCP positive. The mean DAS28 was (5.08 ± 1.00). It indicated that disease activity of the RA patients studied was moderate to high. At the time of measurement, 52.90% of patients were treated with NSAIDs and 6.52% with DMARDs. No patient was treated with corticosteroid (Table 2).

3.3. Patient Characteristics and Comparisons between Two Subgroups of RA Patients. Table 3 shows comparisons of the demographic and clinical characteristics between two subgroup patients divided according to the mean baPWV value of RA group. Compared with low baPWV group, high baPWV group patients were significantly older ($P = 0.008$) and taller ($P = 0.033$). Serum cholesterol ($P = 0.035$), triglycerides ($P = 0.004$), and LDL level ($P = 0.006$) were significantly higher in high baPWV group patients compared

Table 4: The correlations between baPWV and other variables.

Parameters	baPWV	
	r	P
Age (y)	0.439	<0.001
Disease duration (m)	0.159	0.224
Weight (kg)	0.029	0.831
Height (m)	−0.253	0.043
BMI (kg/m^2)	0.108	0.249
SBP (mmHg)	−0.007	0.958
DBP (mmHg)	−0.090	0.508
Cholesterol (mmol/L)	0.231	0.035
Triglycerides (mmol/L)	0.293	<0.001
HDL (mmol/L)	−0.139	0.293
LDL (mmol/L)	0.323	0.003
Glucose (mmol/L)	0.181	0.153
RF-IgG (IU/mL)	−0.066	0.495
RF-IgA (IU/mL)	−0.008	0.938
RF-IgM (IU/mL)	−0.089	0.341
Anti-CCP (RU/mL)	−0.064	0.486
DAS28	0.062	0.483
ESR (mm/h)	0.037	0.651
CRP (mg/L)	0.021	0.809

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; RF: rheumatoid factor; anti-CCP: anticyclic citrullinated peptide; DAS28: disease activity score for 28 joint indices; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

with low baPWV group patients. However, there were no significant differences in RA-related factors between two subgroups ($P > 0.05$).

3.4. Correlations between baPWV and Other Variables. Table 4 shows correlations between baPWV and clinical parameter in RA patients. The baPWV of RA patients was positively correlated with age ($r = 0.439$, $P < 0.001$), serum cholesterol level ($r = 0.231$, $P = 0.035$), serum triglycerides level ($r = 0.293$, $P < 0.001$), and serum LDL level ($r = 0.323$, $P = 0.003$). Meanwhile, baPWV negatively correlated with the height of RA patients ($r = -0.253$, $P = 0.043$). However, no significant correlation was found between baPWV and RA-related factors, such as DAS28, RF, and anti-CCP.

3.5. Multivariate Regression Analysis of Determinants of baPWV. In a stepwise multiple linear regression analysis, we employed baPWV value as dependent variable while using age, height, cholesterol, triglycerides, and LDL as independent variables. Table 5 shows the multivariate linear regression analysis results. Age and serum triglycerides level were independently associated with baPWV ($P < 0.001$, $P = 0.014$, resp.).

4. Discussion

Coronary artery disease is typically silent in RA, with a greater frequency of unrecognized myocardial infarction and sudden cardiac death [15, 16]. RA patients have up to 3 times increased standard mortality rates in comparison to the general population, and today cardiovascular diseases (CVD) are the leading cause of death [17–19]. Vascular disease may account for one-third of all deaths in RA [20]. Early detection of arterial stiffness is useful in primary and secondary prevention of series of major CVD [21]. Several studies have shown that PWV was an independent predictor of future development of CVD [22–24]. The baPWV measurement is noninvasive and convenient and has been used for cardiovascular risk stratification [25, 26]. For the first time, the present study investigated the baPWV level and sought to identify the RA-related and the traditional risk factors for CVD in Chinese patients with RA.

The mean baPWV of our RA patients was 1705.44 ± 429.20 cm/s. The value is significant higher than that of matched control (1386.23 ± 411.09 cm/s) ($P < 0.001$). Wang and coworkers measured baPWV in 2852 participants of North China and found that the mean baPWV of healthy sample is 1339.3±224.8 cm/s for male and 1294.8±241.9 cm/s for female [27]. This is similar to the result of our study. It also demonstrated that RA patients in our study had significant higher baPWV value than healthy controls.

In a univariate model, baPWV in RA patients did not show any significant correlation with RA-related factors. These factors included disease duration, RF-IgG, RF-IgA, RF-IgM, anti-CCP, DAS28, ESR, and CRP. The same results were obtained from the comparisons between two subgroups of RA patients. Kim and associates evaluated the baPWV in Korean patients with RA and also found that the baPWV value of RA patients did not correlate with RA-related factors [28]. Contrary to our findings, Mäki-Petäjä and coworkers found that PWV of RA patients correlated independently with log-transformed CRP ($P < 0.001$). The patients in the study of Mäki-Petäjä and coworkers had a mean age of 57 years, while our patients had a mean age of 50 years [29]. The baPWV is known to increase steeply after age of 50 years [30, 31]. It might be the major reason that caused the difference between the results of Mäki-Petäjä study and ours. Moreover, other factors might also result in the difference, such as the Mäki-Petäjä study that only enrolled 77 RA patients, which means the sample size of their study was smaller than ours; and the mean disease duration of their study was 13 years, which was much longer than ours (24 months); on the other hand, the race of participants was totally different between their study and ours.

In this study, the identified potential risk factors of arterial stiffness based on baPWV measurement were older age and higher serum triglycerides level. It is well known that in elder individuals, arteries are less elastic which induces higher velocity of pulse wave [32–34]. Several studies supported that the detection of increase of PWV is parallel to advanced age [35–38]. In the regression analysis of our study, age was found to be the most important independent variable. Ai and coworkers found that serum level of triglycerides

Table 5: Multiple linear regression analysis of factors associated with baPWV.

	B	SE	Standardized coefficient	t	P
Constant	914.17	215.83		4.23	<0.001
Age	15.82	4.17	0.449	3.80	<0.001
Triglycerides	26.78	16.82	0.236	1.86	0.014

was independent predictor of baPWV in Chinese population [39]. It was similar to our results. The patients' age, as one of the independent associated factors with baPWV, cannot be modified. So the attention should be placed on another modifiable risk factor-serum level of triglycerides. Keeping a normal level of triglycerides should be very important for Chinese RA patients avoiding CVD.

Several limitations of this study must be taken into consideration when interpreting the data. Firstly, the cross-sectional design of our study that resulted from the prognostic significance of baPWV could not be evaluated. Secondly, lack of cardiovascular prognosis factors was also a fact in this study. For better understanding of risk factors of arterial stiffness and cardiovascular risk in Chinese RA patients, longitudinal studies including factors of cardiovascular prognosis are necessary.

5. Conclusions

In summary, this is the first study describing the factors influencing baPWV in Chinese patients with RA. We have shown that RA is associated with increased arterial stiffness in comparison with healthy control subjects. The arterial stiffness, which was assessed by baPWV, had an independent correlation with age and serum triglycerides level. These are undoubtedly important for preventing CVD of Chinese patients with RA.

Authors' Contribution

Ping Li and Cheng-xun Han contributed equally to this paper.

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Clinical Study

Asymptomatic Preclinical Rheumatoid Arthritis-Associated Interstitial Lung Disease

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Objective. Interstitial lung disease (ILD) is a common extra-articular manifestation of rheumatoid arthritis (RA) and a significant cause of morbidity and mortality. The objective of this study was to define high-resolution chest CT (HRCT) and pulmonary function test (PFT) abnormalities capable of identifying asymptomatic, preclinical forms of RA-ILD that may represent precursors to more severe fibrotic lung disease. *Methods*. We analyzed chest HRCTs in consecutively enrolled RA patients and subsequently classified these individuals as RA-ILD or RA-no ILD based on the presence/absence of ground glass opacification, septal thickening, reticulation, traction bronchiectasis, and/or honeycombing. Coexisting PFT abnormalities (reductions in percent predicted FEV1, FVC, TLC, and/or DLCO) were also used to further characterize occult respiratory defects. *Results*. 61% (63/103) of RA patients were classified as RA-ILD based on HRCT and PFT abnormalities, while 39% (40/103) were designated as RA-no ILD. 57/63 RA-ILD patients lacked symptoms of significant dyspnea or cough at the time of HRCT and PFT assessment. Compared with RA-no ILD, RA-ILD patients were older and had longer disease duration, higher articular disease activity, and more significant PFT abnormalities. *Conclusion*. HRCT represents an effective tool to detect occult/asymptomatic ILD that is highly prevalent in our unselected, university-based cohort of RA patients.

1. Introduction

Interstitial lung disease (ILD) is a common extra-articular manifestation responsible for significant morbidity and mortality among patients with rheumatoid arthritis (RA) [1]. These findings parallel the relationship between ILD and other systemic autoimmune diseases such as systemic sclerosis, inflammatory myopathy (polymyositis and dermatomyositis), Sjogren's syndrome, and undifferentiated CTD [1–3].

A recently published population-based study showed that the hazard ratio for development of *clinically evident* ILD in RA (compared to individuals without RA) was 8.96 [4]. Moreover, the risk of death for RA patients with ILD was 3 times higher than in RA patients without ILD, and median survival after ILD diagnosis was only 2.6 years [4]. Given the morbidity/mortality associated with progressive/advanced RA-ILD, earlier diagnosis and aggressive management are critical.

While the majority of cases of RA-ILD occur in patients between the ages of 50 and 60 years, smoking, male gender, and longstanding RA represent additional risk factors for the development of ILD [2]. Patients with established RA-ILD most often present with chronic symptoms of dyspnea and cough. Physical examination may reveal inspiratory crackles, and pulmonary function tests (PFTs) typically demonstrate restrictive physiology marked by a reduced diffusing capacity [2, 5]. Imaging abnormalities are variable, but often demonstrate traction bronchiectasis and/or honeycombing suggestive of usual interstitial pneumonia (UIP) [1].

Based on high-resolution computed tomography (HRCT) scanning, the estimated prevalence of RA-associated ILD (RA-ILD) among patients with RA varies widely from 4 to 68%-depending on patient selection and severity of

pulmonary symptoms [2]. With plain chest radiography, the frequency of ILD detection is only 5%. In a minority of cases, surgical lung biopsy may ultimately be required to establish the diagnosis and histopathological subtype [4, 6]. Because asymptomatic, preclinical ILD demonstrable by HRCT can be progressive and may be more prevalent than expected among patients having RA [7], the objective of this study was to characterize and define the frequency of HRCT and PFT abnormalities capable of identifying "subclinical" RA-ILD.

2. Material and Methods

2.1. Study Population. Patients with RA were consecutively recruited through the Rheumatology Department at The First Hospital of Xiamen University, China, between July 1, 2012 and March 1, 2013. Enrolment into these cohorts was approved by the ethics committee of the First Hospital of Xiamen University and included permission to review all medical records. Demographics, clinical features, medication history, comorbidities, and the disease activity score calculated for 28 joints (DAS28) were carefully recorded. The swollen or tender joint count (0–28) component of the DAS28 included shoulder joints (2), elbow joints (2), wrist joints (2), metacarpophalangeal joints (10), proximal interphalangeal joint (10), and knee joints (2). HRCTs and PFTs were obtained in 100% and 80.6% of study eligible patients, respectively.

2.2. Inclusion Criteria. All enrolled patients were older than 18 years of age and satisfied ACR criteria for definite RA [8]. Eligible patients were further classified into categories of RA-ILD and RA-no ILD based on the following criteria:

(1) RA-ILD-HRCT abnormalities consisting of ground glass opacities, septal lines, reticulation, subpleural fibrosis, traction bronchiectasis, architectural distortion, and/or honeycombing. These radiographic abnormalities could occur with/without clinical symptoms of dyspnea and cough or significant pulmonary function test (PFTs) abnormalities defined as <80% predicted values for the following parameters: forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), total lung capacity (TLC), and diffusion capacity of carbon monoxide (DLCO),

(2) RA-no ILD—absence of significant HRCT or PFT abnormalities as defined earlier.

2.3. Exclusion Criteria. Individuals with active infection, HIV/AIDS, malignancy, or posttransplant immunosuppression were excluded from enrollment due to potential confounding effects on data analysis.

2.4. High-Resolution Computed Tomography. High-resolution computed tomography (HRCT) of the chest without intravenous contrast medium was performed during end inspiration with the patient in a supine position using 1-2 mm collimation at 1-2 mm intervals (Toshiba Medical Systems, Aquilion 16 (Japan)). Images were read independently by 2 blinded pulmonary and radiology specialists (YongHong Shi and XiaoPing Wang), with a focus on the previously outlined parenchymal lung abnormalities. Discrepant readings were rereviewed to determine consensus findings. Using a modification of a previously described quantitative scale [7], reviewers assigned HRCT scores according to the following criteria: 0 (normal), 1 (minimal disease (i.e., 3-4 septal lines)), 2 (mild disease (>5 septal lines, reticulations, subpleural cysts, or ground glass opacities)), 3 (moderate disease (i.e., grade 2 findings as well as traction bronchiectasis, peribronchovascular thickening, or tracheal retraction with one-third to two-thirds lung involvement)), or 4 (severe disease (i.e., grade 2 or 3 findings with more than two-thirds lung involvement)).

2.5. Pulmonary Function Test (PFTs). Pulmonary function was assessed according to American Thoracic Society recommendations [9] and expressed as percent predicted FEV1, FVC, TLC, and DLCO.

2.6. Statistical Analyses. Statistical analyses were performed using SPSS software. All descriptive data were expressed as mean values ± SD. While Student's t-test was used to compare normally distributed quantitative data, chi-squared testing with Yates correction was used to compare frequencies. In all analyses, a two-tailed P value less than 0.05 was considered statistically significant.

3. Results

The clinical data of 103 consecutively enrolled RA patients (76 women and 27 men) are shown in Table 1. For the overall cohort, mean age was 49.1 ± 14.7 years (range 19–81), and the mean disease duration was 4.3 ± 5.7 years. The mean articular disease activity score (DAS28) was 4.4 ± 1.4.

61% (63/103) of RA patients were diagnosed with RA-ILD by HRCT and PFTs, while 39% (40/103) did not meet criteria for ILD and were therefore designated as RA-no ILD. 57/63 RA-ILD patients (90% of RA-ILD subset) lacked symptoms of significant dyspnea or cough at the time of HRCT and PFTs assessment, whereas 6 patients (10% of RA-ILD subset) manifested these clinical features. Compared with patients in the RA-no ILD subgroup, RA-ILD patients were older (53.0 ± 14.8 versus 42.9 ± 12.4 years, $P < 0.001$) and had longer disease duration (5.0 ± 6.7 versus 3.1 ± 3.0 years, $P < 0.05$), greater articular disease activity (DAS 28 scores: 4.9 ± 1.2 versus 3.7 ± 1.3, $P < 0.001$), and more severe respiratory defects (lower percent predicted FVC, TLC, FEV1, and DLCO; all with $P < 0.001$) (Tables 1 and 2). As shown in Table 1, there were no statistically significant differences in the levels of RF or anti-CCP antibodies between the two groups.

Evaluation of chest HRCT scores (Table 3) revealed that the percentage of individuals among the overall RA cohort with radiographic abnormalities was 78.4% (81/103). The main HRCT abnormality was ground glass opacification suggestive of nonspecific interstitial pneumonia (NSIP), a defect that was observed in 57.3% (59/103) of RA patients. Of note, ground glass opacification was detected far more

Table 1: Comparison of clinical characteristics in RA-ILD and RA-no ILD.

	All	RA-ILD	RA-no ILD	*P* value
Total number	103	63	40	
Gender (F/M)	76/27	46/17	30/10	0.823
Mean age (year)	49.1 ± 14.7	53.0 ± 14.8	42.9 ± 12.4	<0.001*
Mean disease duration (years)	4.3 ± 5.7	5.0 ± 6.7	3.1 ± 3.0	0.049*
DAS28	4.4 ± 1.4	4.9 ± 1.2	3.7 ± 1.3	<0.001*
RF	402.0 ± 1268.2	555.8 ± 1599.1	159.8 ± 206.7	0.057
Anti-CCP	218.2 ± 171.7	231.8 ± 178.0	196.8 ± 161.1	0.316

*$P < 0.05$.

Table 2: Pulmonary function tests in RA-ILD and RA-no ILD.

% of predicted	RA-ILD ($n = 47$)	RA-no ILD ($n = 36$)	*P* value
FEV1	74.1 ± 14.6	88.0 ± 12.9	<0.001
FVC	74.9 ± 12.2	86.9 ± 11.3	<0.001
TLC	87.7 ± 15.7	98.4 ± 11.3	0.001
DLCO	68.1 ± 19.5	96.2 ± 17.7	<0.001

Table 3: Lung HRCT scores of RA-ILD and RA-no ILD.

HRCT findings	RA-ILD ($n = 63$)	RA-no ILD ($n = 40$)	*P* value
Ground glass	52	7	<0.001*
Airspace	6	1	NS
Mixed AS/GG	4	0	NS
Honeycombing	4	0	NS
Architectural distortion	20	2	NS
Traction bronchiectasis	11	0	NS

*$P < 0.05$.

frequently in RA-ILD relative to RA-no ILD patients (83% versus 18%, $P < 0.001$). Ten patients manifested mild ground glass opacification in single lung lobe (7/40), airspace abnormalities (1/40), or architectural distortion (2/40). They were identified as RA-no ILD based on associated comorbidities. By comparison, 0/24 age- and sex-matched healthy volunteers had HRCT evidence of ground glass opacification, septal thickening, traction bronchiectasis, or honeycombing.

In this study, all RA patients were treated with combinations of DMARDs that included methotrexate (MTX), with a dose ranging from 10 to 15 mg per week; however, only 7.8% of RA patients (8/103) used biologics or TNF-α blocking agents. In terms of additional environmental risk factors, the prevalence of smoking was very low in both the RA-ILD and RA-no ILD subsets (1/63 and 1/40, resp.), with only one individual in each subgroup having smoking exposure greater than 25 pack-years [10]. Overall, the lack of differences in treatment regimen and the relatively low frequency of smoking in the overall cohort collectively precluded meaningful statistical correlation of these variables with the presence/absence of ILD.

4. Discussion

Asymptomatic/preclinical RA-ILD, which is detectable by HRCT and pulmonary function tests, was observed in 55% of RA patients in our study. The overall prevalence of radiographically defined RA-ILD in this unselected cohort of Chinese RA patients was quite high (61%), though the frequency of *clinically evident* ILD (6%) was consistent with the existing literature [4]. Based on comparative analysis of clinical and demographic variables, older age, longstanding disease, and higher disease activity represent risk factors for the development of RA- ILD [4].

Previous studies have shown that asymptomatic/preclinical RA-ILD is relatively prevalent and often progressive, as evidenced by the analysis of Gochuico et al. showing worsening HRCT scores over a 2-year period in 12/21 individuals with subclinical ILD [7]. Further demonstrating the relatively high prevalence of subclinical RA-ILD, another study reported HRCT evidence of pulmonary disease in 50% of RA patients, with only 10% of these patients manifesting clinical symptoms of lung involvement [11].

In separate analyses, Kim et al. highlighted the wide range of RA-ILD prevalence reported in the literature (as low as 4% and as high as 68%) [2]. To better address this issue and the implications for disease progression/survival, he and his research group defined the HRCT characteristics suggestive of usual interstitial pneumonia (UIP) (basilar predominant reticulation, traction bronchiectasis, and honeycombing, with limited ground glass abnormality) versus NSIP (predominant bibasilar ground glass attenuation with limited to no reticulation and absence of honeycombing) [1], showing a much worse survival in those with radiographic findings indicative of UIP (hazard ratio of 2.3) [1, 4].

Using somewhat different criteria, Biederer et al. demonstrated that patients with RA-ILD and a predominantly ground glass pattern seen on HRCT scans (likely indicative of NSIP) had a shorter mean duration of RA than those patients with a reticular pattern (63 ± 38 months versus 133 ± 112 months, resp.; $P < 0.01$) [12]. In our study where the main HRCT abnormality was ground glass opacification resembling a NSIP pattern, the mean disease duration of 4.3 ± 5.7 years was similar to that reported by Biederer et

al. but shorter than that reported by Zou et al. in a cohort of Chinese RA patients (8.25 ± 9 years) [13]. The latter discrepancy may reflect the wider range of histopathological subtypes included in the analysis of Zou et al. [13], as UIP typically develops in more longstanding disease [4, 12].

What remains unknown is the natural history of subclinical RA-ILD with underlying NSIP histopathology and whether this subtype represents a precursor to end-stage fibrotic lung disease. By our study, however, HRCT is a sensitive measurement to identify asymptomatic preclinical ILD that could reflect pathologic patterns more amenable to immunomodulatory therapy [1, 2, 4, 6]. Therefore, obtaining chest HRCTs early in disease (even in the absence of clinical symptoms) to select patients for more aggressive pharmacologic therapy may represent an effective strategy for halting progression to more fibrotic stages of lung disease (such as UIP) that carry a dismal prognosis.

In our analysis, pulmonary function test abnormalities served as another sensitive indicator for preclinical RA-ILD, paralleling findings of other studies where pulmonary function parameters (lower FEV1, FVC, TLC, and DLCO) in RA-ILD patients correlated with HRCT abnormalities [4]. Of note, Hamblin and Horton found that a reduced DLCO of less than 54% of the predicted value appears to be an independent predictor of ILD progression in RA [10] though the applicability of this finding to subclinical RA-ILD (where impairment of DLCO is generally less severe) remains unclear.

Based on our data, we are unable to conclude whether smoking, MTX, or other unknown medication/environmental exposures increase the risk for development of RA-ILD. Answering these questions will require larger, longitudinal studies incorporating more RA patients. Complementary strategies involving the assessment of serum protein biomarkers should further elucidate disease pathogenesis and provide additional insight regarding the relationship between subclinical RA-ILD and end-stage, fibrotic lung disease that portends a devastating outcome.

The prevalence of asymptomatic, subclinical RA-ILD defined by HRCT and PFT abnormalities was 55% in our cohort of RA patients. The predominant HRCT abnormality among this subset of RA-ILD patients was ground glass opacification most suggestive of NSIP. Older age, longstanding disease, and higher articular disease activity strongly correlated with the development of RA-ILD. Overall, chest HRCT represents a highly sensitive technique for detection of RA-ILD, even in the absence of clinical symptoms, making this procedure an effective screening tool for early ILD that may allow institution of more aggressive therapy geared towards the prevention of end-stage, fibrotic lung disease.

Conflict of Interests

The authors have stated no conflict of interests.

Authors' Contribution

Juan Chen contributed to recruitment and analysis of the validation cohort, data analysis and study design, data analysis, and paper preparation. XiaoPing Wang contributed to data analysis. YongHong Shi contributed to project conceptualization and data analysis. Heqing Huang contributed to processing samples and statistics.

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Review Article

Obstetrical Antiphospholipid Syndrome: From the Pathogenesis to the Clinical and Therapeutic Implications

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Antiphospholipid syndrome (APS) is an acquired thrombophilia with clinical manifestations associated with the presence of antiphospholipid antibodies (aPL) in patient plasma. Obstetrical APS is a complex entity that may affect both mother and fetus throughout the entire pregnancy with high morbidity. Clinical complications are as various as recurrent fetal losses, stillbirth, intrauterine growth restriction (IUGR), and preeclampsia. Pathogenesis of aPL targets trophoblastic cells directly, mainly via proapoptotic, proinflammatory mechanisms, and uncontrolled immunomodulatory responses. Actual first-line treatment is limited to low-dose aspirin (LDA) and low-molecular weight heparin (LMWH) and still failed in 30% of the cases. APS pregnancies should be a major field in obstetrical research, and new therapeutics are still in progress.

1. Introduction

APS is an autoimmune disorder characterized by a high-risk of obstetrical complications affecting both mother and fetus [1, 2]. This condition can either be purely thrombotic, which will not be treated here, or obstetrical or it can combine both aspects of the syndrome. Clinical criteria of obstetrical APS have been revisited in Sydney in 2006 (Table 1) and include a history of three early miscarriages (<10 WG), and/or one stillbirth (>10 WG), and/or one intra-uterine growth restriction or a premature birth before 34 WG due to preeclampsia or eclampsia or placental insufficiency [3]. Furthermore, APS pregnant women have an increased risk of thrombosis [4], thrombocytopenia, and HELLP syndrome [5].

APS can be found as a single disease and is referred as "primary." Secondary APS is associated with other autoimmune diseases, mainly systemic lupus erymathosus (SLE). Women are more commonly affected by APS than men, in primary (3,5 : 1 ratio) as well as in secondary APS (7 : 1) [6]. The prevalence of aPL is estimated to be 5% of the general population, and APS represents 0.5% [6, 7]. However, aPL is commonly found in 15% of women with recurrent pregnancy losses (RPLs), suggesting that APS is one of the most frequent acquired etiology for RPL [8].

aPL is a heterogeneous family of three autoantibodies including lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and antiβ2glycoprotein-1 antibodies (antiβ2GP1 Abs). As β2GP1 seems the main antigen for aPL, antiβ2GP1 Abs are now considered amongst the principal antibodies of the syndrome [9, 10].

During pregnancy, antiβ2GP1 Abs affect trophoblastic cells directly by binding to β2GP1 at the surface of trophoblastic cells [11].

aPL have been incriminated in alteration of trophoblastic cells *via* different mechanisms. Pathogenesis of aPL in pregnancy include thrombotic mechanisms, inflammation, apoptosis and immunomodulatory molecules impairments in trophoblast [12].

Moreover, damages of other cell types such as endometrial cells by aPL during pregnancy have also been involved [13, 14].

Nowadays, pathogenic mechanisms still remain unclear. A better understanding of cellular interactions with aPL is necessary. Because first-line treatments with LDA and

Table 1: Criteria of obstetrical APS [3]. APS is diagnosed when at least one of the following clinical criteria and one of the following laboratory criteria are met.

Clinical criteria	Biological criteria
(i) 3 or more consecutive spontaneous abortions before the 10th WG*, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded	(i) Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis
(ii) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th WG*, with normal fetal morphology documented by ultrasound or by direct examination of the fetus	(ii) Anticardiolipin (aCL) antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer, on two or more occasions, at least 12 weeks apart, measured by standardized ELISA
(iii) One or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia or severe preeclampsia or recognized features of placental insufficiency**	(iii) Antiβ2glycoprotein-1 antibody of IgG and/or IgM isotype in serum or plasma (in titer >99th percentile), present on two or more occasions, at least 12 weeks apart, measured by standardized ELISA

*WG: week of gestation.
**Placental insufficiency features include abnormal or nonreassuring fetal surveillance test, abnormal Doppler flow velocimetry waveform analysis suggestive of fetal hypoxemia, oligohydramnios, and postnatal birth weight less than the 10th percentile for the gestational age.

LMWH fail in about 30% of the cases, new specific therapeutics are in development [15]. The use of other medications is a matter of debate. Thus, hydroxychloroquine (HCQ), an old antimalarial drug used in SLE, has been shown to reduce antiphospholipid titers in the plasma of patients with persistent aPL [16] and to improve fetal outcomes in SLE-treated pregnant patients [17].

In this review of the literature, we discuss the clinical aspects of obstetrical APS on both mother and fetus sides, its pathogenesis, and current treatments as well as future treatment opportunities. In addition to another recent review on the same subject [18], we insist on new clinical and biological aspects of obstetrical APS. Infertility and infant development consequences are detailed as well as the potential impact of antibodies against domain I of β2GP1 on pregnancy. Moreover, special attention for catastrophic APS (CAPS) management is also given.

2. Obstetrical Manifestations of APS

In a European cohort of 1000 patients including 82% of APS women [19], Cervera et al. described the main clinical manifestations related to this syndrome during a 5-year follow-up (from 1999 to 2004). Obstetrical manifestations were very frequent; among them, prematurity and early pregnancy loss (as defined in Sydney's criteria) were the main clinical features (28% and 18%, resp.).

Mean age of disease onset varies between studies (30–40 years), but women of childbearing age are mostly represented.

In APS, pregnancy manifestations, severity of these complications, and maternofetal outcomes vary with aPL. Ruffatti et al. have shown that high titers and triple positivity for aPL were associated with both mother and fetal complications, even when treatment was well conducted [20, 21].

Here, we detailed more specifically the clinical aspects of APS, enlightening its implications on fertility, pregnancy, and fetal development.

2.1. On the Mother's Side. Pathologies linked to APS during pregnancy include recurrent thrombotic events (RTEs) as well as specific obstetrical pathologies. The coexistence of both thrombosis and miscarriage is estimated at 2.5–5% of APS pregnancies [19].

RTEs are major problems during pregnancy because of the management they implicate and the risk of complications, such as pulmonary embolism (PE). However, thrombotic events are usually low under adequate medications in APS patients with ongoing pregnancy. Interestingly, the Nimes Obstetricians and Hematologists Antiphospholipid Syndrome (NOH-APS) observational study compared the incidence of thrombotic events in 517 women with purely obstetrical APS to 796 seronegative women with a history of pregnancy loss. The annual rate of thrombotic complications, defined by deep-venous thrombosis (DVT, 1.46%), PE (0.43%), superficial vein thrombosis (0.44%), and cerebrovascular events (transient ischemic attack and stroke, 0.32%), was found to be higher in obstetrical APS women than in control patients (resp., 0.43%, 0.12%, 0.14%, and 0.09%) [4].

Furthermore, in more than 20% of cases, APS in pregnancy may present with minor symptoms such as thrombocytopenia or livedo reticularis [19]. Low platelet counts (<100 G/L) can be difficult to deal with, especially under LMWH treatment. Therefore, special attention and close follow-up should be considered. Livedo reticularis is an affection of the skin with persistent, not reversible with rewarming, violaceous, red or blue, reticular or mottled, pattern of the skin of trunk, arms or legs [6]. This condition could be explained by a decrease in blood flow in dermic venules, partly due to microthrombosis and inflammation of vessel wall.

More specific obstetrical manifestations include severe preeclampsia, which is defined in Table 2. Preeclampsia generally affects 2–8% of pregnancy [1]. A cross-sectional study conducted in Florida on 141 286 women who delivered in 2001 showed that women with high aPL titers ($n = 88$) had an increased risk of preeclampsia or eclampsia (adjusted odds ratio or AOR 2.93), placenta insufficiency (AOR 4.58), and prolonged length of stay at hospital (>three days, AOR 3.93) [22].

Table 2: Preeclampsia criteria.

Preeclampsia	(i) High blood pressure (>140/90 mmHg) associated with proteinuria (300 mg in a 24-hour urine sample) after 20 WG or (ii) increase in SBP ≥30 mmHg or in DBP ≥15 mmHg after 20 WG, with edema and/or proteinuria
Severe preeclampsia	(i) Presence of preeclampsia as described above and at least one of the following criteria (ii) SBP ≥160 mmHg, or DBP ≥110 mmHg on two occasions at least 6 hours apart (iii) Proteinuria ≥5 g in a 24-hour urine sample collected at least 4 hours apart (iv) Pulmonary edema or cyanosis (v) Oliguria (<400 mL in 24 hours) (vi) Persistent headaches (vii) Epigastric pain and/or impaired liver function (viii) Thrombocytopenia (ix) Oligohydramnios, decreased fetal growth, or placental abruption

Complications of preeclampsia include various rarer conditions such as eclampsia and hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome. Incidence of HELLP syndrome in APS patients is difficult to determine; however it seems more severe and occurs earlier in pregnancy than in patients not affected by APS [1, 5].

Finally, mothers can also be affected by catastrophic APS (CAPS). CAPS represents 1% of APS and can occur outside of pregnancy. CAPS is defined as a "thrombotic storm" secondary to microangiopathic diffuse thrombosis leading to multiorgan failure. 6% of CAPS seems to be associated with pregnancy and postpartum, but this is probably underestimated [23].

CAPS differential diagnosis can be difficult and large during pregnancy, including HELLP syndrome, thrombocytopenic thrombotic purpura (TTP), and disseminated intravascular coagulation (DIC). Since both mother and fetal outcomes are engaged, early diagnosis and management of CAPS are crucial. CAPS is indeed fatal in about 50% of cases even once aggressive therapy is started [1, 23].

2.2. On the Fetus' Side. aPL is responsible for fetal development and growth impairments and can affect any stages of pregnancy.

In the general population, miscarriages affect about 1 to 4-5 pregnancies; however, recurrent pregnancy losses (RPLs) represent only 1% of pregnancy. Although fetal chromosomal abnormalities are the main cause of this condition, aPL is found in 15% of recurrent fetal losses, implicating that APS is one of the main acquired cause for recurrent miscarriages [8].

Stillbirth is a really rare condition in pregnancy in industrialized countries. However, in the "Euro-Phospholipid" project on 1000 patients, it affects up to 7% of APS pregnancies [19]. In the same study, IUGR due to placental insufficiency affected 11% of pregnancies and prematurity was found in 28% of pregnancies.

2.3. Other Manifestations

2.3.1. Implantation Studies. Infertility and APS have been a controversial matter of study through the past years. The incidence of aPL in women with unexplained infertility and *in vitro* fertilization (IVF) failure seems significantly increased compared to control patients [24]. However, because of poorly designed studies, there is still a lack in evidence of aPL prediction on implantation or IVF outcome [25, 26]. Moreover, no study has clearly shown whether aPL could be associated with infertility so far, and precaution should be taken while interpreting positive aPL test results [24, 25].

2.3.2. On the Infant's Side. In a prospective European multicenter registry, 134 babies born from mothers affected by APS have been followed up for 5 years (2005–2010); both clinical and biological parameters were analyzed [27].

If no child presented thrombotic episodes, 3% of them (4/134) had neuropsychological development disorders, among which one autism was diagnosed. The conclusion of the study was that these development disorders were more common in these children and that specific and close follow-up should be given.

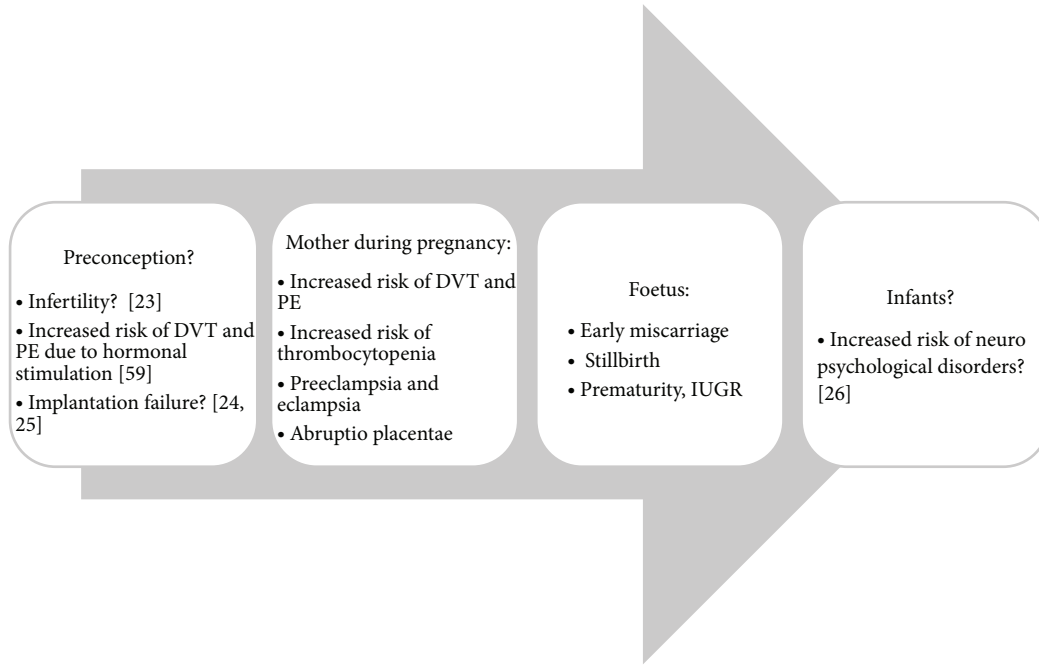
These results should be interpreted with great caution. Because of the difficulty of diagnosis and the frequent changes in the current definition, the general population prevalence in autism is only estimated at around 1% of children [28], suggesting that the association between APS mothers and autistic children is hard to believe.

Moreover, the presence of aPL in these children is estimated at 20%, with no association with any specific clinical manifestation of APS or SLE. Long-term consequences should be evaluated prior to give further conclusion [27]. Figure 1 summarizes the different clinical manifestations of obstetrical APS described above.

3. Pathogenesis of aPL during Pregnancy

In APS, aPL binds to endothelial cells, platelets, and monocytes, inducing a proinflammatory and prothrombotic state responsible for thrombotic complications [29]. During pregnancy, aPL targets the placenta, especially the cytotrophoblastic cells (CT). Initially, the CT differentiates into two cell types. On one hand, the villous trophoblast will fuse to form the syncytiotrophoblast (ST), a barrier of protection between the mother and the fetus. On the other hand, the extravillous trophoblast (EVT) will progressively invade and colonize the maternal endometrium [30].

aPL's main antigen is β2GP1, a cationic protein that is normally in a "closed conformation" when free in the plasma of patients. It is composed of five homologous domains of approximately 60 amino acids each. Domains I and V are the two domains positively charged [31, 32]. During



DVT: deep venous thrombosis; PE: pulmonary embolism; IUGR: intrauterine growth restriction

Figure 1: Obstetrical APS pathologies: more than a single disease.

normal pregnancy and ST formation, anionic phospholipids are externalized at trophoblastic cell surface, leading to the binding of β2GP1 via domain V. This binding offers a potential site of actions for aPL by changing the conformation of the protein from a circular to an open form and exposing domains I to IV to the surface [9, 10, 31, 32]. In 2009, an international multicenter study tested 477 antiβ2GP1 antibody positive plasma samples for antibodies specific for domain I of β2GP1. It showed a stronger association of these specific antibodies with obstetrical morbidity compared to total antiβ2GP1 IgG antibodies (odd ratio 2.4; [1.4–2.5], 95% confidence interval). However, further studies need to be performed to add this test to obstetrical APS criteria [33].

Pathogenesis of aPL on trophoblastic cells is a matter of debate and several hypotheses have been succeeding through time.

It has first been hypothesized that, as a parallel to the "thrombotic APS," obstetrical APS was mainly linked to thrombosis. As proof, histological analysis of placenta collected from spontaneous abortions ($N = 15$), fetal deaths ($N = 13$), and live births ($N = 16$) from APS patients was found to have more thrombotic characteristics as compared to control placenta. However, these findings were not specific for APS, as placenta collected from women with clinical characteristics of APS but without aPL has the same histological findings [34].

Moreover, inflammation, including fibrin deposits, was more represented than thrombosis in histological analysis of placenta of APS women, suggesting another mechanism in pregnancies affected by APS [35].

Studies were then more focused on inflammation processes by aPL on trophoblastic cells and this role was confirmed by *in vitro and in vivo* studies [36–40]. Initiation of complement cascade by aPL and increase in C4 deposition in placenta of mice treated with aPL were strongly linked to adverse fetal outcome [36–39]. Moreover, both C4 and C5 deficient mice were protected from fetal injury when treated with aPL IgG [40].

More recently, immunomodulation has shown to play a critical role in APS. Implications of Toll-like receptors (TLRs) in autoimmune diseases offered a new perspective for the understanding of APS. TLR is a family of 10 different receptors identified in humans and is responsible for the innate immune response. They recognize specific sequences conserved in pathogens; and the main ones are considered to be TLRs 2 and 4 [41]. In thrombotic APS model, TLR 2 and TLR 4 have both been implicated in the pathological activation of endothelial cells, monocytes, and platelets [42–48]. More recently, aPL has been shown to induce both translocations of TLR 7 and TLR 8 in the endosomes of human monocytes, sensitizing both receptors to their specific ligands [49]. In obstetrical APS, TLR 4 has been implicated in the pathological activation of HTR-8 cell line, an EVT cell line, by aPL, leading to an uncontrolled inflammation and apoptosis [50].

Immunomodulation by TLR offered a new insight on how aPL triggered placental alteration. Thus, it has been shown that aPL could mediate a nonthrombotic noninflammatory trophoblast modulation, by altering directly their own properties. Trophoblastic properties implicate three different

mechanisms, defined as (a) migration, (b) invasion, and (c) differentiation [51–53].

First, Mulla et al. showed migration alteration of first trimester trophoblastic cells by monoclonal antiβ2GP1 antibodies by decreasing IL-6 secretion and signal transducer activator of transcription 3 (STAT3) protein expression [54].

Invasion and proliferation impairments by aPL have also been studied *in vitro*. aPL has been shown to prevent HTR-8, a trophoblastic cell line, from invading on matrigel assay and to decrease integrins proteic expressions [55].

Finally, it has previously been described that antiphosphatidylserine antibodies, a type of aPL that is not part of the definition of APS, were responsible for syncytiotrophoblast fusion impairments [56]. A decrease in β-human choriogonadotropin (hCG) secretion, a hormone normally produced by ST, has also been described in term placenta incubated with high doses of anti β2GP1 antibodies [57]. In BeWo cell, a choriocarcinoma cell line, we recently showed that antiβ2GP1 Abs significantly decrease cell differentiation in a dose-dependent way and that this effect was reversed by decreasing TLR 4 membranous expression (manuscript under submission).

Trophoblastic cells seem not the only cell type affected by aPL. Impaired endometrial differentiation in decidual phenotype as well as endometrial angiogenesis inhibition by aPL has also been advocated [58]. Laboratory findings on endometrial cells were different from those found on other cell types. Antiβ2GP1 antibodies purified from APS patients were found to inhibit angiogenesis, VEGF secretion, and NFκB activation in a dose-dependent way in endometrial cells [13, 14]. This implicates that pathological mechanisms of aPL can differ between various cell types which could explain variations in treatment efficiency.

Pathogenesis of Antiphospholipid Antibodies in Pregnancy.

(1) Mechanisms on placental cell

- (i) Thrombosis
  - (a) Aspecific mechanism [30]
- (ii) Inflammation
  - (a) Complement activation [31, 33–36]
- (iii) Immunomodulations
  - (a) TLR 4 activation by aPL [42]
- (iv) Defective placentation
  - (a) Migration: decrease in IL-6 and STAT3 expression [50]
  - (b) Invasion: decrease in integrin expression [51]
  - (c) Differentiation: decrease in β-hCG secretion [52] and decrease in fusion [53]

(2) Mechanisms on endometrial cells [13, 14]

- (i) Angiogenesis inhibition
- (ii) Decrease in VEGF secretion
- (iii) NFκB activation inhibition.

4. Treatments and Future Perspectives

APS pregnancies are real challenges for clinicians and therefore should be planned. Careful counseling is required and multidisciplinary management is the key to a successful pregnancy [2, 59]. APS patients already under oral anticoagulant drugs should be informed of potential teratogenic effects. Once pregnancy is confirmed, oral anticoagulation should be immediately stopped and switched to low-molecular weight heparin (LMWH) for the rest of the pregnancy. Guidelines for first-line APS treatments during pregnancy vary between countries. However, combination of low-dose aspirin (LDA) and LMWH injections is usually admitted and improves both fetal and mother outcomes [60] (Figure 2). Thus, without treatment, the chances of successful pregnancy are around 30%, 50% with LDA alone, and up to 70% with both molecules [61].

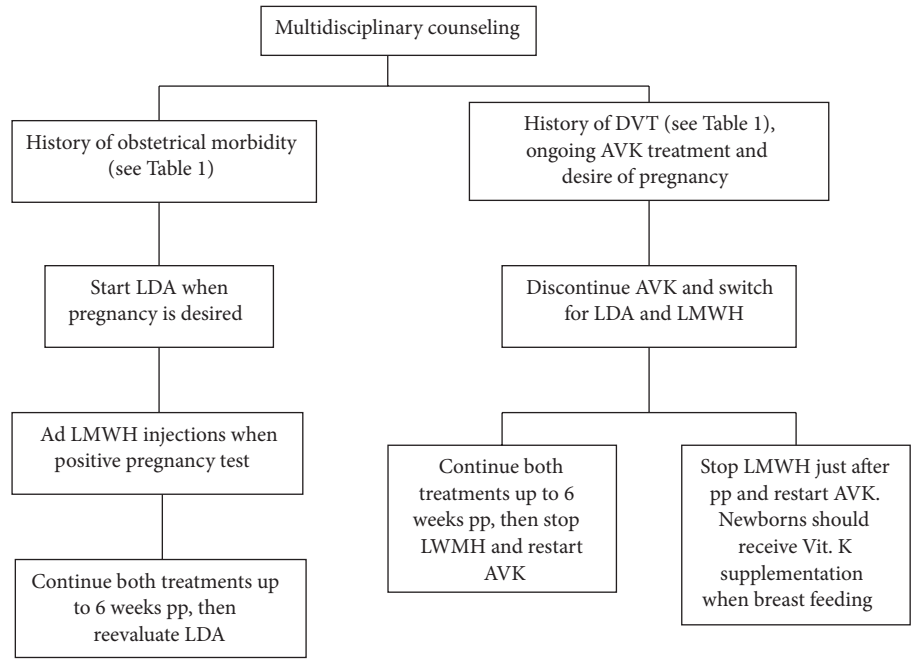
Treating infertile patients with positive aPL is a matter of debate. Studies conducted on LDA and LMWH indications in "aPL infertility" showed contradictory results. Even if heparin seems to improve implantation, there is still no evidence that these two treatments are truly effective for this indication [62, 63].

Biological roles of both aspirin and heparin are large. Nishino et al. have shown that aspirin could decrease thromboxane A2 production and prostaglandin I2 formation, two molecules implicated in pregnancy hypertension and preeclampsia [64]. More recently, aspirin has also been shown to upregulate interleukin-3 (IL-3) production. This molecule seems necessary for trophoblast invasion and placental formation [65].

Heparin actions have been summarized by Kwak-Kim et al. [66]. Heparin as LMWH are anticoagulant molecules that prevent clot formation and can be safely used during pregnancy. However, their roles are not limited to their antithrombotic properties. Among them, they have also been shown to be antiinflammatory and anti-apoptotic molecules.

Both molecules have also their limitations. Mulla et al. showed that neither heparin nor LMWH could reverse the effects of antiβ2GP1 Abs on trophoblast migration [54].

This could partly explain treatment failure in 30% of APS pregnancies. For them, the literature is poor with no evidence-based management defined. Second-line treatments include steroids, hydroxychloroquine (HCQ), intravenous immunoglobulin injections, and plasmaphereses [67, 68]. Among them all, HCQ is the safest molecule used in pregnancy [69]. This antimalarial drug is commonly used in lupus patients and has been shown to improve fetal outcome and to reduce lupus flares [16, 17]. Biologically, HCQ reduces the binding of antiβ2GP1 Abs at the surface of trophoblastic cells [70]. Moreover, the expression of annexin A5, an anticoagulant molecule normally present at the trophoblastic cell surface, is reduced by antiβ2GP1 Abs. HCQ has been shown to restore its expression, preventing the pathological activation of the trophoblastic cells [71]. We also have demonstrated that HCQ restored the effects of antiβ2GP1 Abs on BeWo cell differentiation and decreased TLR 4 expression (manuscript under submission).



DVT: deep venous thrombosis; AVK: oral anti-vitamin K; LDA: low-dose aspirin; LMWH: low-molecular weight heparin; pp: post partum

Figure 2: Obstetrical APS first-line management.

New molecules are also in development. As TLRs have been implicated in the pathological activation of different cell types in APS, specific p38 mitogen-protein kinase (p38-MAPK) and nuclear factor-κB inhibitors, two molecules implicated in intracellular signaling by aPL via TLR, have been developed [72]. However, their uses in pregnancy seem limited by the fact that the suppression of innate immunity could lead to immunosuppression and poor fetal and mother outcomes.

Finally, special attention should be given to prevention of CAPS. As this rare condition can be fatal in about 1/2 of the cases despite any treatment, prophylaxis is still the most important way to avoid IT. Asherson defined special circumstances during which APS patients required special attentions [73]:

(1) infections in APS patients should always be treated carefully;

(2) when surgery is needed, APS patients should receive parenteral anticoagulation;

(3) during postpartum, women should continue anticoagulation for 6 weeks.

5. Take-Home Messages

(i) Obstetrical APS is an entity with high pregnancy complications for both mother and fetus.

(ii) Counseling, multidisciplinary management, and tight follow-up are the keys to successful pregnancy.

(iii) Screening for high-risk APS patients is necessary to improve their pregnancy outcomes.

(iv) A better understanding of pathological mechanisms is necessary for therapeutic improvement.

Abbreviations

TLR 4: Toll-like receptor 4
aPL: Antiphospholipid antibodies
STAT3: Signal transducer activator of transcription 3
hCG: Hormone chorionic gonadotrophin
VEGF: Vascular endothelial growth factor
NFκB: Nuclear factor-kappa B.

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Review Article

Costimulatory Pathways: Physiology and Potential Therapeutic Manipulation in Systemic Lupus Erythematosus

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System lupus erythematosus (SLE) is an immune-complex-mediated autoimmune condition with protean immunological and clinical manifestation. While SLE has classically been advocated as a B-cell or T-cell disease, it is unlikely that a particular cell type is more pathologically predominant than the others. Indeed, SLE is characterized by an orchestrated interplay amongst different types of immunopathologically important cells participating in both innate and adaptive immunity including the dendritic cells, macrophages, neutrophils and lymphocytes, as well as traditional nonimmune cells such as endothelial, epithelial, and renal tubular cells. Amongst the antigen-presenting cells and lymphocytes, and between lymphocytes, the costimulatory pathways which involve mutual exchange of information and signalling play an essential role in initiating, perpetuating, and, eventually, attenuating the proinflammatory immune response. In this review, advances in the knowledge of established costimulatory pathways such as CD28/CTLA-4-CD80/86, ICOS-B7RP1, CD70-CD27, OX40-OX40L, and CD137-CD137L as well as their potential roles involved in the pathophysiology of SLE will be discussed. Attempts to target these costimulatory pathways therapeutically will pave more potential treatment avenues for patients with SLE. Preliminary laboratory and clinical evidence of the potential therapeutic value of manipulating these costimulatory pathways in SLE will also be discussed in this review.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune condition in which autoantibodies against various nuclear and nonnuclear antigens trigger immune-complex-mediated inflammation that can result in organ damage [1]. Similar to other inflammatory and autoimmune conditions, the pathogenic processes that contributed to SLE involve complex interactions between immunocytes and nonimmunocytes such as endothelial and epithelial cells through a number of receptor-ligand systems [2]. Such receptor-ligand association is crucial in initiating and regulating both innate and adapted immunity, from initial presentation of danger signals to naïve immunocytes by professional antigen-presenting cells (APCs) to the moment when communication between T and B cells is driven by specific antigenic epitopes. The cognate T-B-cell interactions which lead to T-cell-dependent autoantibody production by the B cells are central to the pathogenesis of SLE [3].

Generally, antigen presentation is broadly divided into four stages: adhesion, antigen-specific activation, costimulation, and cytokine production and signalling [4]. Instead of initiating cell activation, the process of adhesion which occurs between cells, as well as between cells and extracellular matrix that requires adhesion molecules such as integrins and selectins, is essential in bridging the cells together before subsequent cellular activation takes place [5]. After physically approximating immunologically important APCs and lymphocytes, the APCs capture, internally process, and present peptides of antigen-specific sequences to T lymphocytes through interaction between the major histocompatibility complex (MHC) of the APCs and T-cell receptors (TCRs) on the T cells in an antigen-specific manner. The binding and interaction between the MHC and TCR are, however, insufficient to propagate and sustain downstream proinflammatory signals. Additional intercellular signals in the form of costimulatory pathways are required to perpetuate the ensuing proinflammatory pathways [6]. At this stage, communication

between the cells involved through costimulatory signals is critical in determining if such cell-cell association ensues and terminates. Indeed, besides perpetuating proinflammatory signals, costimulatory pathways are also crucial in attenuating the active immune system or generating peripheral immune tolerance for avoiding unnecessary tissue damage incurred by the overwhelmingly active proinflammatory effector signals [7]. In the subsequent sections, a brief overview of the physiology of well-characterized costimulatory molecules will be highlighted, followed by a discussion of the relevance of some pathophysiologically important costimulatory pathways operant in SLE. Emerging data regarding the therapeutic potential of costimulatory molecule blockades on laboratory benches and randomized controlled clinical trials will also be discussed.

2. An Overview of Costimulatory Molecules and Their Signalling Pathways

2.1. CD80/86-CD28 and ICOS-B7RP1. Costimulatory molecules are broadly divided into those belong to the immunoglobulin (Ig) superfamily (IgSF) and the tumour necrosis factor (TNF) superfamily (TNFSF) based on their phenotypic and signalling features. The CD28 and CD80 (B7-1)/CD86(B7-2) represent one of the most relevant costimulatory pathways of the Ig family. During the very early stage of T-cell activation, CD28 is expressed on T cells and is ligated by CD80/CD86 which are constitutively expressed on dendritic cells (DCs) and inducible in other professional APCs such as B cells and monocytes. CD28 stimulation has been shown to prolong and augment IL-2 production from T cells and prevent the development of peripheral immune tolerance [8]. Consequently, the stimulated T cells further mature, differentiate, and signal the B cells to proliferate and differentiate into antibody-producing plasma cells for production of antibodies. More costimulatory pathways are further activated subsequently to enhance the cognate interactions between APCs and T lymphocytes and intensify the proinflammatory effector signals.

Another recently identified costimulatory system of the same group as CD28-CD80/86 is the inducible costimulator (ICOS)-B7-related protein 1 (B7RP1) pathway. The ICOS which shares similar structural and functional similarities as CD28 was characterized in 1999 [9, 10]. Structurally, ICOS is similar to CD28 in that it is also a transmembrane glycoprotein which is expressed on activated T cells. Functionally, on binding with its ligand B7RP1 which is constitutively expressed on B cells and inducible on monocytes and DC [9, 10], ICOS triggers germinal centre formulation, clonal expansion of T cell, T-cell-dependent antibody production, and isotype switching of B cells [10]. ICOS has been found to be highly expressed on activated CD4+ T cells in patients with autoimmune conditions such as rheumatoid arthritis (RA), SLE, and inflammatory bowel disease [11–13]. On the other hand, ICOS deficiency is found in patients with primary immunodeficiency, with impaired CD4 and CD8 effector functions and reduced memory T-cell population, together with features of common variable immunodeficiency characterized by hypogammaglobulinemia and recurrent bacterial infection [14].

2.2. CD70-CD27. After initial activation of T cells is achieved via MHC-TCR and CD80/86-CD28 interactions, CD70 (TNFSF7) starts to be expressed on the activated T cells and its cognate association with CD27, which is expressed on B and natural killer (NK) cells, serves to strengthen the costimulatory signals between the immunocytes [15]. Activation of CD70 enhances T-cell-dependent antibody production by promoting germinal center formation, B-cell activation, and clonal expansion, as well as differentiation into plasma cells [16]. In the T-cell compartment, cognate interaction between CD70 and CD27 also induces the proliferation of and cytokine secretion by CD4+ and CD8+ T cells and development of cytotoxic T-cell responses by CD8+ T cells [17].

2.3. CD40-CD40L. CD40 belongs to the TNFSF (TNFSF5) and is expressed on APCs, B cells, and traditional nonimmune cells such as endothelial, epithelial, and renal tubular cells [18]. On activation upon ligation with CD40L expressed on activated T cells, CD40 can deliver even stronger activation signals than that of surface bound Ig to B cells. Activated T cells transiently express the CD40L which interacts with CD40 on B cells and further drives B-cell differentiation, maturation, and isotype switching, especially those in the germinal centers [19]. In addition, signal transduction through CD40 upregulates the expression of CD80/CD86 which further intensifies costimulatory signals to the T cells involved in the interaction [20]. Overexpression of CD40L on T cells has been found in a number of autoimmune diseases which are characterized by overproduction of autoantibodies [21, 22]. On the other hand, deficiency of CD40L, such as in patients with CD40 gene mutation, would lead to Ig class-switch recombination deficiency characterized by high IgM but low IgA and IgG levels and recurrent opportunistic infection [23].

During the later stage of T-cell activation, other costimulatory molecules which belong to the TNFRSF such as CD137 and OX40 start to participate in the scene of costimulation. These molecules are only expressed on the activated T cells during the later stage of activation. Expressions of these costimulation molecules are required for further sustaining differentiation of the activated T cells, with an aim to prolong their survival and perform specific effector and memory functions [24].

2.4. CD137-CD137L. CD137 belongs to TNFSF9, and it is a potent costimulatory receptor molecule which is mainly expressed on stimulated T cells and NKT cells [25]. In DCs and CD4+ CD25+ Foxp3+ Treg cells, CD137 is expressed in a constitutive manner and around 48 hours after initial stimulation, CD137 is predominantly expressed on CD4+ and CD8+ T cells [26, 27]. In both murine system and humans, cognate interaction between CD137 and CD137L on activated T cells and B cells, respectively, leads to proliferation and differentiation of both interacting cells and results in

substantial Ig production, before apoptosis of B cells or APCs that occurs upon prolonged stimulation at the terminal stage of immune activation [28]. Indeed, bidirectional signalling ensues in the T and B cells or APCs during CD137-CD137L interaction. Such mutual signalling leads to initial stimulation of both communicating cells followed by their apoptosis, suggesting that CD137 ligation acts as an initial checkpoint to attenuate the over-active proinflammatory effector response [28].

2.5. OX40-OX40L. OX40 (CD134) belongs to the TNFSF4. Similar to CD137, OX40 is expressed on activated CD45RO+ CD4+ T lymphocytes [29]. Signalling of OX40 requires and is controlled by its ligand, OX40L (CD134L), which is constitutively expressed on APCs including B cells, macrophages, DC, and endothelial cells [30]. While OX40 signalling enhances survival of T cells and their subsequent cytokine production and expansion of their memory cell pool, stimulation of OX40L enhances B-cell proliferation and differentiation [31]. A recent study has found that OX40L stimulation inhibited the generation of IL-10-producing CD4+ Tregs cells from memory and naive T cells. In addition, OX40L ligation inhibited IL-10 production and suppressive function of differentiated type 1 regulatory CD4+ T cells (Tr1) [32].

2.6. Downregulation of Proinflammatory Signals during the Late Stage of T-Cell Activation. Costimulation cannot continue unchecked or else overwhelming proinflammatory response will ultimately ruin the host. The costimulatory system consists of peculiar mechanisms which can dampen the proinflammatory signals, achieve immune homeostasis, and assist in the development of peripheral tolerance. CTLA-4 (CD152), a homolog of CD28, starts to be expressed on activated T cell and binds to CD80/86 when T-cell activation has reached its peak [33]. Indeed, the affinity between CTLA-4 and CD80/86 is much higher than that between CD28 and CD80/86 [34, 35]. CTLA-4 activation signals the T cells to attenuate its proliferation and proinflammatory effector signals [36]. Once the CTLA-4 is upregulated, expression of CD28 on T cells will start to be downregulated by endocytosis [36]. Apart from CTLA-4, prolonged interaction between CD137 and CD137L leads to apoptosis of the participating cells which also results in attenuation of the proinflammatory signals [28]. One of the mechanisms postulated is that prolonged activation through CD137 signalling induces large amount of IFNγ production by CD8+ T cells, which in turn stimulates apoptosis of B cells and DCs and results in downregulation of Ig production and CD80/86 expression respectively. Downregulated B7 expression in DCs further weakens the communication with T cells and attenuates the costimulatory signals [28].

2.7. Intracellular Signalling Pathways of Costimulatory Molecules. While a number of downstream signalling pathways after stimulation of costimulatory molecules have been discovered, the functions of many of these pathways are still not fully understood, not mentioning that there are many signalling pathways which have not been discovered or functionally characterized. Nevertheless, Table 1 summarizes the signal transduction pathways of costimulatory molecules in which their functions have been relatively well characterized. Classification of these costimulatory molecules, cells which they are expressed on, and their physiological functions are also briefly described in Table 1. Figure 1 depicts the expression of costimulatory molecules and their concerted effector actions in relation to different stages of immune activation.

3. Costimulatory Molecules and SLE

3.1. CD80/86-CD28 Interaction. Involved in the early stage of T-cell activation, the interaction between CD80/86 and CD28 has been extensively studied amongst various costimulatory molecules in SLE. A number of animal studies attempting to block the CD80/86-CD28 system revealed promising results in terms of amelioration of lupus-related glomerulonephritis, autoantibody production, and class switching, as well as mortality [37, 38]. In a murine lupus model, it has been shown that CTLA4-Ig, a recombinant fusion protein comprising a Fc fragment of human IgG1 which binds either to CD80 or CD86 with a much higher avidity than CD28, contracted the autoreactive B-cell population and reduced autoantibody production and Ig class switching [39]. In clinical studies, CTLA-4Ig (abatacept) has been shown to be able to dampen the interaction between T and B lymphocytes, leading to potential amelioration of autoimmune-driven inflammation [40]. While promising efficacy and safety of abatacept have been demonstrated for the treatment of RA [41], the results of two-phase IIb/III clinical trials testing abatacept in SLE are rather disappointing [42, 43]. In a one-year multicentre, double-blind, placebo-controlled trial of 175 patients with moderately active lupus presenting with arthritis, serositis, and discoid lupus receiving either abatacept (10 mg/kg) or placebo, no statistically significant difference was found between the 2 groups in the primary (new BILAG A or B flare) and the secondary study endpoints [42]. Nevertheless, in *post hoc* analyses while only BILAG A flare was taken into account, significantly more patients receiving placebo experienced flares (54.4%) than those who received abatacept (40.7%). In addition, greatest benefit was seen in patients with arthritis, and improvement of patient-reported outcomes such as sleep disturbance, health-related quality of life, and fatigue was more substantial in the abatacept-treated group [42]. Although more serious adverse events were reported in the abatacept-treated group (19.8% versus 6.8%), *post hoc* analyses did provide evidence that abatacept may confer beneficial effect in severe nonrenal lupus and moderate lupus manifestations [42].

A more recent 52-week phase III clinical trial testing abatacept for its safety and efficacy in 290 patients with ISN/RPS class III or IV lupus nephritis on a background of mycophenolate mofetil and glucocorticoids was published in abstract form [43]. Again disappointingly, no significant difference was found between the treatment and placebo groups in time to achieve complete renal response. However, as in the nonrenal lupus trial [42], *post hoc* analysis revealed that a modified complete response was higher in

Table 1: Summary of the signalling mechanisms and physiological actions of major co-stimulatory molecules in systemic lupus erythematosus.

Costimulation receptor (ref.)	Costimulation ligand	Family	Cells expressing	Signaling molecules involved	Action
*CD80 *CD86 [79, 80]	CD28	IgSF	CD80/86: APCs including monocytes, Mϕ, and DC CD28: mainly naive CD4+ & CD8+ T cells during their initial phase of activation	CD28: phosphorylation by Lck and FYN, and ITK which recruits PI3K and Grb2 PI3K converts PI to PIP_3 and activates Akt and subsequently NF-κB Grb2 binds to SOS1, phosphorylates VAV1 which in turn activates Rac1 and JNK	CD80/86 are constitutively expressed on APCs and B cells CD80/86 are upregulated by inflammation and stimulation of T cells, and they provide costimulatory signals to CD28 and CTLA-4 Stimulation of CD28 prolongs and augments IL-2 production from T cells and prevents the development of peripheral immune tolerance
	CTLA-4	IgSF	T cells during late stage of activation	CTLA-4: phosphorylation by Lck, FYN, and RLK, binds to PI3K, SHP-2, and PP2A. PLCγ, and AKT, and hence proinflammatory effector signals are suppressed	Attenuates further costimulation between communicating immunocytes, dampens proinflammatory signals, and produces anergy. CTLA-4 expression induces CD28 endocytosis on activated T cells
B7RP1 [81]	ICOS	CD28-B7 family	ICOS: activated T cells B71RP1: APCs and B cells	ICOS: phosphorylation of ERK, JNK, and p38	ICOS stimulation induces further activation and clonal expansion of T cells, germinal centre formation, and T-cell-dependent antibody formation
CD137 [82, 83]	CD137L	TNFSF	CD137: activated T cells, NK-T cells CD137L: APCs, B cells	CD137L: Src tyrosine kinase which activates MEK1/2, P38 MAPK, subsequently, and NF-κB (human). Association with TNFR1 which mediates reverse signalling	CD137 enhances proliferation of, and memory as well as cytolytic functions of T cells. It Inhibits CD4+ response and ameliorates autoimmunity due to IFNγ production by CD8+ T cells CD137L induces myelopoiesis, DC maturation, B-cell stimulation, and T-cell proliferation
CD27 [84]	**CD70	TNFSF	CD70: activated T and B cells and Mϕ CD27: resting T and NK cells, some in B cells	CD27 binds to TRAF 2/5 after trimerization and activates NF-κBand the c-Jun pathway CD70 activates PI3K, Erk1/2, and MAPK	CD27 stimulation suppresses Th17 effector function and enhances B-cell activation and Ig production. CD70 signalling may regulate cell cycle of B cells and cytotoxicity of T cells
†OX40 [85, 86]	‡OX40L	TNFSF	OX40: T cells OX40L: APCs, glomerular endothelial cells	OX40L binds to OX40, recruits TRAF 2, 3, 5, and induces phosphorylation of IκBα, and subsequently NF-κB, PI-3K, and protein kinase B	OX40 signalling increases the longevity of T cells and subsequent cytokine production and expansion of memory T-cell population. OX40L signalling enhances B-cell proliferation and differentiation OX40L inhibits the generation of IL-10 producing CD4+ Tregs from naive and memory T cells

Table 1: Continued.

Costimulation receptor (ref.)	Costimulation ligand	Family	Cells expressing	Signaling molecules involved	Action
CD40 [87]	€CD40L	TNFSF	CD40: B cells CD40L: T cells	CD40: TRAF 1/2, 3/5, 5, 6, and induces NF-κB while TRAF 2, 2/6, and 6 induces p38, Akt and JNK Jak 3 and induces STAT5 phosphorylation	Provides a strong activation signal to B cells for their differentiation, proliferation, and hence germinal centre development, and Ig response to T-dependent antigens. CD40 also upregulates CD80/86 expression and provides further stimulation signals to T cells.

Abbreviations: ref: references; CD: cluster of differentiation; IgSF: immunoglobulin superfamily; APCs: antigen-presenting cells; Mϕ: macrophages; DC: dendritic cells; TNFSF: tumour necrosis factor superfamily; Lck: lymphocyte-specific protein tyrosine kinase; FYN: protooncogene tyrosine-protein kinase Fyn; ITK: IL2-inducible T-cell kinase; PI3K: Phosphoinositide 3-kinase; Grb2: Growth factor receptor-bound protein 2; PI: phosphatidylinositol; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; Akt: PKB is a serine/threonine protein kinase; NF-κB: nuclear factor-kappa-light-chain-enhancer of activated B cells; Sos: son of sevenless homolog 1; Vav1: protooncogene vav; Rac1: Ras-related C3 botulinum toxin substrate 1; JNK: c-Jun N-terminal kinase; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; IL: interleukin; RLK: receptor-like kinase; SHP-2: Src homology region 2 domain-containing phosphatase-2; PP2A: Protein phosphatase 2A; PLCγ: Phospholipase Cγ; B7RP1: B7 related protein 1; ICOS: inducible co-stimulator; ERK: extracellular-signal-regulated kinases; NK-T: natural killer T cells; MEK1/2: mitogen-activated protein kinase kinase 1/2; MAPK: mitogen-activated protein kinase; TNFR1: TNF receptor 1; IFNγ: interferon gamma; TRAF: TNF-receptor-associated factors; IkB: inhibitor of κB; Jac3: Janus kinase 3; and STAT5: signal transducers and activators of transcription 5; Ig: immunoglobulin.

*CD80 = B7-1, CD86 = B7-2; **CD70 = CD27L; †OX40 = CD134; ‡OX40L = CD134L; and €CD40L = CD154.

patients who received abatacept [43]. Repeated analyses of these data by less stringent criteria such as the ALMS and LUNAR response criteria also revealed greater difference in complete response favouring the abatacept-treated groups [44]. A randomized controlled phase II trial (ACCESS) comparing the combination therapy of cyclophosphamide (500 mg intravenous infusion fortnightly for 12 weeks) and abatacept (500–1000 mg at 0, 2, and 4 weeks then monthly for 24 to 48 weeks) versus standard cyclophosphamide (500 mg intravenously infusion fortnightly for 12 weeks) in active lupus nephritis is currently underway [45].

Blockade of CD80 by monoclonal antibody has recently been explored in the treatment of lupus-like disease in a mouse model. In a pristane-induced lupus-like disease mouse model, monoclonal antibody against CD80 was shown to be able to attenuate inflammatory response and severity of lupus-like signs [46].

3.2. CD70/CD27 Interaction. Before discussing the impact of the CD70-CD27 interaction on the pathogenesis of SLE, it is useful to highlight the relationship between DNA methylation and SLE. In general, DNA methylation involves the addition of a methyl group to adenosine or cytosine base of the DNA nucleotides and stably alters gene expression [47]. In the early 1990s, global reduction of DNA methylation by 15–20% was first identified in lupus T cells [48]. Subsequently, the link between DNA hypomethylation, T-cell autoreactivity, and development of SLE was further supported by the association between low 5-methylcytosine content of DNA in blood and disease activity in patients with SLE [49]. Thus, methylation status of lupus susceptible genes such as those coding CD70 and CD40L may affect the clinical expression of SLE. In an animal model of lupus, CD70 overexpression on splenic CD4+ cells was observed in 16-week-old MRL/lpr mice with established lupus-like disease but not in their 5-week-old counterparts prior to disease development. Additionally, CD70 expression was found to correlate with DNA hypomethylation of the CD70 gene in activated CD4+ T cells [50]. The expression of DNA methyltransferase 1 (DNMT1) was found to be significantly lower in the 16-week-old mice than that of the 5-week-old mice, and the low DNMT1 level led to T-cell DNA hypomethylation, CD70 overexpression, and consequently age-dependent development of lupus-like disease in the MRL/lpr mice [50].

In humans, CD4+ T cells of patients with SLE were also shown to overexpress CD70 when compared to healthy subjects, although CD70 expression on CD4+ T cells has not been found to be consistently correlated with lupus disease activity [51–53]. Nevertheless, overexpression on T cells in SLE patients was confirmed to be the result of hypomethylation of DNA sequences that flank the CD70 promoter due to reduced expression of DNMT1, which results in failure in downregulating CD70 expression once it is induced by T-cell activation [54]. In a recent study which aimed to unravel the mechanism of DNA hypomethylation of T lymphocytes in patients with SLE, a transcription factor named regulatory factor X1 (RFX1), which functions to recruit DNMT1 to the promoter region of CD70, was shown to be downregulated in human lupus CD4+ T cells, potentially contributing to CD70 overexpression [55]. CD70 overexpression in SLE has been postulated to intensify B cell costimulation with subsequent increase in autoreactive Ig production [52]. In addition to hypomethylation, post-transcriptional modifications on histone protein also play a role in overexpressing CD70 in lupus T cells [56]. *In vitro* treatment of lupus CD4+ T cells with a histone deacetylase inhibitor was demonstrated to overexpress CD70 in these cells by aberrant histone modifications (increase in H3 and

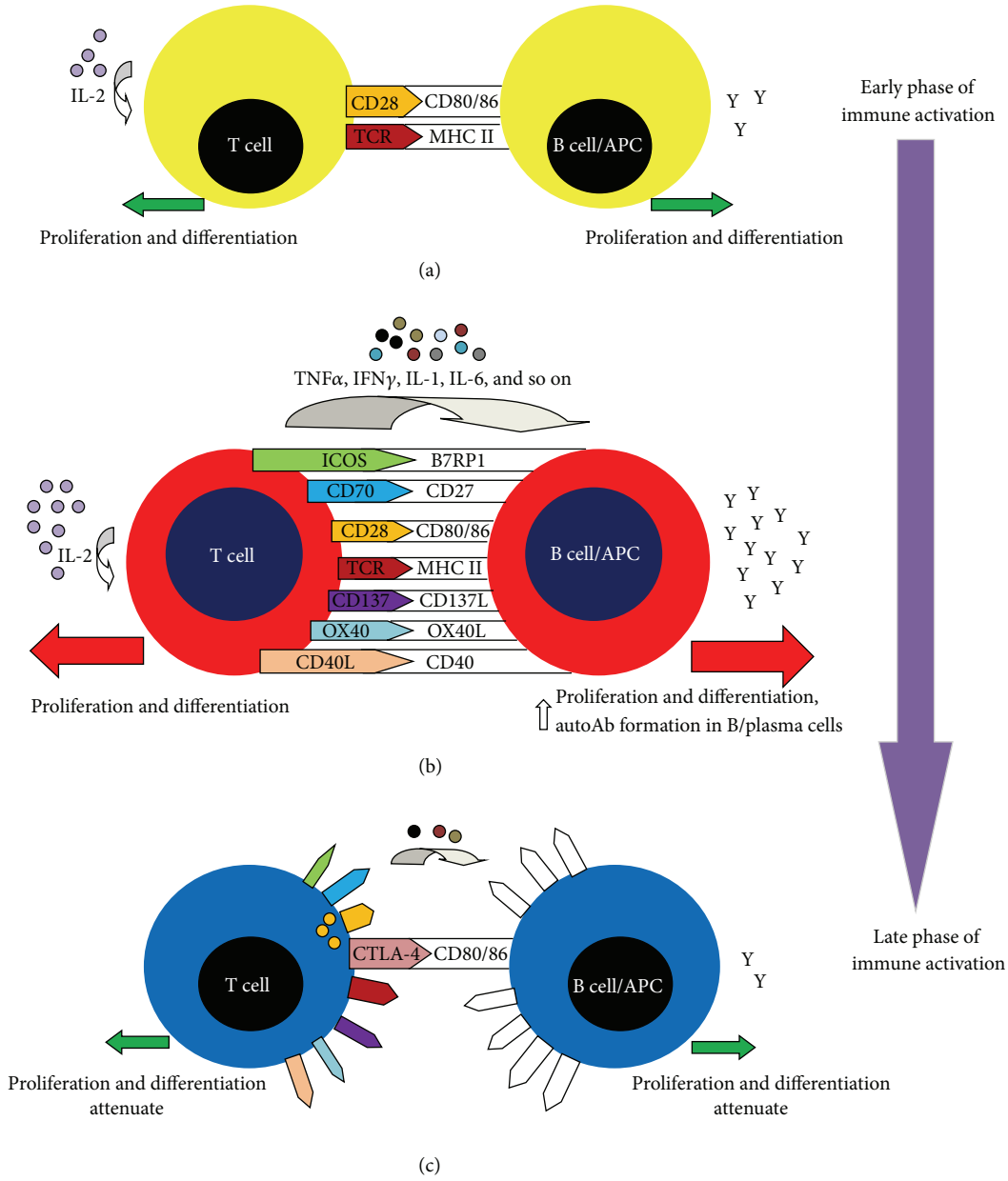

Figure 1: Expression of costimulation molecules in different stages of immune activation and their concerted effector functions. (a) Initial phase of immune activation; (b) maximal phase of immune activation with expression of many costimulatory molecules (figure is for depiction only, costimulation molecules may not be all expressed in a single immune reaction), coupled with proliferation and differentiation of immunocytes, production of proinflammatory cytokines and autoantibodies; (c) after reaching the peak of activation, CTLA4 expressed on T cells interacts with CD80/86 on B cells/APCs with higher affinity than CD28. Immune response attenuates and costimulatory pathways start to disintegrate. CD28 molecules expressed during maximal activation phase are endocytosed, pictorially depicted as intracellular small round yellow vesicles in the T cells of condition (c). Abbreviations: TCR: T-cell receptor; MHC II: class II major histocompatibility complex; APC: antigen-presenting cells; and autoAb: autoantibodies.

H4 acetylation levels) within the TNFSF7 promotor region [56].

High expressions of CD27 in B cells and plasma cells were shown to correlate with SLE disease activity in terms of higher SLEDAI, anti-dsDNA IgG levels, and lower serum complement levels in a cross-sectional study of 59 patients with SLE [57]. However, concurrent infection in these patients with elevated CD27 expression in their plasma cells limits the clinical utility of CD27+ plasma cell level as an ideal marker of lupus disease activity [57].

3.3. CD40-CD40L Interaction. The CD40L gene, similar to that of CD70, is methylation sensitive. The CD40L gene is a SLE susceptible gene located on the X chromosome. Partly as a consequence of demethylated regulatory sequence on the inactive X chromosome, CD40L was found to be

overexpressed on T cells of female lupus patients [58, 59]. CD4+ T cells from female lupus patients treated *in vitro* with DNA methylation inhibitors were shown to overexpress CD40L mRNA, and elevation of CD40L expression has been unanimously shown to promote autoantibody production in B cells in the presence of their strong interaction with CD40 expressed on T cells [58, 59]. Blocking the interaction between CD40L and CD40 is thus a feasible approach to ameliorate autoantibody production and hence the clinical manifestation of SLE [59]. In fact, CD40L blockade has been shown to prevent the development of SLE effectively in a lupus mouse model [60].

Clinical trials testing the blockade of the CD40-CD40L pathway in the treatment of SLE are disappointing [61, 62]. In addition to the failure of meeting the predefined study end-points, the unfavourable side-effect profile of anti-CD40L (BG9588) led to premature termination of a multicentre phase II trial of BG9588 in SLE [62]. In a double-blind, placebo-controlled, multiple-dose study, 85 patients with mild to moderately active SLE were randomized to receive 6 infusions of IDEC-151 of 2.5, 5, and 10 mg/kg and placebo at 0, 2, 4, 8, 12, and 16 weeks [61]. At 20 weeks, although the SLEDAI scores improved in all groups from baseline, statistical significance was not reached amongst the different groups. No difference in the predefined secondary outcomes including BILAG, physician and patient global assessment, Krupp fatigue assessment, serum anti-dsDNA and complement levels, and quality of life was noted. Adverse events were comparable between both groups, and none of the subjects developed anti-CD154 antibody after 16 weeks of treatment [61]. In another smaller phase II, open-label trial of evaluating anti-CD40L antibody (BG9588) in the treatment of 28 patients with proliferative lupus glomerulonephritis (20 mg/kg biweekly for 3 doses then monthly for 4 additional doses), the occurrence of 2 myocardial infarctions 59 and 9 days after infusion, respectively, led to premature termination of the trial, even though patients on BG9588 treatment demonstrated significant reduction of proteinuria, haematuria, and anti-dsDNA titre with increase in serum C3 levels [62].

3.4. CD137-CD137L Interaction. Although CD137 activation upon ligation with CD137L provokes bidirectional signalling which induces proliferation and differentiation of both T and B cells, knocking out CD137 paradoxically induces more autoantibody production, higher level of pathogenic CD4+ and double negative T cells (CD3+CD4-CD8-B220+), more severe cutaneous lupus, glomerulonephritis, and death in the MRL/lpr mouse model [63]. The results led to the hypothesis that if CD137 is agonized, the opposite effects which favour amelioration of SLE might occur. Two separate studies using knockout mice of different backgrounds unanimously demonstrated amelioration of lupus phenotypically and serologically by agonizing CD137 receptor [64, 65]. By injecting agonistic CD137 monoclonal antibody (mAb), reduction in glomerulonephritis in MRL/lpr mice was demonstrated, besides reduction in CD4+ lymphocytes, anti-dsDNA production, germinal centre formation in secondary lymphoid organs, and mortality [65]. The same mAb injected into NZWB/W lupus-prone mice in another study produced similar results, except that no reduction of CD4+ was demonstrated [64]. Instead, an elevation of splenic CD25+ Treg cells was shown in the agonistic CD137 mAb group when compared with injection of isotype control [64]. Of particular note is that while agonizing CD137 has shown benefits in reducing the pathogenic autoantibodies and lymphocytes and amelioration of clinical manifestation of SLE in animal models, a number of *in vitro* studies using nonlupus human samples have revealed that CD137 activation enhances ingression of monocytes and their interaction with ICAM-1 in blood vessels, leading to atherosclerosis and plaque inflammation which may potentially amplify cardiovascular risk [66–68].

3.5. OX40/OX40L. OX40 (CD134) expression in lupus PBMCs is predominantly restricted in CD3+ CD4+ CD45RO+ T lymphocytes, and the level correlates with lupus disease activity [69]. OX40 has also been found to be highly expressed in kidneys of patients with lupus nephritis [70]. While there have been no therapeutic trials performed in animals and humans addressing the safety and efficacy of blocking OX40-OX40L in the treatment of SLE, *in vitro* studies revealed that anti-CD134mAb-treated splenocytes of BXSB mice expressed significantly less markers of active SLE such as anti-dsDNA, IL-6, and IFNγ [71]. In a small mechanistic study of 10 patients with SLE, stimulation of PBMCs under the influence of anti-CD134 mAb resulted in reduction of Th2 cytokine but increase in IFNγ production [72]. Stimulation with CD134-Fc fusion protein further reduced the production of both Th1 and Th2 cytokines including IL-4, IL-10, and IFNγ in patients with lupus nephritis [72], suggesting the potential role of anti-CD134 in reducing IL-4- and IL-10-mediated renal inflammation. In another study, expression of OX40 on IL-17-producing T cells was higher in SLE patients when compared with that of healthy controls [73]. Furthermore, expression of OX40 on T cells was correlated with lupus disease activity, and OX40+ T cells were found in renal biopsies of patients with lupus nephritis, which may signify that OX40+ T cells migrate to the nephritic kidneys and contribute to inflammation and IL-17 production by interaction with CD137L-expressing resident renal cells [73]. Culture of lupus PBMCs with CD134 mAb in another experiment demonstrated inhibition of haemolytic activity and perforin expression on the PBMCs, and the degree of inhibition was associated with the disease activity of SLE and the degree of proteinuria [74].

3.6. ICOS-B7RP-1. Without interaction with B7RP1, ICOS-deficient mice were noted to have reduced total IgG and anti-dsDNA production [75]. In NZB/W F1 mouse model, blockade of B7RP1 with anti-B7h mAb before the onset of renal disease significantly delayed the onset of proteinuria, inhibited all subclasses of IgG autoantibody production, reduced the degree of glomerulonephritis, and prolonged the survival of the mice [76]. Even after the appearance of proteinuria in established disease, anti-B7h mAb consistently improved the renal histopathology and disease progression

Table 2: Major translational studies and clinical trials testing various potential co-stimulatory molecules in the treatment of systemic lupus erythematosus.

Molecule (ref.)	Nature	*In vitro*/animal/observational studies in humans	Clinical trial
CTLA-4Ig [37–40, 42, 43, 45]	Recombinant fusion protein	Reduces autoreactive B cells, autoantibodies, IFNγ production, and class switching, along with amelioration of GN in animal models Dampens cognate interactions between T-B cells and reduction in autoimmune-driven inflammation	No statistically significant difference in new BILAG A or B flares in a RCT of 175 patients. If only BILAG A was assessed in *post hoc* analyses, more patients on placebo flared than those received Tx (54.4% versus 40.7%). More adverse events were noted in placebo than Tx group (19.8% versus 6.8%) In another RCT of 290 patients with class III or IV lupus nephritis, no significant difference was found between both Tx and placebo groups in complete renal response. Using the ALMS and LUNAR response criteria in *post hoc* analyses, more patients on the Tx group than those on placebo had complete renal response The ACCESS trial is currently underway
Anti-ICOS Ab [76]	mAb	No change in Lϕ count and phenotypes in NZB/W F1 mice was noted. Drives production of IFNγ & Th2 cytokine and apoptosis upon T-cell stimulation with OVA in SCID mice. In humans, ICOS expression is elevated in CD4+ and CD8+ T cells	Nil
Anti-B7RP1/anti-B7h Ab [76, 78]	mAb	Delays the onset of proteinuria, inhibits IgG production, reduces GN, and prolongs survival in NZB/W F1 mice. Improves renal histology and disease progression in NZB/W F1 mice with established disease	Phase 1b trial (AMG557) for the treatment of stable lupus has just been completed. Data are being awaited
Anti-CD137Ab [64, 65]	mAb	Agonistic to CD137, leading to reduction of GN, splenic CD4+ T cells, anti-dsDNA production, germinal center formation, and reduced mortality in MRL/lpr mice. In NZW/B F1 mice, similar effect as in MRL/lpr mice yet no reduction in splenic CD4+ count but elevation of splenic CD25+ Treg cells In nonlupus human samples, CD137 agonization induces vascular inflammation, plaque formation, and atherosclerosis	Nil
CD134-Fc [72]	Recombinant fusion protein	Reduces Th1 and Th2 cytokine and IFNγ production from PBMCs in patients with lupus nephritis	Nil
Anti-CD134Ab [71, 72]	mAb	Reduces IL6, anti-dsDNA and IFNγ levels in CD134mAb-treated splenocytes of BXSB mice Reduces Th2 but increases IFNγ production in PBMCs of patients with SLE	Nil
Anti-CD40L Ab [60–62]	mAb	Reduces anti-DNA autoantibody production and renal disease and significantly prolongs survival in NZB/W lupus-prone mice. No renal damage and even absence of immune depositions are noted in mice that responded to treatment	A 20-week phase II RCT of 85 patients with mild to moderate SLE receiving IDEC-131 or placebo did not reach both primary and secondary endpoints A phase II open-label trial of 28 patients with proliferative GN receiving BG9588 was terminated prematurely due to 2 cases of cardiac events although significant reduction of proteinuria, haematuria and serum anti-dsDNA titre, and elevation of serum C3 were achieved with BG9588

Abbreviations: ref: references; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; IFNγ: interferongamma; GN: glomerulonephritis; BILAG: British Isles Lupus Assessment Group index; RCT: randomized controlled trial; Tx: treatment; mAb: monoclonal antibody; ALMS: Aspreva Lupus Management Study; LUNAR: Lupus Nephritis Assessment with Rituximab; ICOS: inducible costimulator; Lϕ: lymphocyte; OVA: ovalbumin; SCID: severe combined immunodeficiency; CD: cluster of differentiation; B7RP1: B7-related protein 1; NZW/B: New Zealand white/black; Treg cells: regulatory T cells; and PBMC: peripheral blood mononuclear cells.

in the animals [76]. Interestingly, in contrast to anti-B7h mAb treatment, anti-ICOS mAb treatment did not affect lymphocyte count and phenotypes of the animals. Instead, anti-ICOS mAb treatment of mice with severe combined immunodeficiency induced antigen-specific T-cell activation which led to the production of large amount of IFNγ and Th2 cytokines, more apoptosis, and cell death [76]. Based on these observations, monoclonal antibodies against ICOS functionally trigger ICOS signalling instead of inhibiting it. The timing and immunological milieu when ICOS signalling is manipulated are postulated to be crucial in determining the effect of ICOS blockade. For example, delayed but not early, ICOS blockade of a mouse cardiac allograft model was shown to enhance cardiac graft survival where CD28 costimulation was absent and while CD8+ T cells, CTLA-4, and the STAT-6 pathway were functionally active [77].

In humans, ICOS expression on CD4 and CD8+ T cells has been shown to be elevated in lupus patients, while ICOS-L (B7RP1) expression is downregulated in peripheral blood memory B cells after cognate interaction with ICOS+ T cells in coculture systems. In lupus kidney samples, it was found that ICOS+ T and B cells which infiltrated the kidneys were in close contact, suggesting that T-B interactions may also take place in peripheral tissues [12]. A phase Ib trial assessing anti-B7RP1 mAb (AMG 557) in the treatment of stable lupus has just been completed, and results are eagerly awaited [78].

Table 2 summarizes the major translational studies and clinical trials manipulating various costimulatory pathways in the treatment of SLE.

4. Conclusion

Costimulatory pathways initiate and perpetuate proinflammatory processes by sustaining cognate interaction between activated T cells and APCs which is central to the pathogenic process of SLE. Equally important, costimulatory pathways are capable of attenuating the ensuing proinflammatory response which is overwhelmed, as exemplified by the interaction between CTLA-4 and CD80/86 and prolonged CD137 stimulation.

While most studies involving murine lupus models have concluded the superior efficacy and safety of costimulatory blockade in treating lupus, most clinical trials which evaluated costimulatory blockers in SLE to date, such as CD154 and CTLA-4Ig, did not meet the predefined therapeutic endpoints. Nevertheless, reanalyses of these clinical trial data with the use of less stringent clinical response criteria and *post hoc* analyses may still advocate superior therapeutic value of these costimulatory blockades in moderate and severe lupus manifestations. Based on what we have learnt from the lessons of failure, there are 2 concomitant directions which we should proceed for reevaluating promising pharmacological agents and exploring potential biologic therapies for SLE. First, more realistic study design for clinical trials with less stringent and more practical endpoints can be employed to restudy medications such as abatacept and anti-CD154 mAb. Second, a number of existing background treatments of SLE such as glucocorticoids and cyclophosphamide would obscure the potential therapeutic effects of the biologics being evaluated in clinical trials. As such, more thorough understanding of the molecular aspects of the existing treatments and their potential impact on the signalling mechanisms of the medications under evaluation will be beneficial. While the current data from clinical trials testing the efficacy and safety of anti-CD154 and CTLA-4Ig for the treatment of SLE have not been promising, novel yet potentially safer targets based on our current knowledge need to be explored. For example, instead of targeting CD137, antagonizing CD137L may be of potential to reduce disease severity and cardiovascular complications because agonizing CD137 may induce atherosclerosis which is potentially detrimental to patients with SLE [66–68]. Similarly, antagonizing B7RP1 may be safer because targeting at ICOS may actually stimulate it and potentially triggers more severe autoimmunity [76].

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Review Article

The Immune Factors Involved in the Pathogenesis, Diagnosis, and Treatment of Sjogren's Syndrome

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Sjogren's syndrome (SS) is a systemic, autoimmune disorder characterized by salivary insufficiency and lymphocytic infiltration of the exocrine glands. Even though the mechanism of its pathology and progression has been researched ever since its discovery, the roles of different parts of immune system remain inconclusive. There is no straightforward and simple theory for the pathogenesis and diagnosis of Sjogren's syndrome because of the multiple kinds and functions of autoantibodies, changing proportion of different T-lymphocyte subsets with the progression of disease, unsuspected abilities of B lymphocytes discovered recently, crosstalk between cytokines connecting the factors mentioned previously, and genetic predisposition that contributes to the initiation of this disease. On the other hand, the number of significant reports and open-label studies of B-cell depletion therapy showing clinical efficacy in sjogren's syndrome has continued to accumulate, which provides a promising future for the patients. In a word, further elucidation of the role of different components of the immune system will open avenues for better diagnosis and treatment of SS, whose current management is still mainly supportive.

1. Introduction

Xerostomia is a common and primary symptom during clinical practice, which could be caused by various factors, mainly Sjogren's syndrome. Sjogren's syndrome (SS) is an autoimmune disease with the hallmark of clinical features of salivary insufficiency and pathological features of focal, periductal, and perivenular lymphocytic infiltrates. The association between dry eyes and dry mouth was first noticed by Hadden in 1888, who introduced the term xerostomia [1]. In 1933, Dr. Henrik Sjogren published the most comprehensive article on this subject describing a cluster of women in a small Swedish town presenting with keratoconjunctivitis, lymphoid infiltrations of the conjunctiva, cornea, lacrimal glands, and parotid glands, a history of arthritis, swelling of the salivary glands, and dryness of the oronasopharynx. Two years later, this series of observation was connected with Mikulicz's disease and together formed the general basis for this syndrome [2]. In 1936, Duke Elder honored Sjogren by naming the disease Sjogren's syndrome. Sjogren's syndrome can be classified into two forms: primary Sjogren's syndrome characterized by keratoconjunctivitis and xerostomia without an associated autoimmune disease and secondary Sjogren's syndrome characterized by keratoconjunctivitis and xerostomia associated with an autoimmune disorder, for example, rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis. The immunological mechanism of this disease has long been studied and still been an active subject for investigation. In this review, we will discuss the function of different components of the immune system involved in the pathogenesis, progression, and treatment of this disease.

2. The Autoantibodies

A large number of autoantibodies have been detected in the serum of patients with Sjogren's syndrome. According to Tzioufas et al., these antibodies possess three different abilities: serving as disease markers; indicating the association with other autoimmune diseases; and exhibiting possible pathogenetic role [3].

2.1. Anti-Ro/SSA and Anti-La/SSB. Anti-Ro/SSA and anti-La/SSB, antibodies directed against Ro/La ribonucleoprotein complexes, can serve as a diagnostic hallmark of Sjogren's syndrome. Depending on the method applied for their identification, anti-Ro/SSA and anti-La/SSB antibodies are detected in approximately 50 to 70% of pSS patients [4]. Interestingly, anti-Ro/SSA antibodies may be found either solely or concomitantly with anti-La/SSB antibodies, whereas exclusive anti-La/SSB positivity is rare [5].

The resources of these antibodies remain sophisticated even with improved identification methods. Using a novel technique, Tengner et al. have demonstrated the presence of Ro and La autoantibody producing cells in salivary gland biopsy tissues from patients with SS [6]. And previous studies have demonstrated that anti-Ro/SSA and anti-La/SSB autoantibodies are enriched in saliva of pSS patients. It seems that the affected salivary glands are the major site of autoantibody production. These findings indicate that anti-Ro/SSA and anti-La/SSB autoantibodies are produced and assembled at sites of inflammation and imply their potential involvement in the autoimmune exocrinopathy of this disease. However, Hammi and his colleagues considered that the leakage of anti-Ro/SSA and anti-La/SSB antibodies from blood into saliva could be the main resource of autoantibodies, with the evidence that serum was shown to be significantly more sensitive than parotid saliva for the detection of Ro and La antibodies [7].

As a hallmark of sjogren's syndrome, it seems that the level of Ro/La antibody should remain unaltered during the subsequent course of the disease. A long-term follow-up study in the North East England by Davidson and his colleagues claimed that the serological pattern of the majority of patients remained constant throughout the follow-up period [8]. However, Fauchais et al. indicated that in a cohort of 445 pSS patients, anti-Ro/SSA and anti-La/SSB antibodies were not present at baseline but emerged secondarily in 8% of these cases, while in several cases the antibodies disappeared during followup. Some patients may exhibit pSS systemic complication during followup despite the disappearance of both hypergammaglobulinemia and anti-SSA antibodies. It is suggested that patients suspected to suffer from pSS, negative for anti-Ro/La antibodies though, should be referred for salivary gland biopsy [9].

2.2. Antibodies Indicating the Association with Other Autoimmune Diseases. The presence of more than one autoimmune disease in the same patient is common in clinical studies, and earlier investigations have discovered some commonalities among autoimmune diseases. For instance, as reported by Selmi et al., primary biliary cirrhosis may be considered a Sjogren's syndrome of the liver, whereas Sjogren's syndrome can be equally discussed as primary biliary cirrhosis of the salivary glands [10]. The existence of autoantibodies like rheumatoid factors and cryoglobulins, which correlate with other autoimmune diseases, may indicate the occurrence of polyautoimmunity in the prognosis of the patient. Table 1 shows the antibodies indicating the association with other autoimmune diseases [3].

Table 1: Prevalence and associated diseases of autoantibodies.

Antibodies	Prevalence (%)	Related autoimmune diseases
Rheumatoid factors (RF)	40–50	Hypergammaglobulinemia
Cryoglobulins	10–15	Lymphoma development and death
Anti-centromere antibodies (ACA)	4–17	Systemic sclerosis
Anti-mitochondrial antibodies (AMA)	5–6.5	Primary biliary cirrhosis
Anti-cyclic citrullinated peptide antibodies (anti-CCP)	7–10	Articular manifestations
Anti-smooth muscle antibodies (ASMA)	6.5–62	Autoimmune hepatitis

2.3. Anti-Muscarinic Receptor Antibodies. Whether salivary glands in patients with SS produce less saliva because of their functional or structural disturbance, induced by anti-M3R antibodies, has been a hot spot for research in recent years. Muscarinic receptors (MRs) are acetylcholine receptors coupled to G-proteins; they can be detected in plasma membranes of certain neuronal and nonneuronal cells. How the anti-M3R antibodies influence the salivary glands and which part of the receptor plays the crucial role are still controversial. A recent study by Passafaro et al. showed that anti-M3R antibodies act as a partial muscarinic agonist, which increase prostaglandin E2 (PGE2) and cyclic AMP production through modifying Na^+/K^+-ATPase activity and also interfere with the secretory effect of the parasympathetic neurotransmitter [11]. Using muscle strip and whole organ functional assays, Smith and Dawson showed that IgG with anti-M3R activity from patients with SS disrupted neurotransmission in tissues throughout the mouse gastrointestinal tract [12]. Several researchers have selected the second extracellular loop as the crucial part involved in the progression of pSS since it is an epitope of anti-receptor antibodies for many G-protein coupled receptors in other autoimmune conditions. Another recent study by Tsuboi et al. proved that autoantibodies against the second extracellular loop of M3R could be involved in salivary dysfunction in patients with SS by generating two hybridomas producing anti-M3R monoclonal antibodies against the second extracellular loop of M3R (anti-M3R2nd mAbs) and analyzing their function by Ca^{2+}-influx assays, using a human salivary gland (HSG) cell line [13].

3. T Cells

It was noted in 1983 that T cells constituted the majority (>75%) of lymphocytes infiltrating the salivary glands and that CD4 T cells constituted the majority of these cells [14].

3.1. Th1 and Th2 Cells. Traditional opinion about these two Th subsets is that there is a dynamic balance between the two families of cytokines; while uncontrolled Th1 cells determine autoimmune states, unusual deviation to Th2 cells leads to

allergic disorders [15]. The observation of Fox and Kang that increased levels of IL-1b, IL-6, tumor necrosis factor (TNF)-α, and IFN-γ have been reported in saliva from patients with SS in comparison with controls with histologically normal salivary glands, suggests that Th1 responses predominate in SS autoimmune lesions [16]. In some autoimmune cases, however, reduction of the Th1 response or a Th2 type shift may alleviate disease; many apparent exceptions are now well documented. And Th2-derived cytokines such as IL-10 have been found to be elevated in the saliva of Sjogren's syndrome patients and correlate with the severity of the disease [17]. The results of Mitsias and his colleagues reported that Type II microenvironment prevails in low-grade infiltration, while type I pattern increases in patients with definite SS, and patients with advanced lymphocytic infiltration may suggest that the cytokine pattern may shift from Th1 to Th2 as the immunopathological lesions progress [18]. Detailed mechanism of how steroid hormone and cytokines regulate the Th1/Th2 is a complex issue that needs more research, and, hopefully, some new treatment options may be drawn from this.

3.2. Th17 and Tregs. In spite of the role of Th1 and Th2 cells in sjogren's syndrome which remains contradictory, several data show that some abnormalities that first ascribed to Th1 cells were instead engendered by Th17 cells [19]. Th17 cells produce a family of cytokines from IL-17A through IL-17F, and, to a lesser extent, TNF-α and IL-22, which have been detected in the serum and saliva of SS patients, and IL-17 expression correlated with the severity of lesions. On the other hand, Tregs are thought to exert a countervailing suppressive effect on Th17 mediated cellular immunity. The reports are contradictory in that the blood of SS patients contains too many or too few Treg cells. Nguyen et al. [20] and Cornec et al. [21] found that the Th17/IL-23 system is upregulated in C57BL/6.NOD-Aec1Aec2 mice and SS patients at the time of disease and proposed the hypothesis that in the presence of IL-6, the T-cell differentiation might switch from Treg-cell pathway to Th17 pathway. As well as rheumatoid and systemic lupus erythematosus, shown by earlier investigations, IL-6 has been detected in the salivary glands of patients with SS, which not only participates in the generation of Th17 but also fosters their proliferation. IL-18, TGF-β, and other cytokines participate in the regulation of T cells in a complicated way, and more investigations are needed to be clarified.

4. B Cells

Although T cells were originally considered to play the initiating role in the autoimmune process, while B cells were restricted to autoantibody production, recent years have seen growing evidence that the roles of B cells in pSS pathophysiology are multiple and that these cells may actually play a central role in the development of the disease [20, 21].

Hansen and his colleagues reveal that patients with pSS show a general reduction of their CD27+ memory B-cell levels in peripheral blood, while an accumulation/retention of these cells is observed in their salivary glands [22, 23]. It has been reported in previous studies that the abnormal differentiation of B cells in pSS leads to a decline in circulating memory B cells and a subsequent increase in levels of plasma cells and long-lived plasma cells. In a word, the distribution and function of B cells have been reported to be altered in pSS. Aqrawi et al. observed similar phenomenons and took progress in confirming that the decrease in memory B-cell levels in the peripheral blood of patients with pSS is specific to anti-Ro and anti-La memory B cells, while whether the increase in memory B-cell infiltrates in salivary glands is also caused by anti-Ro- and anti-La-specific memory B cells needs further studies [24].

The respective membrane expression of IgD and CD38 distributes mature B cells (Bm) into sequential stages from Bm1 through Bm5 cells. These subsets can also be detected in peripheral blood, suggesting that a part of the cells maturating in secondary lymphoid organ can escape from GC and rejoin circulation. B-cell subset distribution is altered in the blood of patients with pSS, with an increase in Bm2/Bm2′ and a decrease in eBm5/Bm5. The ratio (Bm2/Bm2′)/(eBm5/Bm5) could even be considered as a diagnostic tool for the pSS [25].

Despite producing autoantibodies and cytokines, B lymphocytes can induce epithelial cells of salivary glands into apoptosis in sjogren's syndrome. By using coculture experiments with human salivary gland (HSG) cell line cells and tonsilar B lymphocytes, Varin et al. observed that direct HSG-cell-B-lymphocyte contacts were able to induce apoptosis in epithelial cells. This B-lymphocyte-mediated cell death could not be ascribed to Fas-FasL ligand interactions but required translocation of protein kinase C delta (PKC δ) into the nucleus of epithelial cells [26].

4.1. BAFF. BAFF (for B-cell activating factor of the TNF family), which is produced by all sorts of macrophages and DCs and from epithelial cells and activated T lymphocytes, has been demonstrated to be key in B-cell homeostasis. BAFF is critical for B cells to survive in the periphery and is also involved in B-cell selection by dictating set points for mature primary B-cell numbers and adjusting thresholds for specificity-based selection during downstream differentiation. As shown by many researchers, Daridon et al., for example, BAFF level is increased in the saliva of the patients and may correlate with the severity of pSS [27]. In addition, BAFF transgenic mice manifest T2-cell hyperplasia in their exocrine glands, as reported by Groom et al., and developed systemic lupus erythematosus and SS-like disease. However, there are also reports about the serum levels of BAFF that remain within, or even below, normal levels in a proportion of SS patients, indicating that the BAFF levels may fluctuate with inflammatory activity, the variations of the detection assays, and the progression of pSS [28]. Since this cytokine is crucial at several levels in the pathogenesis of pSS, it could serve as an ideal target for treatment, and precise examination methods will be needed. Two different classes of BAFF antagonists are at various stages of clinical development, selective BAFF blockers (anti-BLyS, belimumab, or Lymphostat B), and nonselective BAFF blockers (human Ig Fc), and given the

importance of BAFF, further clinical trials are needed for its use in human.

4.2. B-Cell Depletion Therapy. Overexpression of soluble factors, such as antibodies, IFN-α, and BAFF, as described previously, plays an important role in the initiation and continuation of pSS. Depletion of circulating B cells may provide an opportunity to reset the immune tolerance and regulate the abnormal functions of B cells, as well as, if possible, influence the T-cell compartment, since B cells participate in the antigen prestation and produce several important cytokines in the differentiation of T cells. The most widely studied target for achieving B-cell depletion is the CD20 antigen, a transmembrane protein found on pre-B and mature B cells but not on pro-B cells and normal plasma cells. The only anti-CD20 biologic agent tested in pSS is rituximab. Although the direct pathophysiological role of B cells in glandular tissue destruction in pSS has not been fully elucidated, B-cell depletion therapy with rituximab appears to be successful and improves salivary flow, which was reported by Meijer and his groups in 2010 [29, 30]. Meiners et al. reviewed 20 reported trials evaluating the effect of rituximab in pSS patients and found great discrepancy among the results. It seems that the result we can conclude from these trials is that pSS patients with early, active disease with extraglandular manifestations are most likely to benefit the most from rituximab treatment [31].

As detailed before, the cytokine BAFF plays a key role in B-cell differentiation, survival, and activation. As reported by Pers and his colleagues, following placebo-controlled RTX trial in pSS after B-cell depletion therapy with RTX showed an increase of BAFF in serum levels, indicating the role of BAFF in the repopulation of B cells after treatment [32]. In conclusion, targeting BAFF in combination with B-cell depletion seems logical and may hold therapeutic promise for pSS.

5. Immunogenetics of pSS

Immunogenetic mechanism of pSS is another issue that has been heatedly investigated, using a different kind of mouse models, including MRL/lpr, NZB/NZW F1-hybrids, nonobese diabetic (NOD), NFS/sld, and several new strains like Id3 gene knock-out (KO) mouse, the aromatase gene KO mouse, the Baff gene knock-in mouse, and the IQI/Jic mouse and the C57BL/6.NOD-Aec1Aec2 congenic line. Not surprisingly, studies using animal models of sjogren's syndrome indicate that disease susceptibility is multigenic, encompassing many critical causal elements [2].

Observations reported by Harley et al. suggest that pSS susceptibility is linked to HLA-DQ genes, specifically DQ1 and DQ2, when associated with the presence of anti-SS-A/Ro and anti-SS-B/La autoantibodies [33]. A study by Tzioufas using sera from 90 pSS patients from different countries in Europe showed a significant association of anti-Ro/SSA anti-La/SSB with the presence of HLA-DRB1$*$03 and DQB1$*$02, while the strength of this association varied with countries in which the data was collected. However, different MHC genetic linkages should be expected in different ethnic groups. The discrepancy among the results from different countries may be merely because of the relative homogeneity of the populations studied [34, 35].

Since abnormalities of the NF-κB signal transduction pathway and phosphorylation of IκBα in several autoimmune diseases are observed, analysis of the gene and protein expression profiles of SS monocytes has been carried out by Lisi et al. Their findings clearly demonstrate changes in the levels of IκBα in SS monocytes, suggesting that the attenuated expression of IκBα could contribute to the deregulation of NF-κB pathways in the SS pathogenesis. Decreased expression of IκBα may specifically amplify cytokines production and inflammatory response linked to Sjogren's syndrome [36].

Polymorphisms in genes encoding cytokines or factors implicated in cytokine signaling are also thought to participate in disease pathogenesis. As shown by Cay and his colleagues, polymorphism in the TNF-alpha gene promoter at position-1031 is associated with increased circulating levels of TNF-α, myeloperoxidase, and nitrotyrosine in primary Sjogren's syndrome [37].

6. Conclusion

Sjogren's syndrome is a multifaceted autoimmune disease. Even though the mechanism of its pathology has been studied since its discovery, the roles of different populations of immune cells remain inconclusive. How the cells and cytokines interact to promote the development of SS is a highly promising field of investigation. With the development of techniques, the complicated underlying genetic factors involved in pSS are promising to be identified. Although investigations about treatments targeting the immune factors participating in the progression of pSS show some positive outcome, more clinical trials are required before their application in human.

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Research Article

Correlation of Increased Blood Levels of GITR and GITRL with Disease Severity in Patients with Primary Sjögren's Syndrome

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Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a type I transmembrane protein belonging to the TNFR superfamily. After activated by its ligand GITRL, GITR could influence the activity of effector and regulatory T cells, participating in the development of several autoimmune and inflammatory diseases included rheumatoid arthritis and autoimmune thyroid disease. We previously reported that serum GITRL levels are increased in systemic lupus erythematosus (SLE) patients compared with healthy controls (HC). Here, we tested serum soluble GITR (sGITR) and GITRL levels in 41 primary Sjögren's syndrome (pSS) patients and 29 HC by ELISA and correlated sGITR and GITRL levels with clinical and laboratory variables. GITR and GITRL expression in labial salivary glands was detected by immunohistochemistry. pSS patients had significantly increased serum levels of sGITR and GITRL compared with controls (GITR: 5.66 ± 3.56 ng/mL versus 0.50 ± 0.31 ng/mL; $P < 0.0001$; GITRL: 6.17 ± 7.10 ng/mL versus 0.36 ± 0.28 ng/mL; $P < 0.0001$). Serum sGITR and GITRL levels were positively correlated with IgG (GITRL: $r = 0.6084$, $P < 0.0001$; sGITR: $r = 0.6820$, $P < 0.0001$) and ESR (GITRL: $r = 0.8315$, $P < 0.0001$; sGITR: $r = 0.7448$, $P < 0.0001$). Moreover, GITR and GITRL are readily detected in the lymphocytic foci and periductal areas of the LSGs. In contrast, the LSGs of HC subjects did not express GITR or GITRL. Our findings indicate the possible involvement of GITR-GITRL pathway in the pathogenesis of pSS. Further studies may facilitate the development of targeting this molecule pathway for the treatment of pSS.

1. Introduction

Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a type I transmembrane protein belonging to the TNFR superfamily, which was originally discovered by Nocentini et al. as a gene upregulated in dexamethasone-treated murine T cell hybridomas [1]. GITR has low basal expression on naive murine $CD4^+$ and $CD8^+$ T cells as well as human T cells, similar to other TNFR family members such as 4-1BB and OX40 [2–5]. However, both murine and human regulatory T cells (Tregs) constitutively express GITR [6]. GITR is activated by its ligand GITRL (TNFSF18), a type II transmembrane protein belonging to the TNF superfamily. GITRL is expressed at low levels by antigen-presenting cells such as macrophages, dendritic cells, and B cells and is upregulated upon activation, consistent with the ligands of OX40 and 4-1BB [5–8]. Although GITRL has also been found on endothelial cells and activated T cells, the role is unclear [9]. The GITR/GITRL pathway has been reported to modulate dendritic cell function and promote T-cell mediated immunity [10]. Like most TNFR family members, human GITR-GITRL interaction appears to be trimeric while the murine GITRL binds GITR through a dimeric fashion [11, 12]. Currently, the significance of the differential ligand binding GITR-GITRL between human and murine cells remains largely unclear.

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by keratoconjunctivitis sicca, xerostomia, and extraglandular abnormalities [13]. However, the precise etiology remains unclear. At the immunological

level, numerous studies have indicated that both T-cell activation and proinflammatory cytokine production are pivotally involved in pSS pathogenesis. Previous studies have demonstrated that the functional interaction of GITR with its cognate ligand GITRL can provide a costimulatory signal to both $CD4^+$ and $CD8^+$ naive T cells, enhancing proliferation and effector function [3, 6, 14, 15]. There is increasing evidence indicating that GITR and GITRL are involved in the pathogenesis of autoimmune disease. Several studies have reported that the GITR/GITRL pathway plays an important role in autoimmune diseases, as demonstrated by experimental models of experimental autoimmune encephalomyelitis, collagen-induced arthritis and autoimmune diabetes [15–17]. Our recent studies have revealed a close association of increased GITRL expression with disease activity in patients with SLE (Lei et al.) [18]. To determine whether GITR and GITRL system participates in pSS pathogenesis, we measured the serum levels of sGITR and GITRL in pSS patient and investigated correlation of their expression levels with clinical and laboratory variables. Moreover, the expression of the GITR and GITRL in the salivary glands of patients with pSS was also examined by immunohistochemistry.

2. Materials and Methods

2.1. Patients and Serum Samples. The study group comprised 41 patients (39 women and 2 men) with a mean age of 36.42 ± 18.71 years. All patients were recruited from the Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University between September 2011 and September 2012 and diagnosed with primary SS fulfilled the American-European Consensus Group Criteria for this diagnosis [19], and individuals with other rheumatic diseases, infections, or malignant tumors were excluded from the study. Sera were also collected from 29 healthy controls at the same hospital, and all recruited healthy controls were excluded from having any autoimmune diseases. There were no significant differences in the ages or sex ratios between the two groups. In addition, labial salivary gland (LSG) biopsy specimens were collected from 10 female patients that matched the histological criteria for a diagnosis of SS [20] and had severe cellular infiltration (focus index ≥ 1). The biopsies were performed for routine diagnostic purposes after obtaining the patient's consent. There were also 6 control LSG specimens from subjects who did not fulfill the classification criteria for pSS but had sicca symptoms, such as dry mouth or dry eye. The controls were matched for sex and age to the pSS patients and had been examined for the presence of rheumatic disease including secondary SS. Both the research protocol and the consent forms were approved by the Research Ethics Committee of Jiangsu Province Hospital.

2.2. Clinical and Immunological Data. All patients underwent extensive medical examinations and serological evaluations, including measurements of antinuclear antibodies (ANA), anti-Ro/SSA antibody (A-SSA), anti-La/SSB antibody (A-SSB), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), and rheumatoid factor (RF). In addition, the numbers of cellular infiltration per 4 mm^2 of tissue (focus index, FI) were measured. The clinical data from the patients were recorded in Table 1.

Table 1: Characteristics of pSS patients and the control subjects.

	pSS (n = 41)	Control (n = 29)
Age (years)	36.42 ± 18.71	34.53 ± 21.47
Sex (female/male)	39/2	28/1
Disease duration (months)	36.33 ± 42.25	—
Arthritis (%)	6 (14.63)	—
Fever (%)	8 (19.51)	—
Anemia (%)	12 (29.27)	—
Leukopenia (%)	20 (48.78)	—
Thrombocytopenia (%)	4 (9.76)	—
Renal disease (%)	7 (17.07)	—
Pulmonary interstitial changes (%)	5 (12.20)	—
Autoimmune liver disfunction (%)	4 (9.76)	—
ANA positive (%)	36 (87.80)	—
A—SSA positive (%)	34 (82.93)	—
A—SSB positive (%)	19 (46.34)	—
ESR (mm/H)	56.75 ± 39.38	—
CRP (mg/L)	4.86 ± 3.21	—
IgG (g/L)	24.76 ± 8.12	—
IgM (g/L)	1.97 ± 1.87	—
IgA (g/L)	3.76 ± 1.51	—
RF (IU/L)	67.79 ± 32.47	—
Focus index (1–3)	1.56 ± 0.77	—

pSS: primary Sjögren's syndrome; ANA: antinuclear antibody; A-SSA: anti-Ro/SSA antibody; A-SSB: anti-La/SSB antibody; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; IgG: immunoglobulin G; IgM: immunoglobulin M; IgA: immunoglobulin A; RF: rheumatoid factor; Focus index: the number of foci per 4 mm^2 of tissue.
Values are expressed as mean ± standard deviation.

A volume of 5 mL peripheral venous blood was collected from each patient and health control subject and waited to clot at room temperature for 2 hours. Samples were then centrifuged for 10 minutes at 800 g. The serum samples were separated and frozen at −80°C until further analysis.

2.3. Labial Salivary Gland Biopsy. LSG biopsies were taken from patients with sicca symptoms. For this, local anesthetic was injected into the lower lip and a small incision to the right or left of the lip midline was made. Four or five LSG lobules were harvested and placed into Carnoy's fixative for 24 hours. Standard paraffin preparations were prepared for sectioning at a thickness of 3 μm and stained with hematoxylin and eosin. The slides were examined for the presence of lymphocytic infiltrates and/or foci using standardized criteria. The focus index (FI) was recorded as the number of foci per 4 mm^2 of LSG tissues.

2.4. Measurement of Serum sGITR and GITRL. Serum sGITR and GITRL levels were measured using ELISA kits (RayBiotech Inc.) according to the manufacturer's protocols. Briefly, serum samples (1 : 50 dilution) and standards were added to the 96-well plate and incubated overnight at 4°C.

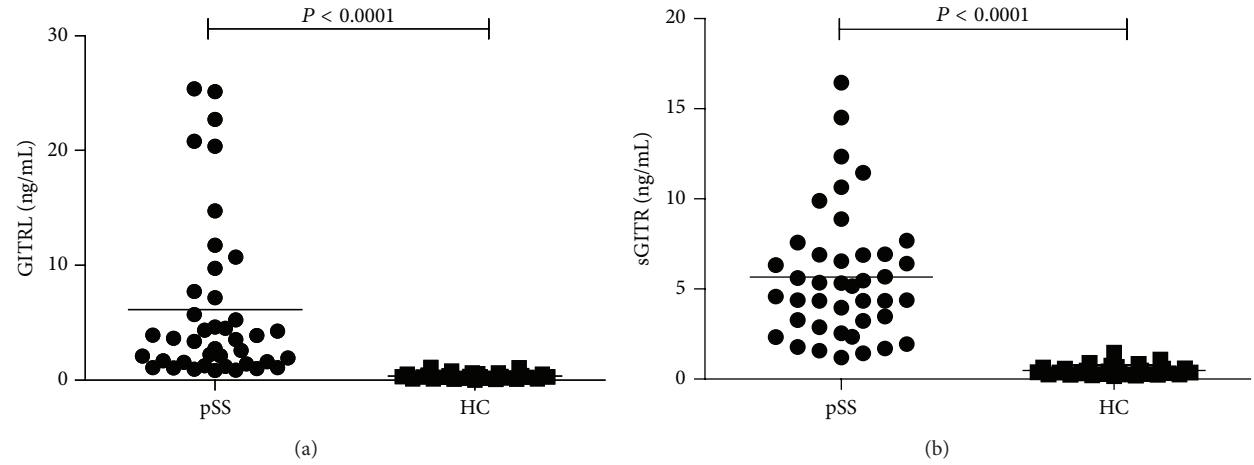

Figure 1: Comparison of serum GITRL and sGITR levels between pSS and HC. (a) Serum GITRL levels were significantly elevated in SS patients versus HC. (b) Serum sGITR levels were also significantly elevated in SS patients versus HC. Each symbol represents an individual patient and healthy donor. Horizontal lines indicate median values. GITRL: glucocorticoid-induced TNFR-related protein ligand; sGITR: soluble glucocorticoid-induced TNFR-related gene; SS: Sjögren's syndrome; HC: healthy control.

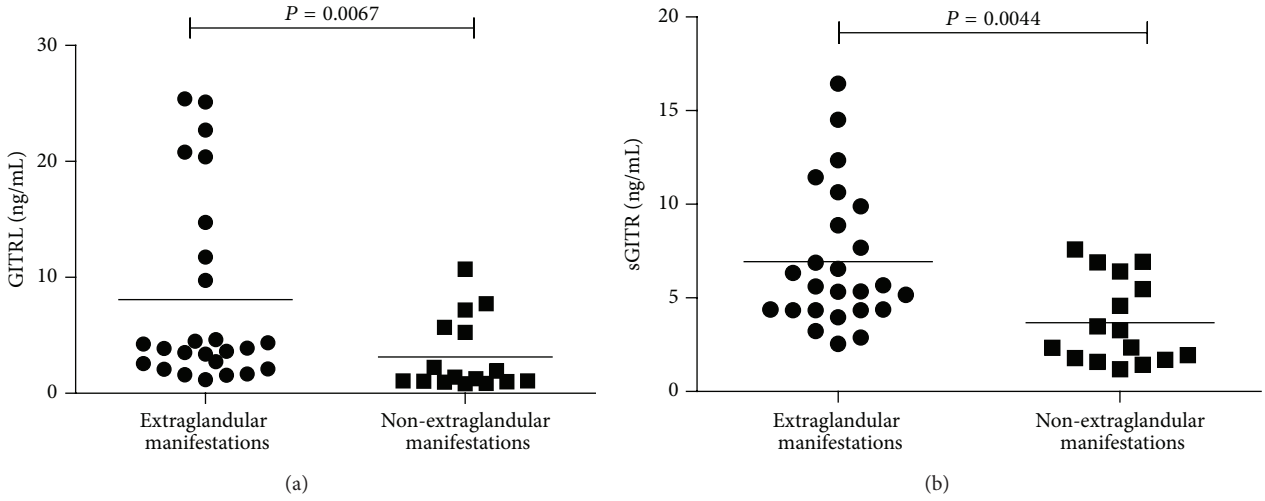

Figure 2: Comparison of serum GITRL and sGITR levels between pSS patients with or without extra-glandular manifestations. (a) Serum GITRL levels exhibited elevation in pSS patients with extra-glandular manifestations ($n = 25$) relative to patients without extra-glandular manifestations ($n = 16$). (b) Serum sGITR levels were also higher in pSS patients with extra-glandular manifestations ($n = 25$) than patients in the absence of extra-glandular manifestations ($n = 16$). Each symbol represents an individual patient; horizontal lines indicate median values.

After incubation for 2 hours and washing 3 times, biotinylated antihuman GITRL antibodies were added, followed by incubation with HRP-conjugated streptavidin and color development with TMB substrate solution. The intensity of the color reaction was measured by a microplate reader (Bio-Rad, Beijing, China) at a wavelength of 450 nm. Concentrations of sGITR and GITRL were determined by a standard curve according to the manufacturer's instructions.

2.5. Immunohistochemical Staining for GITR and GITRL. The fixed LSG biopsy specimen slides were fixed in Carnoy's fixative and embedded in paraffin wax. Paraffinized LSG tissues were sectioned to 3 μm thickness, deparaffinized in xylene, and rehydrated through a series of concentrations of ethanol. After inactivation of endogenous peroxidase, sections were blocked by incubation with 5% bovine serum album for 30 minutes at room temperature then incubated with either rabbit anti-human GITRL (Santa Cruz) or mouse anti-human GITR (R&D Systems) at 4°C overnight in a humidified chamber and both of which were diluted 1 : 50. The slides were washed for 5 minutes; sections were then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody for 1 hour at room temperature; both of which were diluted 1 : 200. The reactions were developed using a DAB substrate kit, with hematoxylin as counterstain. Each slide was evaluated by one of the authors (Ms. Xiaoke Feng) under a microscope (Nikon, Tokyo, Japan).

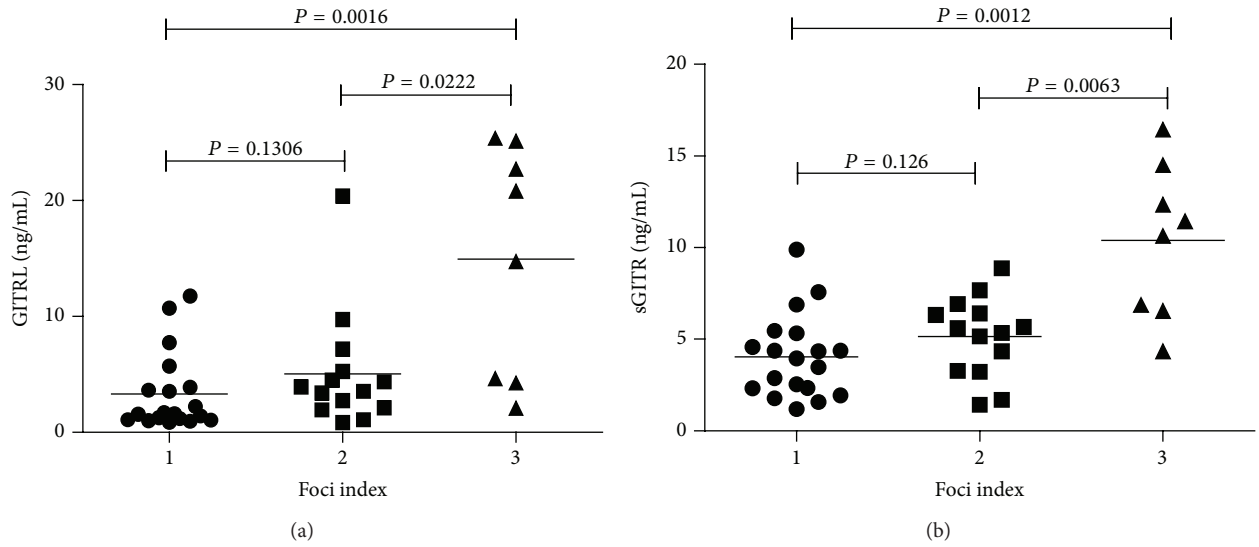

Figure 3: Comparison of serum GITRL and sGITR levels among pSS patients with different foci index. pSS patients were divided according to the foci index (1~3) in labial salivary glands (LSGs) of lymphocytic infiltration (see Methods). (a) Serum GITRL levels were significantly higher in pSS patients with serious sialadenitis. (b) Serum sGITR levels were also elevated as foci index increased in pSS patients.

2.6. Statistical Analysis. Data were presented as mean ± standard deviation unless specified otherwise. Statistical analysis was performed using SPSS for Windows (version 11.5). The Mann-Whitney rank sum test or Kruskal-Wallis tests were used to compare sGITR and GITRL levels. The correlation between GITRL/sGITR levels and various values were analyzed by Spearman's rank correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Primary SS Patients Showed Higher Serum GITRL and sGITR Levels Than Healthy Controls. Serum GITRL and sGITR levels in pSS ($n = 41$) and healthy controls ($n = 29$) were measured by ELISA analysis. As shown in Figure 1, serum levels of GITRL were significantly higher in pSS patients than in HC (6.17 ± 7.10 ng/mL versus 0.36 ± 0.28 ng/mL; $P < 0.0001$). In addition, serum sGITR levels were also markedly elevated in pSS patients when compared with HC (5.66 ± 3.56 ng/mL versus 0.50 ± 0.31 ng/mL; $P < 0.0001$). The results suggest that GITRL and sGITR overexpression is possibly involved in the pathogenesis of pSS.

3.2. Serum GITRL and sGITR Levels Were Markedly Higher in Primary SS Patients with Extraglandular Manifestations. We further divided pSS patients into groups with or without extraglandular manifestations according to their symptoms. pSS patients with sicca symptoms only such as dry mouth or dry eye were defined as the non-extraglandular manifestations group, while other patients with fever, arthritis, anemia, leukopenia, thrombocytopenia, renal disease, pulmonary interstitial changes, or autoimmune liver dysfunction were extraglandular manifestations group. As shown in Figure 2, both serum GITRL and sGITR levels in pSS patients with extra-glandular manifestations were significantly higher than those without extra-glandular manifestations (GITRL: 8.60 ± 8.25 ng/mL versus 3.15 ± 3.13 ng/mL; $P = 0.0067$. sGITR: 6.92 ± 3.70 ng/mL versus 3.68 ± 2.27 ng/mL; $P = 0.0044$).

3.3. Elevated Serum GITRL and sGITR Levels Were Associated with Increased Foci Index (FI) in Primary SS Patients. The FI was recorded as the number of foci per 4 mm^2 of LSGs. We divided pSS patients into various groups according to their FI. As shown in Figure 3, elevated GITRL and sGITR levels were closely associated with increased severity of lymphocytic infiltration. Serum GITRL levels were significantly higher in FI 3 group of pSS patients (FI 3 versus FI 2: 14.97 ± 9.95 ng/mL versus 5.16 ± 5.20 ng/mL; $P = 0.0222$. FI 3 versus FI 1: 14.97 ± 9.95 ng/mL versus 3.31 ± 3.33 ng/mL; $P = 0.0016$). Similarly, serum sGITR also markedly elevated in FI 3 group (FI 3 versus FI 2: 10.39 ± 4.18 ng/mL versus 5.19 ± 2.16 ng/mL; $P = 0.0063$. FI 3 versus FI 1: 10.39 ± 4.18 ng/mL versus 4.04 ± 2.27 ng/mL; $P = 0.0012$). However, the FI 2 group did not differ significantly from the FI 1 group.

3.4. Correlation of Serum GITRL and sGITR Levels with Other Laboratory Observations. To further determine the relationship between serum GITRL/sGITR levels and laboratory test results including the titers of ANA, A-SSA, A-SSB, ESR, CRP, RF, and Ig levels, it was found that both serum GITRL and sGITR levels were positively correlated with ESR (GITRL: $r = 0.8315$, $P < 0.0001$; sGITR: $r = 0.7448$, $P < 0.0001$, Figures 4(a) and 4(c)) and IgG (GITRL: $r = 0.6084$, $P < 0.0001$; sGITR: $r = 0.6820$, $P < 0.0001$, Figures 4(b) and 4(d)). However, no significant correlations were found between serum GITRL or sGITR levels and the other laboratory values (data not shown). Interestingly, when patients were grouped according to test results normal or abnormal,

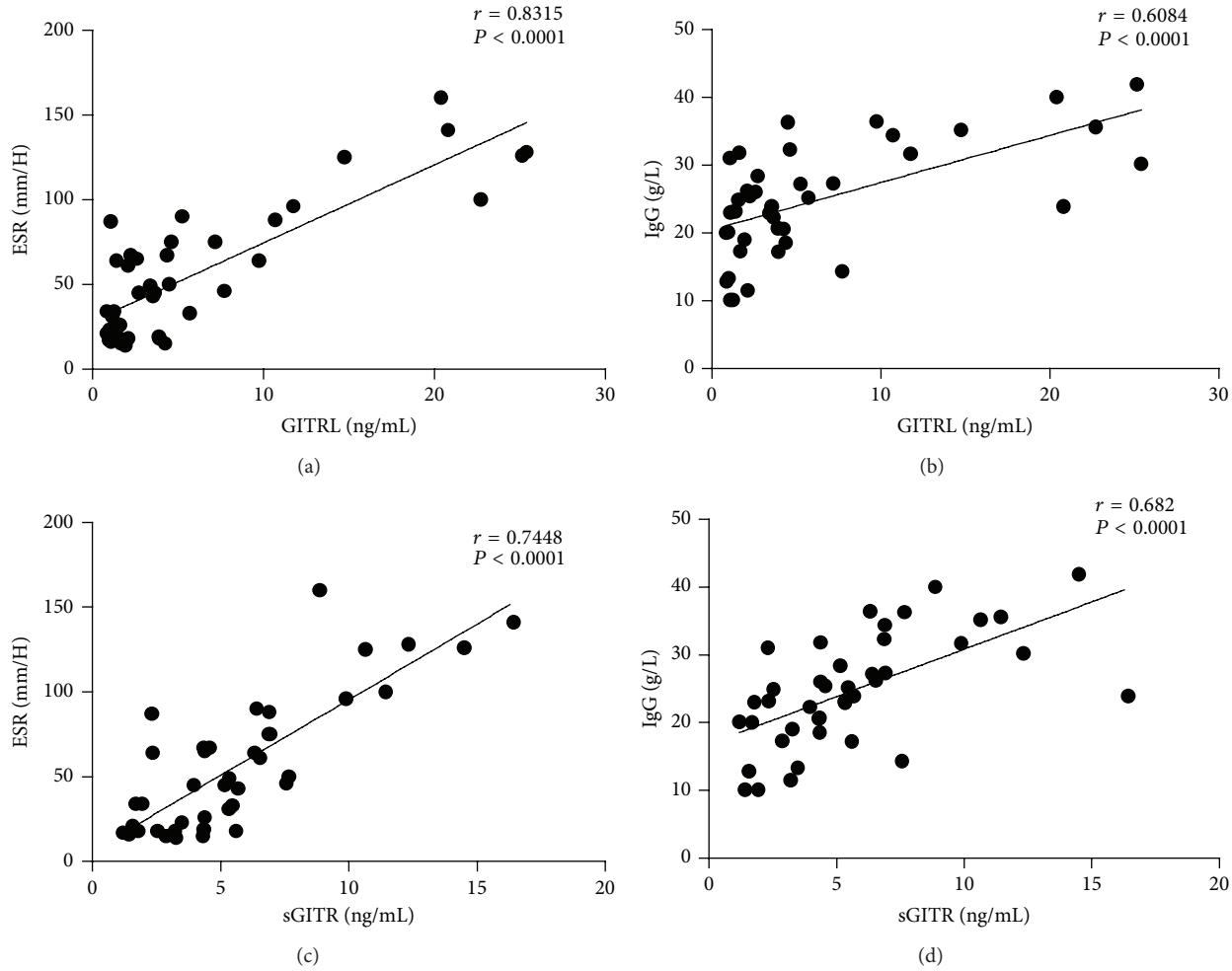

Figure 4: Correlation of serum GITRL and sGITR levels with laboratory values. (a), (b) Positive correlation was observed between serum GITRL levels and ESR, IgG. (c), (d) Positive correlation was also seen between serum sGITR levels and ESR, IgG. ESR: erythrocyte sedimentation rate; IgG: immunoglobulin G.

elevated serum GITRL levels exhibited were found in the groups with high titers of ANA (6.85 ± 7.32 versus 1.22 ± 0.42, $P = 0.0050$) or high concentration of CRP (15.87 ± 8.76 versus 4.41 ± 4.75, $P = 0.0078$), RF (8.98 ± 8.64 versus 2.47 ± 1.72, $P = 0.0050$), IgM (10.48 ± 8.96 versus 5.01 ± 6.30, $P = 0.0431$), and IgA (7.93 ± 7.92 versus 2.34 ± 1.94, $P = 0.0069$), while serum sGITR levels were significantly elevated in the patients with high titers of ANA (6.09 ± 3.58 versus 2.51 ± 0.83, $P = 0.0114$) or high concentration of CRP (7.05 ± 3.60 versus 4.76 ± 3.305, $P = 0.0231$), RF (6.93 ± 3.95 versus 3.85 ± 1.85, $P = 0.0075$), and IgA (6.48 ± 3.81 versus 3.87 ± 2.14, $P = 0.0381$). In addition, patients with leukopenia were observed having markedly increased serum GITRL and sGITR levels (GITRL: 9.18 ± 8.91 versus 3.29 ± 2.96, $P = 0.0158$; sGITR: 7.28 ± 4.03 versus 4.10 ± 2.18, $P = 0.0110$), as shown in Table 2.

3.5. High Expression of GITRL and GITR in the Labial Salivary Glands (LSGs) from Primary SS Patients. To evaluate the local effect of GITRL and GITR in LSGs, we applied immunohistochemical staining to determine the expression of GITRL and GITR in LSGs. The freshly explanted lower lip biopsy specimens were sectioned and stained with anti-GITRL and anti-GITR antibodies. All 10 pSS samples exhibited distinct expression of GITRL, while 6 of the 10 samples were positive for GITR. In contrast, none of the LSGs from the sicca complainers exhibited GITRL or GITR expression. Six specimens from pSS patients stained for GITR showed similar pattern of GITRL and GITR expression; that is, GITRL was prominent in infiltrating lymphocytes and ductal cells, while GITR was mainly expressed in infiltrating lymphocytes with a weak expression observed on ductal cells. Overall, the expression of GITRL was stronger and more widely distributed than GITR. Both GITRL and GITR were expressed on lymphocytic infiltrates and to a lesser degree in the acinar components (Figure 5).

4. Discussion

In this study, we determined the serum levels of sGITR and GITRL in pSS patients and further revealed the positive

Table 2: Comparison of serum GITRL and sGITR levels between pSS patients with normal or abnormal laboratory values.

Parameter	GITRL (ng/mL)			sGITR (ng/mL)		
	Normal Mean ± SD (*n*)	Abnormal Mean ± SD (*n*)	*P* value	Normal Mean ± SD (*n*)	Abnormal Mean ± SD (*n*)	*P* value
A—SSA	3.23 ± 3.63 (7)	6.76 ± 7.52 (34)	0.1362	3.91 ± 2.14 (7)	6.01 ± 3.71 (34)	0.1657
A—SSB	6.41 ± 7.38 (22)	5.95 ± 7.03 (19)	0.5830	5.71 ± 3.57 (22)	5.60 ± 3.63 (19)	0.8857
ANA	1.22 ± 0.42 (5)	6.85 ± 7.32 (36)	**0.0050	2.51 ± 0.83 (5)	6.09 ± 3.58 (36)	*0.0114
CRP	4.41 ± 4.75 (25)	15.87 ± 8.76 (16)	**0.0078	4.76 ± 3.30 (25)	7.05 ± 3.60 (16)	*0.0231
RF	2.47 ± 1.72 (17)	8.98 ± 8.64 (24)	**0.0050	3.85 ± 1.85 (17)	6.93 ± 3.95 (24)	**0.0075
IgM	5.01 ± 6.30 (33)	10.48 ± 8.96 (8)	*0.0431	5.31 ± 3.39 (33)	7.08 ± 4.13 (8)	0.2428
IgA	2.34 ± 1.94 (13)	7.93 ± 7.92 (28)	**0.0069	3.87 ± 2.14 (13)	6.48 ± 3.81 (28)	*0.0381
WBC	3.29 ± 2.96 (21)	9.18 ± 8.91 (20)	*0.0158	4.10 ± 2.18 (21)	7.28 ± 4.03 (20)	*0.0110

$P < 0.05$ means significant difference ($^{*}P < 0.05$; $^{**}P < 0.01$).
Laboratory values such as A-SSA, A-SSB, ANA, and RF positive were defined as abnormal, while laboratory parameters such as CRP, IgM, and IgA above limit values were defined as abnormal. In addition, WBC abnormal means leukopenia.
A-SSA: anti-Ro/SSA antibody; A-SSB: anti-La/SSB antibody; ANA: antinuclear antibody; CRP: C-reactive protein; RF: rheumatoid factor; IgM: immunoglobulin M; IgA: immunoglobulin A; WBC: white blood count.

correlation of their expression levels with IgG and ESR, which were in association with disease severity of pSS. In addition, we have shown for the first time the expression pattern of GITR and GITRL in the salivary glands of patients with pSS.

Several studies including our recent findings have suggested that GITR-GITRL system is involved in the pathogenesis of autoimmune disease including rheumatoid arthritis (RA) and SLE [18, 21]. In several mouse autoimmune disease models including experimental autoimmune encephalomyelitis, collagen-induced arthritis, inflammatory bowel diseases, gastritis, thyroiditis, and autoimmune diabetes, GITR inhibition resulted in an anti-inflammatory effect. In accordance, GITR triggering by GITRL or anti GITR antibody exerted an increased inflammatory response [15–17, 22–24]. GITR and GITRL system also participates in the development of autoimmune diseases in human as demonstrated in RA recently, which was found that GITRL protein levels in the serum samples of RA patients were significantly higher than those in samples from healthy control subjects. Furthermore, the increased levels of GITRL in RA patients were positively correlated with the DAS-28 scores of these patients [21]. Recently, we have reported that serum GITRL levels are markedly increased in patients with SLE compared with healthy controls, especially in patients with active disease. Moreover, serum GITRL levels are found to be positively correlated with SLEDAI, titers of anti-dsDNA antibody, ESR, and IgM but negatively correlated with C3. In addition, serum GITRL levels elevated in SLE patients with renal involvement and vasculitis compared with patients that the above-mentioned manifestations were absent [18]. However, the role of GITR-GITRL system in the pathogenesis of SS is poorly understood. Saito et al. have demonstrated that Sjögren's syndrome-like autoimmune sialadenitis in MRL-Fas^{lpr} mice was associated with expression of GITRL in salivary gland duct epithelial cells [25], consistent with our current observations. Together, these results indicate a proinflammatory role of GITR/GITRL pathway in driving autoimmune progression in various autoimmune diseases.

Recent studies have suggested that in most of inflammatory and autoimmune diseases, the activation of GITR-GITRL pathway promotes leucocyte extravasation, increases T lymphocyte activation, and partially reverses the immunosuppressive function of Treg [26–28]. Several lines of evidence support the notion that the insufficiency or dysfunction of Treg may contribute to the breakdown of immune tolerance, leading to the procession of autoimmune diseases along with the interruption of immune homeostasis [29–31]. Since previous studies have reported that the numbers of Treg are positively correlated with higher grade of infiltration at the salivary glands in pSS [32], we speculate that GITR/GITRL activation may participate in pSS pathogenesis by inhibiting Treg immunosuppressive activity and increasing T lymphocyte activation. It has been reported that signaling downstream of GITR/GITRL pathway results in the activation of NF-κB as well as the members of the MAPK pathway including p38, JNK, and ERK [1, 3, 33], which in turn enhances T cell survival by upregulating IL-2Ra, IL-2, and IFN-γ [3]. Further studies are warranted to investigate how GITR/GITRL pathway modulates the homeostatic regulation

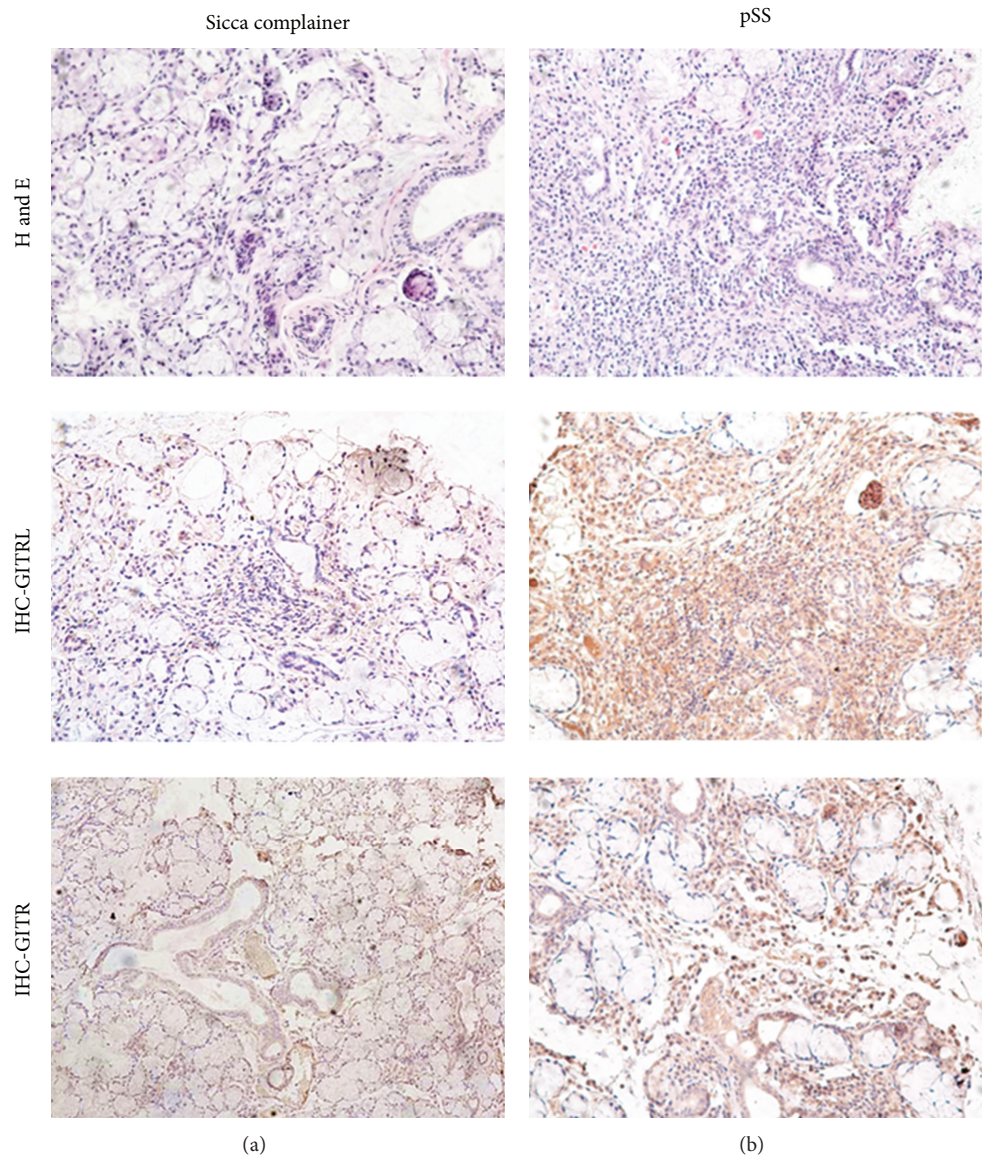

Figure 5: High expression of GITRL and GITR in labial salivary glands from patients with pSS. The labial salivary glands (LSG) of pSS patients exhibit increased GITRL and GITR expression. Shown is the expression of the GITRL and GITR in the labial salivary glands of sicca complainer ($n = 6$, left column) and patients with pSS ($n = 10$; right column), as determined by immunostaining using specific antibodies. The cells that stained with the antibodies appeared in brown. The infiltrating lymphocytes and periductal areas of LSG from the patients with pSS exhibited intense GITRL and GITR staining, whereas the expression of GITRL was stronger and more widely distributed than GITR. In contrast, the sicca complainers did not exhibit any GITRL or GITR expression in their labial salivary glands.

of Treg/Th17 cells during the pathogenesis of autoimmune diseases.

In this study, we have demonstrated a close correlation of serum elevated GITRL and sGITR with the increased degree of lymphocytic infiltration in patients with pSS. Moreover, GITR and GITRL were readily detected in the lymphocytic foci and periductal areas of the LSGs. In contrast, the LSGs of healthy control subjects did not express GITR or GITRL. Moreover, we have further detected a sharp decrease of the stimulated salivary flow in the pSS patients with glandular cells positively stained for GITRL (data not shown). Our findings suggest that GITR and GITRL may play an important role in the sialadenitis suffered by patients with pSS. In addition, our investigations have revealed a close correlation of circulating sGITR and GITRL levels with the disease activity and severity in pSS patients. Our data have clearly shown that serum levels of sGITR and GITRL positively correlate with ESR and IgG, and exhibit elevation in the groups with extra-glandular manifestations as well as abnormal laboratory parameters such as ANA, CRP, IgM, IgA, and WBC. We provide new evidence indicating involvement of GITR/GITRL overactivation in the disease pathophysiology

of pSS, which may serve as a new biomarker to assess the disease activity and severity of pSS.

5. Conclusion

Our findings indicate the possible involvement of GITR-GITRL pathway in the pathogenesis of pSS. Further investigations on the systemic and localized effects of GITR and GITRL in pSS may facilitate the development of targeting this molecule pathway for the treatment of pSS.

Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work, and there is no professional or other personal interest of any product, service and/or company that influence them.

Acknowledgments

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Research Article

The Correlations of Disease Activity, Socioeconomic Status, Quality of Life, and Depression/Anxiety in Chinese Patients with Systemic Lupus Erythematosus

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The prevalence of psychological problems is frequent in systemic lupus erythematosus (SLE) patients and appears to be increasing. The current study investigated the relationship among disease parameters, quality of life, and the psychological status in Chinese patients with SLE. A self-report survey design was administered to 170 SLE patients and 210 healthy individuals using the Self-Rating Anxiety Scale, the Self-Rating Depression Scale, and the Short Form 36 health survey (SF-36). Our results showed that 20.3% SLE patients had anxiety, and 32.9% had depression, which were significantly higher than the control group (7.1%, 14.3%, resp.). And there were significant correlations among socioeconomic status (SES), disease activity, and anxiety/depression in SLE patients. Meanwhile, SF-36 analysis results revealed that VT, PF, and RP scales were the most powerful predictors of anxiety of SLE patients, and SLEDAI, VT, PF, SF, and RE domains were significantly accounted for anxiety. In summary, there were significant relationships among disease parameters, quality of life, and anxiety/depression in Chinese SLE patients. Therefore, it is necessary to have psychiatric and psychological evaluations and formulate an integrated approach for managing mental health in Chinese lupus patients, especially those who have high disease activity, low SES, and poor quality of life.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that may affect multiple organ systems, including the central nervous system (CNS) [1]. Psychiatric symptoms are present in the majority of patients with SLE, among which major depression is the most common psychiatric manifestations [2]. Depression presented in 11%–39% of patients may be the initial symptom before the diagnosis of SLE [3]. It was reported that there were 4 times higher prevalence of depression in SLE compared to a matched, non-SLE population. In addition, anxiety is quite common in SLE patients, often as a reaction to the illness. Ainiala and colleagues have reported that the anxiety disorders were twice as prevalent among SLE patients as compared to the controls [4].

Even though SLE presents accompany with a wide variety of treatable psychiatric symptoms, such as depression and anxiety, they rarely seek and receive adequate treatment [5]. Overlooking anxiety and depression may have severe consequences for the patients, such as increased incidence of cardiovascular disease [6], myocardial infarction [7], suicidal ideation [8, 9] and death [10], decreased quality of life [11, 12], disability, and the loss of employment. Anyone, in turn, can worsen anxiety and depression symptoms [5].

The pathogenesis of psychiatric symptoms in lupus is still not well understood, but in which genetic and environmental factors may play a pivotal role. Depression and anxiety

may also be present as a reaction to a serious recurring, painful illness, which is associated with visible symptoms such as insomnia, fatigue, and limited functioning [13, 14]. Socioeconomic status (SES) is broadly employed in health research, signaling the importance of socioeconomic factors for health outcomes. Low SES is generally associated with high psychiatric morbidity, depression [15], and mortality [16]. Whether depression and anxiety are associated with lupus activity remains debatable.

There are several studies focus on psychological problems in China lupus patients. A study from Hong Kong has found that anxiety disorder was present in 22% SLE patients, and 18.2% had depression [17]. A study from Anhui medical university has reported that the prevalence of depression was 59.3% and correlated with suicidal ideation in SLE patients [8]. But there are few studies that focus on disease parameters, quality of life, and depression/anxiety in SLE patients from China mainland.

Thus, the aim of this study was to examine the relationship among disease parameters, quality of life, and the psychological status in Chinese patients with SLE. Moreover, we wished to ascertain the possible risks of anxiety and depression.

2. Patients and Methods

2.1. Participants. SLE patients were recruited from Affiliated Hospital of Nantong University between January 2010 and July 2011. A total of 170 SLE patients and 210 healthy individuals were consecutively invited to participate in a single-center cross-sectional study. Healthy individuals were used as the control group. All patients fulfilled the 1997 American College of Rheumatology (ACR) revised criteria for the classification of SLE. Patients were excluded based on the following: (1) they did not complete questionnaire; (2) they had comorbidities (e.g., serious infections or cardiac, respiratory, gastrointestinal, neurological, or endocrine diseases) that could influence SLE activity. Control subjects were excluded if they exhibited current or history of other systemic diseases or psychiatric disorders. This study was approved by the Ethics Committee of Affiliated Hospital of Nantong University, and written informed consent was obtained from all participants.

2.2. Measures of Clinical Variables. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure disease activity [18].

2.3. The Revised Self-Rating Anxiety Scale (SAS) [19]. SAS was used to evaluate the level of anxiety-related symptoms during the week prior to the survey. This self-administered test has 20 questions, with 15 items reflecting increasing anxiety levels and 5 questions reflecting decreasing anxiety levels. Each question was scored on a scale from 1 to 4 (rarely, sometimes, frequently, and always). The scores ranged between 20 and 80: scores greater than 70 suggest severe anxious symptoms, scores between 60 and 69 indicate moderate to marked anxiety, scores between 50 and 59 suggest minimal to mild anxiety, and scores less than 50 indicate no anxious symptoms.

2.4. The Revised Self-Rating Depression Scale (SDS) [20]. SDS is a 20-item questionnaire designed to assess mood symptoms over the past week (e.g., "I feel downhearted, blue and sad"). Each item is scored on a Likert scale ranging from 1 to 4; scores greater than 70 suggest severe depressive symptoms, scores between 60 and 69 indicate moderate to marked depression, scores between 50 and 59 suggest minimal to mild depression, and scores less than 50 indicate no depressive symptoms.

2.5. Measure of the Quality of Life [21]. The patient's general health status was measured using the Short Form- (SF-) 36 questionnaire, which measured eight multi-item dimensions: physical functioning (PF, 10 items); role limitations due to physical problems (RP, four items); role limitations due to emotional problems (RE, three items); social functioning (SF, two items); mental health (MH, five items); energy/vitality (VT, four items); body pain (BP, two items); and general health perception (GH, five items). For each dimension, item scores were coded, summed, and transformed on a scale from 0 (worst possible health state measured by the questionnaire) to 100 (best possible health state).

2.6. Statistical Analysis. The data were expressed as means ± SDs for continuous variables and as frequencies (%) for categorical variables. The Statistical Package for SPSS 18.0 was used for all data management and analyses. Descriptive analyses were performed to investigate the participants' characteristics. Student's t-test was used in independent groups for parametric variables, and the Spearman's correlation analysis was used to assess the correlation between parametric variables. Stepwise regression analyses were conducted for SAS and SDS scores separately to explore the significant predictors of dimorphic concerns. We considered $P < 0.01$ and $P < 0.001$ to be highly statistically significant and $P < 0.05$ to be statistically significant.

3. Results

3.1. Sample Characteristics. 12 SLE patients and 14 healthy individuals did not complete the questionnaire, resulting in the enrollment of 158 SLE patients (14 males and 144 females) and 196 healthy individuals (20 males and 176 females) in the current study. Table 1 showed their demographic data, medical and psychological variables. There was no significant difference in the ages, genders, marital status, education, work status, income/person, and menstrual history between the SLE patients and the controls. The SAS and SDS scores were significantly higher in the SLE group compared to the control group. According to the cut-off scores, anxiety disorder was present in 32/158 (20.3%), and 52/158 (32.9%) had depression, which were significantly higher than the healthy group ((14/196, 7.1%) and (28/196, 14.3%), resp.) ($P < 0.01$). As shown in Table 2, the scores of all the 8 scales were lower in SLE patients compared with healthy individuals.

Table 1: Demographic, psychological, and disease characteristics in SLE patients and controls.

Variables	SLE patients ($N = 158$)	Control subjects ($N = 196$)	P
Female gender[a]	144 (91.2)	176 (89.8)	0.76
Age, years[b]	32.9 ± 10.2	35.0 ± 11.4	0.20
SAS (≥50)[a]	32 (20.3)	14 (7.1)	<0.01
SDS (≥53)[a]	52 (32.9)	28 (14.3)	0.003
SLEDAI	11.8 ± 9.5		
Marital status[b]			
Single	32 (20.3)	56 (18.6)	0.20
Married	126 (79.7)	140 (71.4)	
Education[b]			
<9 years	86 (54.4)	96 (49.0)	0.47
≥9 years	72 (45.6)	100 (51.0)	
Work status[b]			
Working	30 (19.0)	44 (22.5)	0.57
Unemployed	128 (81.0)	152 (77.5)	
Income/person[b]			
≤2000 yuan	100 (63.3)	118 (60.2)	0.68
>2000 yuan	58 (36.7)	78 (39.8)	
Menstrual history[b]			
Normal	96 (66.7)	102 (58.0)	0.26
Abnormal	48 (33.3)	74 (42.0)	

[a]Mean ± SD. [b]Number (percentage).
SAS: revised Self-Rating Anxiety Scale; SDS: revised Self-Rating Depression Scale; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Table 2: Correlations between psychological scores, disease parameters, and quality of life in SLE patients.

Variables	SAS		SDS	
	r	P	r	P
Domains of SF-36				
PCS	−0.53	<0.0001	−0.53	<0.0001
MCS	−0.68	<0.0001	−0.73	<0.0001
PF	−0.49	<0.0001	−0.54	<0.0001
RP	−0.55	<0.0001	−0.52	<0.0001
BP	−0.05	0.66	0.05	0.65
GH	−0.36	0.001	−0.37	0.0009
VT	−0.4	0.0003	−0.43	0.0001
SF	−0.49	<0.0001	−0.57	<0.0001
RE	−0.63	<0.0001	−0.64	<0.0001
MH	−0.31	0.005	0.34	0.003

SAS: revised Self-Rating Anxiety Scale; SDS: revised Self-Rating Depression Scale; PCS: physical components summary; MCS: mental components summary; PF: physical functioning; RP: role limitations due to physical problems; RE: role limitations due to emotional problems; SF: social functioning; MH: mental health; VT: energy/vitality; BP: body pain; GH: general health perception.

There was significant difference between physical functioning (PF), role limitations due to physical problems (RP), role limitations due to emotional problems (RE), mental health (MH), and energy/vitality (VT) in SLE and control group ($P < 0.05$).

3.2. Correlations between Psychological Scores, Disease Parameters, and Quality of Life in SLE Patients. Previous studies have shown that low socioeconomic factors (SES) were generally associated with high psychiatric morbidity, depression, and anxiety [22]. As show in Table 3, we have found that there were significant correlations between SES (low education, work status, and income) and anxiety/depression in SLE patients. In addition, gender and menstrual history were some examples of depression risk factors. There was a significant positive correlation between anxiety/depression severity (assessed using SAS/SDS score) and disease activity (SLEDAI score). Previous studies have found that impaired quality of life and functional disability were independent risk factors of psychological disorders [12]. In the present study, we found that all the 8 scales of SF-36 domains and PCS/MCS were significantly correlated with SAS and SDS scores except body pain (BP) scale ($P < 0.05$).

3.3. Stepwise Regression Analysis for Anxiety and Depression. Multiple stepwise regression analysis revealed that VT, PF, and RP scales of SF-36 were the most powerful predictors of anxiety of SLE patients (Table 4). Meanwhile, SLEDAI, VT, PF, SF, and RE domains of SF-36 were significantly accounted for anxiety (Table 5).

4. Discussion

The present study confirmed that Chinese SLE patients were more likely to suffer from anxiety and depression than healthy individuals. Psychological problems significantly correlated with SES, disease status, and quality of life. SLE patients with anxiety and depression were in low SES and had worse disease status, lower quality of life. Among the assessed parameters, VT, PF, and RP scales of SF-36 were major contributors to anxiety in SLE patients, while disease activity and VT, PF, SF, and RE domains of SF-36 contributed to depression.

SES is broadly employed in health research, signaling the importance of socioeconomic factors for health outcomes. Previous study has found that poorer coping styles, ongoing life events, stress exposure, and weaker social support were some examples of depression risk factors that were more prevalent in lower SES groups [23]. Regarding the direction of the association for SES and depression, results more consistently supported the idea that causation (low SES increases risk of depression) outweighed selection (depression hinders social mobility), although both directions may operate simultaneously [17]. It is well known that SES is multifactor. Occupation [5, 24, 25], education, and income [26] were frequently used as measures of SES. With notable exceptions, there were significant relationships between anxiety/depression and SES [5]. There was a substantial body of research linking SES, anxiety/depression, and SLE. Waheed et al. found that educational qualification had significant association with anxiety and depression. Marital status, gender, economic activity, and monthly family income had no

Table 3: Disease status and quality of life in the anxious and depressed subgroups.

Variables	SAS			SDS		
	<50	≥50	*P*	<53	≥53	*P*
Age[a]	32.3 ± 10.3	35.2 ± 9.6	0.32	32.4 ± 10.6	34.0 ± 9.4	0.51
Sex[b]						
Male	6 (9.5)	1 (6.3)	0.68	7 (13.2)	0 (0.0)	0.05
Female	57 (90.5)	15 (92.7)		46 (86.8)	26 (100.0)	
BMI	21.2 ± 2.7	21.1 ± 2.9	0.93	21.1 ± 2.8	21.4 ± 2.6	0.61
Marital status[b]						
Single	15 (23.8)	1 (6.3)	0.25	14 (26.4)	2 (7.7)	0.11
Married	48 (76.2)	15 (93.7)		38 (73.6)	24 (92.3)	
Education[b]						
<9 years	29 (46.0)	14 (87.5)	0.003	23 (43.4)	20 (76.9)	0.005
≥9 years	34 (54.0)	2 (12.5)		30 (56.6)	6 (23.1)	
Work status[b]						
Working	15 (23.8)	0 (0)	0.03	13 (24.5)	2 (7.7)	0.07
Unemployed	48 (76.2)	16 (100.0)		40 (75.5)	24 (92.3)	
Income/person[b]						
≤2000 yuan	36 (57.1)	14 (87.5)	0.02	28 (52.8)	22 (84.6)	0.006
>2000 yuan	27 (42.9)	2 (12.5)		25 (47.2)	4 (15.4)	
Menstrual history[b]						
Normal	39 (68.4)	9 (60.0)	0.54	36 (79.2)	12 (46.2)	0.006
Abnormal	18 (31.6)	6 (40.0)		10 (20.8)	14 (53.8)	
Years since diagnosis of SLE[b]						
<1	11 (17.5)	2 (12.5)	0.85	10 (18.9)	3 (11.5)	0.63
1–5	31 (49.2)	9 (56.3)		27 (50.9)	13 (50.0)	
>5	21 (33.3)	5 (31.2)		16 (30.2)	10 (38.5)	
SLEDAI[a]	10.4 ± 7.3	17.1 ± 14.7	0.01	10.3 ± 7.7	14.8 ± 12.1	0.046
Domains of SF-36						
PCS[a]	256.7 ± 60.7	158.1 ± 51.7	<0.0001	264.0 ± 58.6	181.1 ± 61.5	<0.0001
MCS[a]	282.8 ± 67.0	161.7 ± 60.1	<0.0001	295.1 ± 56.9	183.3 ± 72.9	<0.0001
PF[a]	86.8 ± 14.1	61.3 ± 28.8	<0.0001	88.9 ± 12.2	66.9 ± 26.1	<0.0001
RP[a]	52.4 ± 42.5	4.7 ± 13.6	0.0001	57.1 ± 43.1	13.5 ± 23.7	<0.0001
BP[a]	63.8 ± 26.8	54.0 ± 20.0	0.18	63.6 ± 26.6	58.2 ± 24.4	0.38
GH[a]	53.6 ± 14.0	38.1 ± 13.3	0.0001	54.4 ± 12.6	42.5 ± 16.8	0.0007
VT[a]	66.6 ± 16.1	48.1 ± 19.5	0.0002	68.8 ± 15.0	50.8 ± 18.7	<0.0001
SF[a]	86.7 ± 29.3	64.1 ± 34.4	0.01	91.7 ± 26.8	62.5 ± 31.6	0.0001
RE[a]	67.2 ± 38.1	0 ± 0	<0.0001	71.7 ± 36.0	16.7 ± 33.0	<0.0001
MH[a]	62.3 ± 15.1	49.5 ± 19.4	0.006	62.9 ± 13.9	53.4 ± 20.3	0.017

[a]Mean ± SD. [b]Number (percentage).
SAS: revised Self-Rating Anxiety Scale; SDS: revised Self-Rating Depression Scale; PCS: physical components summary; MCS: mental components summary; PF: physical functioning; RP: role limitations due to physical problems; RE: role limitations due to emotional problems; SF: social functioning; MH: mental health; VT: energy/vitality; BP: body pain; GH: general health perception.

Table 4: Stepwise regression analyses of medical and psychological variables and their relationship to SAS in SLE patients.

SAS	Coef.	Std. Err.	*t*	*P*	[95% CI]
VT	−0.15	0.05	−3.00	0.004	−0.25, −0.05
PF	−0.11	0.05	−2.27	0.026	−0.22, −0.01
RP	−0.09	0.02	−3.64	<0.001	−0.14, −0.04

SAS: revised Self-Rating Anxiety Scale; VT: energy/vitality; PF: physical functioning; RP: role limitations due to physical problems.

Table 5: Stepwise regression analyses of medical and psychological variables and their relationship to SDS in SLE patients.

SDS	Coef.	Std. Err.	t	P	[95% CI]
SLEDAI	0.19	0.09	2.08	0.04	0.01, 0.37
VT	−0.19	0.05	−3.48	0.001	−0.30, −0.08
PF	−0.13	0.05	−2.60	0.01	−23.8, −0.03
SF	−0.09	0.03	−2.69	0.009	−0.15, −0.02
RE	−0.08	0.02	−3.13	0.003	−0.12, −0.03

SDS: revised Self-Rating Depression Scale; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; VT: energy/vitality; PF: physical functioning; SF: social functioning; RE: role limitations due to emotional problems.

effect on the frequency of anxiety and depression [27]. In the present study, we have found that SLE patients who had low education, unemployed, and low income were prone to anxiety/depression. Female gender and younger age have well-known associations with depression and confound the SES anxiety/depression relationship in SLE [28]. In the present study, gender was independently associated with depression in SLE. Interestingly, we have found that there were significant correlation between abnormal menstrual history and depression. Whether anxiety/depression is associated with lupus activity remains debatable. Walker SE and colleagues reported that the anxiety severity did correlate positively with SLE activity [29]. Nery et al. reported a significant positive correlation between depression and disease activity [30]. In contrast, other studies reported that there was not relationship between lupus activity and presence of a major depressive episode [31]. In the present study, our group found significant positive correlation between anxiety/depression and disease activity. Health-related quality of life in lupus was found to be significantly worse in comparison with control group. Recent study reported that SLE patients who had significantly poorer health-related quality of life were significantly more depressed and anxious than their healthy counterparts [11]. We have also found the PCS and MCS and all 8 domains of SF-36 except BP were significantly worse than control subjects. The results were similar with the study from Hong Kong. We have also found that the SLEDAI scores were a strong predictor of depression in patients with SLE.

Notably, the results of the present study demonstrated that anxiety in Chinese SLE patients differs from SLE patients in other countries. This could be explained by some cultural features which may influence mental disease diagnosis and management in China. We have found that the prevalence of depression was higher than Hong Kong, and it might be due to cultural differences and SES such as income and medical insurances policy.

In order to identify which variables were most significantly correlated with anxiety and depression, stepwise regression analysis was used. We have found that VT, PF, and RP scales of SF-36 were the most powerful predictors of anxiety of SLE patients. Meanwhile, SLEDAI, VT, PF, SF, and RE domains of SF-36 were significantly accounted for anxiety. It could be explained that impaired quality of life and functional disability were independent risk factors for psychological disorders.

A possible limitation of the present study was that all patients involved in the survey were from only one center and its failure to differentiate between men and women; the gender differences in SLE patients require further analysis in a future study. Another limitation of the study was that we did not detect the impact of proinflammatory cytokines on depression. Recent study reported that higher serum TNF-α level was independently associated with poorer health-related quality of life and more severe depressive symptoms in SLE patients in Singapore [11].

In summary, our study indicated that psychological problems were frequent in Chinese SLE patients. Severe disease status and reduced quality of life significantly correlated with anxiety and depression. Disease activity was higher in anxious and depressed subgroups. Quality of life was decreased in depressed subgroups. Impaired mental health and pain were the most powerful predictors of anxiety and depression. Low SES was independently associated with poor mental health. These findings confirmed the importance of psychosocial interventions in combination with medical therapy for SLE patients.

Authors' Contribution

Biyu Shen and Wei Tan contribute equally to this work.

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